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Nitric Oxide Synthase-2 and Expression of Perforin in Uterine NK Cells

Tim G. Burnett* and Joan S. Hunt†

In human, mouse, and rat pregnancy, maternal NK cells accumulate and differentiate at implantation sites. These cells, termed uterine NK (uNK) cells, express NO synthase (NOS)-2 and develop cytolytic molecules such as perforin and granzymes during differentiation in situ. In this study, relationships between expression of the NOS-2 gene, uNK cell population density and tissue distribution, and synthesis of perforin were investigated. Uteri from wild-type (WT) and NOS-2−/− mice were collected at gestation days (g.d.) 8, 10, 12, 14, and 16 (n, >2/g.d.). Histochemical staining failed to reveal any differences between the population densities or tissue distributions of uNK cells in WT and NOS-2−/− uteri at any stage of gestation. By contrast, immunohistochemical staining with anti-perforin Abs demonstrated significantly fewer perforin-positive uNK cells in two uterine compartments of NOS-2−/− mice in comparison to the same compartments in WT mouse uteri. Perforin-positive uNK cells were reduced in NOS-2−/− metrial glands at g.d. 8, 10, and 12 and in decidua basalis at g.d. 12 (p < 0.05). Analysis of perforin protein by immunoblotting confirmed this observation. Northern blot hybridization studies showed that loss of perforin protein in NOS-2−/− mice was accompanied by decreased steady-state levels of perforin mRNA. These results demonstrate that migration of uNK cells into the uterus, selection of residency sites, and proliferation in situ are independent of NOS-2. By contrast, their differentiation, including transcription and translation of the cytotoxic molecule perforin, was shown to rely on normal expression of the NOS-2 gene. The Journal of Immunology, 2000, 164: 5245–5250.

During pregnancy in humans and rodents, a unique subset of lymphocytes predominates at implantation sites (1). The uterine lymphocytes, known in humans as large granular lymphocytes and in mice and rats as granulated metrial gland cells or uterine NK (uNK)2 cells, are subsets of the NK cell lineage. They express cell surface markers consistent with this subset (Thy-1, asialo-GM1, and large granular lymphocyte-1) and contain NK cell cytolytic mediators (2–5). Evidence for this lineage relationship is also found in studies showing that uNK cells are absent in mice lacking T cells and NK cells (6), but are present and morphologically normal in mice lacking T and B cells (7).

Mouse uNK cells differentiate in situ from bone marrow-derived precursor cells (8). Following implantation, the cells increase dramatically in size and gradually develop prominent intracellular granules containing glycoconjugates and cytolytic molecules such as perforin, granzymes, TNF-α, and IFN-γ (4, 9, 10). Recent studies in our laboratory show that in mice, these cells also contain NO synthase (NOS)-2, an enzyme that is responsible for synthesizing the potent free radical NO (11). NO is used as an effector molecule in tumor cell killing and is also a potent regulator of cell function in many physiological processes (12, 13).

The functions of NOS-2 and other uNK cell molecules in processes important to pregnancy are essentially unknown, but could include both autocrine and paracrine activities. In this study, we postulated autocrine function(s) for uNK cell NOS-2 that might include changes in the density or anatomical location of uNK cells or their differentiation pathways. Differentiation was analyzed by evaluating perforin, which appears in uNK cells following implantation and increases in abundance until mid-gestation, which is approximately gestation days (g.d.) 10–12 in mice (14). We assessed NOS-2 by comparing uNK cells in wild-type (WT, C57BL/6 × 129 SvEv) and NOS-2-deficient (NOS-2−/−) mice as a function of stage of pregnancy. The results demonstrate a critical role for NOS-2 in uNK cell differentiation.

Materials and Methods

**Mice and tissue collection**

Breeding colonies of NOS-2−/− (gift from Dr. Carl Nathan, Weill Cornell Medical College, New York, NY) and strain-matched background C57BL/6 × 129 SvEv (The Jackson Laboratory, Bar Harbor, ME) (WT) mice were maintained following guidelines established by the Animal Use and Care Committee of the University of Kansas Medical Center. Pregnancy (day 1) was identified by the presence of a vaginal plug. Tissue was obtained for histochemical staining and immunohistochemistry by deeply anesthetizing the pregnant mouse and removing the uterus. The uterus was manually dissected into implantation sites containing embryo, extraembryonic membranes, placenta, decidua, metrial gland, and uterine smooth muscle (uteroplacental tissue) and then fixed in 4% paraformaldehyde in PBS overnight. Fixed tissue was embedded in paraffin and 5-mm-thick cross-sections were obtained. Uteroplacental tissue was obtained from at least two pregnant NOS-2−/− and two WT mice at g.d. 8, 10, 12, 14, and 16. To obtain tissue for RNA and protein isolation, pregnant uteri were dissected to isolate the maternal components of implantation sites which included decidua, metrial gland, and uterine smooth muscle. Brain from nonpregnant female mice was also collected to serve as negative control tissue. The dissected tissues were flash frozen in liquid nitrogen and stored at −80°C until used. Pregnant uterus was collected from four NOS-2−/− and WT mice each at g.d. 10, 12, and 14.

