

Positional Relationship between Natural Killer Cells and Distribution of Sympathetic Nerves in Decidualized Mouse Uterus

Abdolkarim Sheikhi^{1*}, Hossein B. Ganji², Razieh Sheikhi³

¹Departments of Immunology ²and Anatomy, School of Medicine, Zanjan University of Medical Sciences, Zanjan, Iran, ³Department of Environmental Health, Faculty of Health Sciences, Tehran University of Medical Sciences, Tehran, Iran

ABSTRACT

Background: Uterine natural killer (uNK) cells are the most abundant leukocytes in pre-implantation endometrium and early pregnancy deciduas in humans and rodents. They are associated with structural changes in maternal spiral arteries but regulation of their recruitment and activation is incompletely understood. The major subpopulation of uNK cells in humans expresses CD56, the neural cell adhesion molecule (NCAM)-1 while their counterpart in mouse expresses asialoGM1, a brain ganglioside. Sympathetic nerves express NCAM-1 which mediates homotypic binding. Sympathetic fibers innervate the mesometrial vasculature but their relationship to the myometrial and decidual uNK cell recruitment is unknown. **Objective:** The present study aims to explore positional relationship between natural killer cells and distribution of nerves in decidualized mouse uterus. **Methods:** Immunohistochemistry and mRNA expression for the enzyme tyrosine hydroxylase were used to map sympathetic nerve fibre distribution within C57BL/6 implantation sites and to address a relationship with uNK cells. **Results:** Tyrosine hydroxylase positive neurons were identified in the mesometrium closely associated with uterine arteries. Staining became gradually vanished as the nerves crossed the myometrium and entered the decidualized uterus. No neuronal stain was associated with the spiral arteries. Periodic Acid Schiff's reactive uNK cells were absent from the mesentery, but abundant in decidua basalis where they are associated with non-innervated vessels. **Conclusion:** Data suggest that the recruitment of uNK progenitor cells to the uterus is unlikely to be dependent on signaling by the sympathetic nervous system

Keywords: Uterine NK cell, Deciduas, Spiral Artery, Tyrosine Hydroxylase, Sympathetic Nerves

*Corresponding author: Dr. Abdolkarim Sheikhi, Department of Immunology, School of Medicine, Shahrak Karmandan, Zanjan University of Medical Sciences, Zanjan, Iran. Tel: (+) 98 241 4240301-ext. 296, Fax: 98 241 4249553, e-mail: sheikhi@queensu.ca

INTRODUCTION

Uterine Natural Killer (uNK) cells are the predominant leukocytes (more than 70% of decidual lymphocytes) in implantation sites of a wide variety of species, including mice and humans and are strongly associated with endometrial decidualization (1- 6). The lymphocyte composition of early human decidua differs significantly from that of peripheral blood with only 10% of blood lymphocytes (PBL) being NK cells. Most blood NK cells are characterized by high expression of the CD16 receptor (FcγRIII) and moderate to low expression of CD56, the neural cell adhesion molecule (N-CAM). In contrast, decidual NK cells are CD56^{bright}CD16^{dim}. Studies in mice have shown that self-renewing precursors of NK cells do not reside in the uterus but home from primary and secondary lymphoid organs once implantation has been initiated (7- 8). Homed progenitors proliferate, differentiate and become activated within restricted mesometrial regions of implantation sites. In the decidua basalis, they are central in location and associated with arteries, including the major spiral arteries from gestation day (gd) 6. In the wall of the uterus, they form a structure between the two uterine smooth muscle coats from gd8 which will surround the vessels supplying each implantation site. This structure, known variously as the mesometrial lymphoid aggregate of pregnancy (MLAp); metrial gland (MG) and mesometrial decidua, is enriched with less mature uNK cells that are smaller, less heavily granulated and actively proliferating. The cytoplasmic granules acquired by differentiating uNK cells are glycoprotein-rich and react with Periodic Acid-Schiff's (PAS) reagent (9). About gd8, as cytoplasmic granules begin to accumulate in mouse uNK cells, the cells acquire the marker asialo GM1, a brain ganglioside, indicative of their activation (10). The mechanisms regulating uNK cell homing, positioning, accumulation and activation within the decidua basalis and MLAp are still largely unknown. uNK cells, through interferon (IFN)-γ production, are essential for spiral arterial modification (11), a normal physiological process of pregnancy that structurally changes the major arteries that feed into the placenta.