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3 Abbreviations used in this paper: uNK, uterine NK; NOS, NO synthase; g.d., gestation day; WT, wild type; PAS, periodic acid-Schiff.
Histochemistry and immunohistochemistry

Unless otherwise noted, all reagents for these and all other protocols described below were obtained from Sigma (St. Louis, MO). Cross-sections of tissues taken from the centers of implantation sites were deparaffinized and stained with periodic acid–Schiff reagent (PAS) to reveal the dense glycoprotein-containing granules of uNK cells (15). Tissue sections were also stained by immunohistochemistry using two rabbit anti-perforin polyclonal Abs: whole serum (a gift from Dr. Chau Ching Liu, University of Pittsburgh School of Medicine, Pittsburgh, PA) and IgG fraction (Torrey Pines Bio Labs, San Diego, CA). Immunostaining was performed by deparaffinizing and blocking in 10% normal goat serum for 1 h at room temperature. The sections were then incubated with rabbit anti-perforin Ab at a dilution of 1:400 or with an equal concentration of normal rabbit serum (Vector Laboratories, Burlingame, CA) for 1 h at 37°C. After incubation with the primary Ab or control serum, endogenous peroxidase activity was blocked by treating the sections with 0.5% H2O2 in methanol for 30 min at room temperature. The sections were then incubated with rabbit anti-perforin Ab at 1:10,000 dilution for 1 h at room temperature. The sections were then incubated with rabbit anti-perforin Ab at a 1:10000 dilution of HRP-labeled goat anti-rabbit IgG for 1 h at room temperature, followed by incubation with streptavidin–HRP conjugate (Zymed, South San Francisco, CA) for 10 min. The color was developed by treating the sections with 3-amino-9-ethylcarbozole in 0.5% dimethyl-formamide (Zymed) which yields a red substrate where Ag is present.

Image analysis

Tissue sections stained by immunohistochemistry were visualized by light microscopy and digital images were captured using a charge-coupled device video camera. The digitized images were analyzed using Optimas 5 imaging software (Optimas, Bothell, WA). Three areas in the metrial gland and/or decidua basalis were analyzed to determine the frequency of positive staining (average positive areas/mm2) in each area. Positive staining areas were defined as areas with pixel intensity and size within set thresholds. Extensive testing was performed to establish threshold limits consistent with values obtained by visual inspection. At least two implantation sites from two to three mothers of each strain were analyzed at g.d. 8, 10, 12, 14, and 16 (n = 4/g.d.) The two strains were analyzed for statistical differences at each g.d. by Student’s t test. Two implantation sites from two mothers of each strain were analyzed at g.d. 8, 10, 12, 14, and 16 (n = 4). The two strains were analyzed for statistical differences at each g.d. by Student’s t test.

Perforin protein detection by immunoblot analysis

Frozen tissue was solubilized in PBS containing 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 0.5 M EDTA, 0.1 mg/ml PMSF, 0.01 mg/ml aprotonin, and 0.01 mg/ml leupeptin. Protein concentrations were determined for each sample using the Bio-Rad DC protein assay (Bio-Rad, Hercules, CA). Solubilized samples (40 μg/ml) were separated by SDS-PAGE under nonreducing conditions using a mini-gel apparatus (Bio-Rad). The separated proteins were then transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH) in transfer buffer containing 25 mM Tris base (Bio-Rad), 192 mM glycine (Bio-Rad), 0.1% SDS, and 20% methanol. The blots were blocked for 2 h in 3% nonfat dry milk (Bio-Rad) in PBS and then incubated with one of the polyclonal Ab specific for perforin or normal rabbit IgG diluted 1:5000 in PBS containing 0.05% Tween 20 and 3% nonfat dry milk (Bio-Rad). After washing the membranes in PBS with 0.05% Tween 20, the blots were incubated with a 1:10000 dilution of HRP-labeled goat anti-rabbit IgG for 1 h at room temperature. After washing five times with PBS-Tween 20, bound Ab was detected by chemiluminescence following the manufacturer’s instructions (enhanced chemiluminescence kit; Amersham, Pharmacia Biotech, Piscataway, NJ). The blots were washed and exposed to Hyperfilms ECL (Amersham). The blots were then exposed to Hyperfilms ECL and developed with autoradiography film (Kodak) and analyzed using a Storm 820 Phosphorimager (Molecular Dynamics). The bands were quantified by densitometry of ethidium bromide-stained 28S rRNA. The membranes were then probed with a 32P-labeled cDNA probe for perforin (Ref. 16; a gift from Dr. Chau Ching Liu, University of Pittsburgh School of Medicine). Prehybridization was performed for 30 min at 68°C using Perfecthyb hybridization buffer. The same buffer was used for hybridization with the addition of salmon sperm DNA (100 μg/ml) and radiolabeled probe at 3 x 106 cpm/ml. The blots were incubated at 68°C for 1 h and then washed twice with a solution containing 0.3 M NaCl and 0.03 M trisodium citrate (2× SSC) at room temperature for 15 min. The final wash solution contained 0.1× SSC and was performed three times for 15 min each at 60°C. The membranes were used to expose x-ray film (Amersham Pharmacia Biotech). Relative abundance of perforin mRNA was determined by normalizing the OD of perforin bands to the OD of ethidium bromide-stained 28S rRNA.

Results

Mouse implantation sites

The mouse implantation site at mid-gestation is composed of three distinct areas. The metrial gland lies between the circular and longitudinal muscles of the uterine myometrium. Beneath the circular muscle is the decidua basalis, which is formed from uterine endometrium following implantation. Both the metrial gland and decidua basalis are derived from maternal tissue. The placenta is derived from embryonic tissue and is separated from the decidua basalis by a layer of trophoblast cells called giant cells. The boxes in Fig. 1 illustrate locations at which quantitative image analysis was performed on tissue sections.

Frequency of uNK cells in WT and NOS-2−/− mice implantation sites

To determine whether or not maternal uNK cells at implantation sites differed between WT and NOS-2−/− mice, uteroplacental tissues from g.d. 8, 10, 12, 14, and 16 were visually inspected for large granular PAS-positive cells. Subjective analysis by light microscopy failed to identify any major differences in the distribution and frequency of uNK cells between NOS-2−/− and WT mice (Fig. 2. A and D).

To verify this observation, the number of uNK cells per mm2 was assessed at each g.d. from color photomicrographs. The frequency of PAS-positive cells in WT and NOS-2−/− mice did not differ significantly at any g.d. (p > 0.05) in either the metrial gland

FIGURE 1. Schematic diagram of mouse implantation site at g.d. 12. MG, metrial gland; LM, longitudinal muscle; CM, circular muscle; DB, decidua basalis; GC, giant trophoblast cells; P, placenta; LEp, luminal epithelium.
area or decidua basalis (Fig. 3). These data indicated that the distribution and proliferation of uNK cells at implantation sites is not altered by lack of NOS-2.

**Localization of perforin protein in pregnant NOS-2<sup>-/-</sup> mouse uterus**

Since the distribution and density of uNK cells appeared to be normal in NOS-2<sup>-/-</sup> mice, the next step was to determine whether their differentiation was altered. To assess differentiation, we investigated the expression of perforin, a cytotoxic molecule that appears in uNK granules in a gestation-dependent manner. Mouse uteroplacental tissue sections from g.d. 8, 10, 12, 14, and 16 were stained using two different rabbit anti-perforin Abs to localize the expression of perforin at implantation sites. The Ab obtained from Dr. Liu was used on all tissues and the commercial Ab was used to verify staining patterns.

As expected, perforin was specifically localized to the metrial gland area and decidua basalis of implantation sites at g.d. 8, 10, 12, 14, and 16 in both WT and NOS-2<sup>-/-</sup> mice (g.d. 12, Fig. 2, B and E). Positive cells were virtually absent in the placenta (data not shown). Adjacent sections were incubated with normal rabbit serum to control for nonspecific immunoreactivity, and positive staining was absent (Fig. 2, C and F). The expression of immunoreactive perforin was limited to the uNK cells as indicated by the large size, granular appearance, and PAS positivity of the same cells in adjacent sections (Fig. 2, A and D). Perforin staining within uNK cells was localized to cytoplasmic granules in both WT and NOS-2<sup>-/-</sup> mice.