Neural cell adhesion molecules, which mediate homophilic binding, are important in embryonic development. CD56 (Leu 19/ NKH-1) and asialo GM1 are expressed on sympathetic nerves as well as on uNK cells (12- 16). This suggests that a component of uNK cell recruitment, positioning and activation could be due to homotypic interactions between these lymphocytes and the sympathetic axons and fibres that innervate the uterus. The uterus is supplied by the sympathetic nervous system that travels with the uterine arteries and veins. To determine whether sympathetic innervation reaches the regions where uNK cells accumulate, we compared the distribution of PAS positive uNK cells to the distribution of uterine sympathetic neurons with tyrosine hydroxylase (TH), a marker for noradrenergic sympathetic innervation in C57BL/6 mice (17).

MATERIALS AND METHODS

Animals. Adult male and female C57BL/6 (B6) mice were mated for timed gestations. The day of plug detection was called as 0.5 gd. Two pregnant mice were studied at each gd 6.5, 8.5, 10.5 and 12.5 using 3 healthy implantation sites for each mother. Uteri from two virgin females were also investigated. All procedures were conducted under animal utilization protocols approved by the Animal Care Committee of Queen's University (Canada).

UNK Cell Isolation by Magnetic Bead Separation Method. To obtain uNK cells, decidua basalis were dissected from PBS-perfused gestation day (gd) 12.5 B6 females.

Tissues were finely minced and briefly dissociated in 1000 IU DNase in 2% BSA. The suspension was filtered through an 80- μ m mesh filter and washed two times with Hank's balanced salt solution containing 2% BSA. The cells were counted by trypan blue dye exclusion method. Beads (4×10^8 / ml) were conjugated to biotinylated *Dolichos biflorus* (DBA) lectin (a ligand specific for uNK cells) (18), for 30 minutes. The cell suspension was incubated for 30 min with these DBA lectin-conjugated magnetic beads. The retained cells were washed three times using PBS and detached from the beads using a rinse of 0.1 M N-acetyl-D-galactosamine (Sigma, USA), which is a specific sugar ligand of DBA lectin. The purity of uNK cells isolated by this method was >98%. RNA was extracted from uNK cells as described below.

Histological Studies. Mice were anaesthetized followed by perfusion with 4% paraformaldehyde (PFA) in 0.2 M phosphate buffer saline (PBS). Two hundred ml were perfused at 20 ml/min, with drainage from the right atrium. Uteri were then removed and immersed in the same fixative overnight (20°C). Uteri were transferred to 30% sucrose in 0.1 M PBS for 3 days (20°C). Uteri were transected between each implantation site, and each was mounted in OCT compound (Fisher Scientific, Ottawa, ON) and frozen using Methylbutane cooled by dry ice. Frozen specimens were wrapped and stored at -80°C until study. Specimens were cryosectioned at 10 μ m mounted onto Superfrost gelatin coated slides (Fisher Scientific, Ottawa, ON) for the study. Primary antibody was rabbit anti- Th42 (Chemicon International; AB1542; 1/1000), a sympathetic nerve marker. Standard procedures were followed for avidin-biotin—horseradish peroxidase immunohistology using no primary antibody as the negative controls and no counterstains(19). uNK cells were stained in adjacent frozen sections (to those immunostained) using a standard PAS reaction followed by counterstaining with methylene blue.

Gene Expression Studies. RNA prepared from dissociated decidua basalis or highly purified gd12.5 B6 uNK cells harvested from dissociated decidua basalis using magnetic beads coated with *Dolichos biflorus* lectin, was evaluated for transcription of NCAM-1.NK1.1 (Klrb1c), was used as a positive NK cell amplification control. Primers for NCAM-1 were F-ACGTCCGGTTCATAGTCCTG; R-CACACACCAGGGTGACAGAC; giving a 219 bp product and primers for NK1.1 (Klrb1c) were F-TGACCCTGATTGGGATGAGT; R-TTGAATGAGCAGCAAAGTGG; giving a 224 bp product. Negative controls lacked cDNA (see18).

RESULTS

Antibodies identified sympathetic nerve fibers in all sections of virgin and pregnant uteri gd 6.5, 8.5, 10.5 and 12.5. The nerves were embedded in the walls of uterine artery branches located in the mesometrium (Figure 1C). As the vessels crossed the myometrium, reactive fibers were noticeably reduced within vessel cross-sections. No reactive nerve fibres were present in decidualized endometrium (Figure 1D). PAS reactive uNK cells were present in decidua basalis and many associated with non-innervated segments of uterine artery branches (Figure 1B) gd 6.5, 8.5, 10.5 and 12.5. uNK cells were absent from the uterine mesentery (not shown). uNK cell distribution in the MLAp was uniform and did not appear to be altered by the infrequent nerve bundles seen in some myometrial segments of the uterine vessels. To evaluate whether murine uNK cells are capable of NCAM-1 transcription, RT-PCR was employed. Figure 1F illustrated the RT-PCR results of NCAM-1 and NK1.1 (Klrb1c), the positive control marker

for murine NK cell identification. As it is shown in figure 1F, the dissociated decidua basalis and highly purified uNK cells express NCAM-1 and NK1.1 markers.