The localization pattern of anti-perforin immunostaining was identical between the WT and NOS-2<sup>-/-</sup> mice at all g.d. that were assessed. That is, immunoreactivity was observed in the metrial gland and decidua basalis but not in other areas of the implantation sites. However, the NOS-2<sup>-/-</sup> mice appeared to have a lower overall staining intensity during mid-gestation (Fig. 2, B and E) despite having a normal density of uNK cells. To quantify this, image analysis was performed on the anti-perforin immunostains. Highly immunopositive areas were detected and the frequency of these areas per unit area was quantified by image analysis in four to eight implantation sites. A significant decrease ($p < 0.05$) of staining intensity was observed in the decidua on g.d. 8, 10, and positive staining was absent (Fig. 2, C and F). The expression of immunoreactive perforin was limited to the uNK cells as indicated by the large size, granular appearance, and PAS positivity of the same cells in adjacent sections (Fig. 2, A and D). Perforin staining within uNK cells was localized to cytoplasmic granules in both WT and NOS-2<sup>-/-</sup> mice.

**FIGURE 2.** NK cells in metrial glands (MG) from g.d. 12 WT (A–C) and NOS-2<sup>-/-</sup> (D–F) mice. Subjacent sections were stained with PAS (A and D) or immunostained using anti-perforin Ab from Dr. Liu (B and E). Normal rabbit serum was used as a negative control (C and F). Arrows indicate positive staining uNK cells. Original magnifications, ×200.

**FIGURE 3.** Frequency of PAS-positive cells in metrial gland area (A) and decidua basalis (B). Cell density is expressed as the number of positive cells per mm<sup>2</sup> ± SEM. Two implantation sites were analyzed from each of two mothers on each day of gestation ($n = 4$).
Perforin protein is decreased in the pregnant uterus of NOS-2−/− mice

To verify the results of the immunostains and to quantify the relative amounts of perforin, immunoblotting was performed on metrial gland tissue taken from WT and NOS-2−/− during mid-gestation, the time at which the highest levels of perforin protein are detected by immunohistochemistry. As shown in Fig. 5A, chemiluminescent detection of protein derived from pregnant uteri at g.d. 10, 12, and 14 revealed decreased levels of perforin in pregnant NOS-2−/− mice compared with WT mice. Our immunoblots revealed up to four protein bands specifically binding anti-perforin Ab with apparent molecular masses of 81, 76, 67, and 63 kDa. The largest band (81 kDa) was absent in some samples and the 67-kDa band was the most prominent. The anti-actin Ab produced a single band at 52 kDa. Nonspecific binding was absent when blots were probed with normal rabbit IgG. The ratio of the OD obtained from the 67- and 63-kDa perforin-specific bands to the OD of specific bands reacting with anti-actin Ab was used to compare the relative amounts of perforin protein (Fig. 5B). At each g.d., the average perforin:actin ratio was lower in the NOS-2−/− mice than in WT mice. Immunoblots were repeated three times with tissue from additional mothers with similar results. The relative perforin levels were always higher in the WT mice compared with the g.d.-matched NOS-2−/− mice. Peak levels of perforin expression were observed on either g.d.12 or 14 in both strains.

Pregnant NOS-2−/− mice have decreased levels of perforin mRNA

The steady-state levels of perforin mRNA in uteri from pregnant NOS-2−/− and WT mice were analyzed and compared by Northern blot analysis. Total RNA was collected from uterine tissue of pregnant WT and NOS-2−/− mice at g.d. 10, 12, and 14. The total amount of perforin mRNA was assessed by scanning densitometry of the exposed autoradiogram and densitometry OD values were normalized to the relative amount of 28S rRNA in each lane. A single band of ~2.9 kb was detected in all samples (Fig. 6A). Perforin mRNA was not detected in total RNA from brain tissue, which was utilized as a negative control. Normalized band intensity indicated that the relative abundance of perforin mRNA was decreased in pregnant NOS-2−/− mice compared with WT counterparts at each g.d. assessed (Fig. 6B). This experiment was repeated four times. In all cases, NOS-2−/− mice had lower steady-state levels of perforin mRNA than WT mice at each g.d. These results indicate that levels of perforin mRNA are related to NOS-2 expression, either directly or indirectly.

Discussion

In this study, we report for the first time that NOS-2 is likely to play an active role in promoting the expression of the gene encoding perforin or increasing the stability of the perforin message in...
uNK cells. The first set of studies, where we compared the absolute numbers of uNK cells in NOS-2−/− and WT mouse implantation sites, failed to identify any relationship between NOS-2 expression and the densities of NK cells in the pregnant uterus. Thus, migration of this cell type into the pregnant uterus and/or proliferation in situ is unrelated to NOS-2 expression. In striking contrast, major differences in immunoreactive perforin in the uNK cells of NOS-2−/− and WT mice were identified at mid-gestation using both immunohistochemical analysis and immunoblots. Both methods showed that less perforin was present in the uNK cells of NOS-2−/− than in WT mice. Perforin is known to contain two potential glycosylation sites (17), and the occurrence of multiple bands is likely due to alternative glycosylation and/or proteolytic cleavage. These alternative forms may represent different stages of perforin biosynthesis and maturation (18). Analysis by Northern blot hybridization demonstrated that reduction of perforin protein was accompanied by a reduction in steady-state levels of perforin mRNA, thus demonstrating a link between NOS-2 and availability of perforin mRNA for processing into protein.

The reproductive effects of NOS-2 gene ablation have not been previously investigated partly because NOS-2−/− mice reportedly produce normal numbers of embryos in each litter and do not demonstrate any reduction in the frequency of births (19). However, we and others have shown that in mice, humans, and rats, NOS-2 is appropriately positioned in both spatial and temporal terms to have a major influence on pregnancy (11, 20, 21). Biosynthesis of NO is clearly critical; when NOS inhibitors are administered to pregnant rats, the result is severe maternal hypertension and fetal growth restriction, symptoms resembling pre-eclampsia in humans (22). These results suggest that one of the functions of NOS at the maternal-fetal junction may be to modulate the uterine vasculature.

Here, we report that NOS-2 is instrumental in programming production of perforin in uNK cells. Perforin synthesis in this lineage is a major feature of their differentiation in the pregnant uterus following implantation. Thus, NOS-2 is strongly associated with at least one feature of the differentiation process. The function of perforin in this cell type is unknown. It has been postulated to assist in killing of stry trophoblast cells from the placenta (23) and in killing virus-infected cells (24), but there is at present little experimental evidence for either activity. Perforin-deficient mice reproduce normally and do not differ from WT mice in the morphology of the fetal-maternal interface. Curiously, these mice appear to have a greater frequency of uNK cells (25). Lacking evidence for a paracrine function, it is tempting to speculate that perforin may have an autocrine function, being part of the apoptotic cell death pathway that characterizes these cells as gestation proceeds to termination (26). Such an immunoregulatory role for perforin has recently been proposed based on observations that showed persistent accumulation of CD8+ T cells in perforin-deficient mice during acute viral infection (27).

Lack of perforin protein was linked to reduced levels of specific message encoding NOS-2 mRNA in uNK cells; steady-state levels of perforin mRNA in NOS-2−/− mice were markedly decreased relative to perforin message in WT mice. This supports the idea that NO regulates either the rate of perforin transcription or the stability of specific message in uNK cells.

NO could regulate perforin transcription through any of several signaling mechanisms (13). In the best-studied mechanism, NO activates soluble guanylyl cyclase, causing an increase in concentrations of cGMP. NO reacts with the heme group of guanylyl cyclase to alter the conformation and promote the conversion of GTP to cGMP (28). Intracellular signaling proceeds via a cGMP-dependent protein kinase, which transduces the signal through a phosphorylation cascade (29). NO has also been shown to activate G proteins (30) which are involved in inducing transcription of a number of genes. Through these mechanisms, NO could influence the expression of the binding partners for regulatory sites that control perforin expression. In NK cells and CTL, the expression of perforin is dependent upon an Ets binding site motif and its binding protein, NF-P2 (31). Moreover, the perforin promoter region contains other putative regulatory elements such as CG box, AP-2 binding site, and cAMP-responsive elements (32, 33).

Alternatively, NO may impinge on external signals that modulate perforin expression. Several cytokines have been implicated in the induction of perforin in cytotoxic cells, including IL-2 (34), IL-6 (35), and IL-15 (36). Of these, IL-15 has been identified as a modulator of perforin expression in mouse uNK cells (37), and it is not inconceivable that NOS-2 acts through IL-15 to regulate perforin expression. NOS-2 has been shown to be a required component of IL-12 signaling in NK cells during early Leishmania major infection (38). Since IL-15 and IL-12 signaling both operate by activating STAT (39), it is possible that NO is also required for IL-15 signaling. Indeed, a STAT binding site has been suggested to play a major role in the expression of the gene encoding perforin in human cells (40). Alternatively, NO may be required for the availability of IL-15 or the expression of the IL-15 receptor.

This study shows that lack of NOS-2 in mice results in reduced perforin production by uNK cells. Although the function(s) of uNK cells remains to be identified, as does the function(s) of perforin in these cells, the results of this study provide some insight into the regulatory pathways that control the nature of uNK cells. It will be of interest to learn whether NOS-2/NO has regulatory roles on other subsets of NK cells or CTL, which are also known to produce perforin.

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