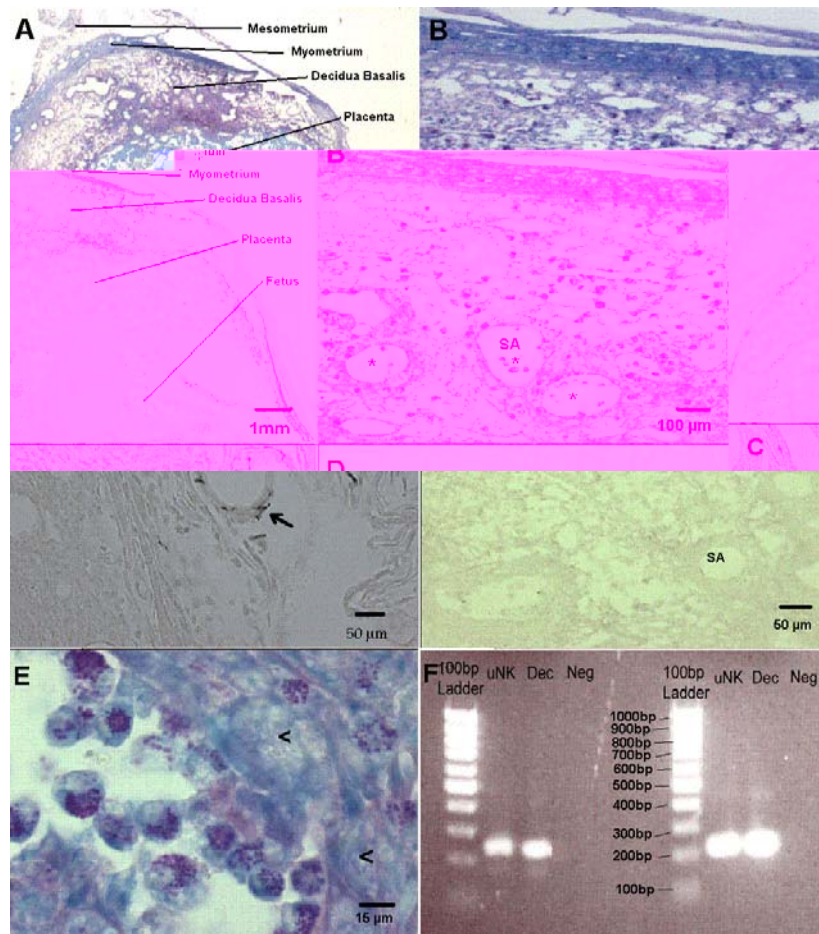


FIGURE 1. Photomicrographs (A-E) of cryostat sections of gd10.5 B6 implantation sites. A: Illustrates the mesometrium in relation to the myometrium, decidua basalis and placenta for orientation (PAS counterstained with methylene blue). B: Illustrates the numerous PAS-reactive uNK cells (example illustrated by arrow) in the central decidua basalis. Asterisks mark cross sections of the non-innervated coils of the spiral artery (SA) branches from the innervated arcuate branches of the uterine artery. Intravascular uNK cells are found within these non-innervated endometrial arterial coils. C: Illustrates immunoreactivity to anti-tyrosine hydroxylase in the walls of the uterine artery branches (UA) in the mesentery (arrows) as they approach the myometrium (lower right of image). D: Shows the central decidua basalis of the same tissue section where no immunoreactivity to anti-tyrosine hydroxylase was found. The spiral artery (SA) in (D) is a branch from the immunoreactive uterine artery imaged in (C). E: Illustrates uNK and trophoblast cells, co-localized in a spiral artery. F: Illustrates the electrophoresis of amplicons from gd12.5 B6 uNK cells and decidua basalis (Dec) probed for expression of NCAM-1(Left panel) and NK1.1 (Right panel). Neg indicates the negative water amplified control.

DISCUSSION

Dynamic, ovarian hormone-related changes in location, distribution and density of nerve bundles have been previously reported in rats and mice during the estrous cycle, pregnancy and parturition. Uterine sympathetic nerve bundles are associated with arteries. In rats, the highest nerve fiber density has been reported in the mesometrial triangle of the caudal uterus (20-23). We localized the sympathetic fibers to arterial walls within

the mesentery and myometrium. The nerve fibers were absent from modifying and fully modified spiral arteries, where uNK cells were abundant both surrounding and within the vessels. This suggests that uNK cells within decidua basalis are not sympathetic neurotransmitter regulated.

Mouse uNK cell progenitors are thought to home from blood (24). Their point of uterine entry is unknown but pro-uNK cells would flow through innervated mesometrial arteries and, if marginating, could experience concentration-dependent neurotransmitter regulation. The sympathetic mediator norepinephrine blocks target cell lysis by human NK and IL-2 stimulated lymphocytes but promotes migration (25). Immature, proliferative uNK cells congregate in the MLAp, a transient structure in the uterine wall that separates the myometrial layers. Here, occasional arterial wall-associated nerve bundles were found but histological differences could not be identified in uNK cells between regions of MLAp having arterioles with and without immunoreactive nerve fibers. Intravascular uNK cells are reported to be extremely rare in MLAp (26) making it possible that pro-, pre- or immature stages of uNK cells, like decidual stages, do not experience neurotransmitter regulation. Alternatively, current techniques may not adequately identify earlier cell stages committed to uNK cell differentiation and intravascular neurotransmitter activation of these cells may promote their terminal differentiation and activation. It is also possible that uNK cells contribute to the cyclic, ovarian steroid-related regression of arterial nerve fiber bundles during their promotion of structural changes within gestational spiral arteries. Study of possible neurotransmitter contributions during uNK cell differentiation could be undertaken in mice with specific chemical or genetic neurotransmitter blockade.

Early gestational endometrium is a site of elevated angiogenesis. In other tissues, the pattern and branching of new arterial tissue is guided and regulated by the release of vascular endothelial cell growth factor (VEGF) from nerves (27). Because uNK cells produce VEGF (28-31), we hypothesize from our study that uNK cells provide the guidance for arterial growth and branching in early gestational endometrium where there is a lack of neuronally-produced VEGF (29). The mobile uNK cells carry receptors that recognize molecules expressed by trophoblast. Thus, uNK cells would be able to guide maternal vascular development towards each implantation site.

This study suggests that mouse uNK cells transcribe CD56, the neural cell adhesion molecule and have the potential to participate in homotypic binding with sympathetic nerve fibres using this molecule and/or others. However, the absence of sympathetic nerve fibres at the sites in which uNK cells were located suggested that sympathetic nerves are unlikely associated with the positioning and activation of these transient uterine cells.

ACKNOWLEDGEMENTS

We thank Dr. B. A. Croy and Dr. C. Tayade for lab facilities, Dr. M. Kawaja for helpful discussions and Ms. V. Norkum for technical assistance. This work was supported by NSERC of Canada and Zanjan University of Medical Sciences.

REFERENCES

- 1 Loke YW, King A. Human implantation. cell biology and immunology. United Kingdom: Cambridge University Press; 1995.p. 1–40.
- 2 Whitelaw PF, Croy BA. Granulated lymphocytes of pregnancy. *Placenta*. 1996; 17:533-43.
- 3 Croy BA, He H, Esadeg S, Wei Q, McCartney D, Zhang J et al. Uterine natural killer cells: insights into their cellular and molecular biology from mouse modelling. *Reproduction*. 2003 Aug;126:149-60.
- 4 Moffett-King A. Natural killer cells and pregnancy. *Nat Rev Immunol*. 2002; 2: 656-63.
- 5 Widayati DT, Ohmori Y, Fukuta K. Distribution patterns of immunocompetent cells in the pregnant mouse uteri carrying allogeneic mouse and xenogeneic vole embryos. *J Anat*. 2004; 205:45-55.
- 6 Stewart JJ. Granulated metrial gland cells in 'minor' species. *J Reprod Immunol*. 1998; 40:129-46.
- 7 Croy BA, Kiso Y. Granulated metrial gland cells: a natural killer cell subset of the pregnant mouse uterus. *Micro. Res. Tech*. 1993; 25:189-200.
- 8 Peel S. Granulated metrial gland cells. *Adv Anat Embryol Cell Biol*. 1989; 115:1-112.
- 9 Croy BA. Granulated metrial gland cells—interesting cells found in the pregnant uterus. *Am J Reprod Immunol*. 1990; 23:19-21.
- 10 Parr EL, Parr MB, Zheng LM, Young JD. Mouse granulated metrial gland cells originate by local activation of uterine natural killer lymphocytes. *Biol Reprod*. 1991; 44:834-41.
- 11 Ashkar AA, di Santo JP, Croy BA. Interferon gamma contributes to initiation of uterine vascular modification, decidual integrity, and uterine natural killer cell maturation during normal murine pregnancy. *J Exp Med*. 2000;192:259-70.
- 12 Edelman GM. Cell adhesion molecules in the regulation of animal form and tissue pattern. *Ann Rev Cell Biol*. 1986;2: 81-116
- 13 Cunningham BA, Hemperly JJ, Murray BA, Prediger EA, Brackenbury R, Edelman GM. Neural cell adhesion molecule: structure, Immunoglobulin-like domains, cell surface modulation, and alternative RNA splicing. *Science*. 1987;236:799-806.
- 14 Edelman GM. Morphoregulatory molecules. *Biochemistry*. 1988; 27:3533-43.
- 15 Lanier LL, Testi R, Bindl J, Phillips JH. Identity of Leu-19 (CD56) leukocyte differentiation antigen and neural cell adhesion molecule. *Exp Med*. 1989; 169:2233-8.
- 16 Young WW Jr, Hakomori SI, Durdik JM, Henney CS. Identification of ganglio-N-tetraosylceramide as a new cell surface marker for murine natural killer (NK) cells. *J Immunol*. 1980; 124:199-201.
- 17 Guidry G, Landis SC. Absence of cholinergic sympathetic innervation from limb muscle vasculature in rats and mice. *Auton Neurosci*. 2000; 82: 97-108.
- 18 Borzychowski AM, Chantakru S, Minhas K, Paffaro VA, Yamada AT, He H et al. Functional analysis of murine uterine natural killer cells genetically devoid of oestrogen receptors. *Placenta*. 2003; 24:403-11.
- 19 Walsh GS, Kawaja MD. Sympathetic axons surround nerve growth factor-immunoreactive trigeminal neurons: Observations in mice overexpressing nerve growth factor. *J Neurobiol*. 1998; 34:347-60.
- 20 Dyce KM, Sack WO, Wensing CJG. *Textbook of Veterinary Anatomy*. 2nd ed. Philadelphia (PA): WB Saunders Company; 1996.
- 21 Houdeau E, Rossano B, Prud'homme MJ. Regional and muscle layer variations in cholinergic nerve control of the rat myometrium during the oestrous cycle. *Auton Neurosci*. 2003; 104:1-9.
- 22 Haase EB, Buchman J, Tietz AE, Schramm LP. Pregnancy-induced uterine neuronal degeneration in the rat. *Cell Tissue Res*. 1997; 288:293-306.
- 23 Zoubina EV, Smith PG. Axonal degeneration and regeneration in rat uterus during the estrous cycle. *Auton Neurosci*. 2000; 84:176-85.
- 24 Chantakru S, Miller C, Roach LE, Kuziel WA, Maeda N, Wang WC et al. Contributions from self-renewal and trafficking to the uterine NK cell population of early pregnancy. *J Immunol*. 2002;168:22-8.
- 25 Lang K, Drell TL, Niggemann B, Zanker KS, Entschladen F. Neurotransmitters regulate the migration and cytotoxicity in natural killer cells. *Immunol Lett*. 2003;90:165-72.
- 26 Chantakru S, Kuziel WA, Maeda N, Croy BA. A study on the density and distribution of uterine Natural Killer cells at mid pregnancy in mice genetically-ablated for CCR2, CCR 5 and the CCR5 receptor ligand, MIP-1 alpha. *J Reprod Immunol*. 2001; 49:33-47.
- 27 Carmeliet P, Tessier-Lavigne M. Common mechanisms of nerve and blood vessel wiring. *Nature*. 2005; 436:193-200.
- 28 Wang C, Tanaka T, Nakamura H, Umesaki N, Hirai K, Ishiko O et al. Granulated metrial gland cells in the murine uterus: localization, kinetics, and the functional role in angiogenesis during pregnancy. *Microsc Res Tech*. 2003; 60:420-9.
- 29 Tayade C, Black GP, Fang Y, Croy BA. Differential gene expression in endometrium, endometrial lymphocytes, and trophoblasts during successful and abortive embryo implantation. *J Immunol*. 2006; 176:148-56.
- 30 Li XF, Charnock-Jones DS, Zhang E, Hiby S, Malik S, Day K et al. Angiogenic growth factor messenger ribonucleic acids in uterine natural killer cells. *J Clin Endocrinol Metab*. 2001;86:1823-34.
- 31 Hanna J, Goldman-Wohl D, Hamani Y, Avraham I, Greenfield C, Natanson-Yaron S et al. Decidual NK cells regulate key developmental processes at the human fetal-maternal interface. *Nat Med*. 2006; 12:1065-74.