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PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 10 (17): 2885-2889, 2007
ISSN 1028-8880

Are Natural Killer Cells Distributed in Relationship to Nerve Fibers in the Pregnant Mouse Uterus?

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Abstract: Specialized lymphocytes, called uterine Natural Killer (uNK) cells, appear in human and rodent uteri and become abundant at implantation sites during decidualization and early pregnancy. The hallmark of human uNK cells is intense expression of CD56, a neural cell adhesion glycoprotein (NCAM-1) while mature (granulated) mouse uNK cells express asialoGM1, a brain ganglioside. Murine uNK cells initiate the normal structural changes induced in maternal spiral arteries by pregnancy but regulation of their recruitment, localization and activation is incompletely understood. To address whether uNK cell distribution is co-localized with nerve fiber distribution, sections of gestation day (gd) 6-12 implantation sites from C57BL/6 (B6) mice were studied. Nerve fibers reactive with antibodies to pan neurofilament 150 kD or with tyrosine hydroxylase, an enzyme restricted to sympathetic fibers, were present the walls of branches from the uterine artery in the mesentery. Reactivity was lost as the vessels crossed the myometrium and entered endometrium/decidua. Periodic Acid Schiff's reactive uNK cells were absent from the mesentery and enriched in decidua basalis where they transcribed NCAM-1 and associated with non-innervated segments of the uterine arteries, including spiral arteries. These data suggest that the localization and activation of mature uNK cells are unlikely to be neurotransmitter regulated.

Key words: Uterine NK cell, decidua, spiral artery, tyrosine hydroxylase, neurofilament

INTRODUCTION

Uterine Natural Killer (uNK) cells are transient endometrial cells that become the dominant lymphocytes within early implantation sites of many species. Mechanisms regulating their recruitment to and localization within implantation sites are unknown. In humans and rodents, uNK cells appear during endometrial decidualization and are strongly associated with decidual vessels (Peel, 1989; Stewart, 1998; Chantakru *et al*., 2001; Trundley and Moffett, 2004; Bulmer and Lash, 2005). The hallmark of human uNK cells is their intense expression of CD56, the neural cell adhesion molecule (NCAM-1). Most blood NK cells are $CD16^{+}$ (FcγRIII), $CD56^{dim}$. It has not been established if mouse uNK cells express NCAM-1 but, during differentiation from immature, agranular to heavily granulated cells, mouse uNK cells gain expression of asialoGM1, a brain ganglioside (Parr *et al*., 1990). Since CD56 binds homotypically (Lanier *et al*., 1991), we postulated that interactions between uNK cells and nerve fibers might regulate positioning or activation of uNK cells within the endometrium.

In mice, self-renewing progenitors of uNK (pro-uNK) cells do not reside *in utero* but home from primary and secondary lymphoid organs (Chantakru *et al*., 2002). Homed progenitors proliferate, differentiate and become activated within highly restricted areas of the mesometrial endometrium and uterine wall that surround the major blood vessels of each implant site. This mural region known variously as the mesometrial lymphoid aggregate of pregnancy (MLAp), metrial gland (MG) or mesometrial decidua, is enriched for smaller, less heavily granulated, less mature uNK cells (Peel, 1989; Paffaro *et al*., 2003). Murine uNK cells require progesterone and Interleukin-15 to mature (Peel, 1989; Ashkar *et al*., 2003) and during this process they acquire glycoprotein-rich cytoplasmic

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granules that react histochemically with Periodic Acid-Schiff's (PAS) reagent (Chantakru *et al*., 2001). Murine uNK cells, through interferon (IFN)-γ production, initiate physiological changes in the structure of the endometrial spiral arteries permitting their dilation and gain in capacity to transport blood to the placenta (Ashkar *et al*., 2000).

Like mouse uNK cells, human uNK cells produce IFN-γ and have potent angiogenic actions (Hanna *et al*., 2006). Human uNK cells are postulated to have important functions in implantation and in regulation of trophoblast invasion and may be dysregulated in miscarriage and pre-eclampsia. Although neurotransmitters alter the rate of migration and activation of human peripheral blood NK cells in culture (Lang *et al*., 2003), no studies have asked if uNK cell distribution or activation has a neuronal component. Nerves accompany uterine artery branches in the mesometrium. We asked if RNA from purified C57Bl/6J uNK cells contained NCAM-1 transcripts using RT-PCR and if PAS-reactive uNK cells co-distribute with immuno-reactive fibers from the uterine nerves in serial histological sections of implantation sites from these mice.

MATERIALS AND METHODS

Animals: Adult male and female C57Bl/6J (B6) mice were purchased from the Jackson Laboratory, Bar Harbor ME and paired for timed matings. The day of copulation plug detection was called gd 0.5. For histology, conducted at Queen's University, two pregnant mice were studied at gd 6.5, 8.5, 10.5 and 12.5 using 3 healthy implantation sites/dam. Uteri from two virgin females were also investigated. RNA prepared in other studies from dissecte decidua basalis or from highly purified gd 12 B6 uNK cells harvested from dissociated decidua basalis using magnetic beads coated with *Dolichos biflorus* lectin, a ligand specific for uNK cells (Borzychowski *et al*., 2003), was also evaluated at the University of Guelph. All animal handling procedures were conducted under animal utilization protocols approved at the appropriate institution.

Gene expression studies: To address expression of NCAM-1 by mouse uNK cells, 50 μg cDNA (Borzychowski *et al*., 2003) and 0.5 U Red Taq (Sigma, St. Louis MO) were used for amplification (94°C, 5 min; 32 cycles of 94°C, 30sec; 58°C, 30 sec; 72°C, 30 sec; 7 min at 72°C. Products from three independent experiments were electrophoresed in 1% agarose gel stained with ethidium bromide. NK1.1 (Klrb1c), was used as a positive NK cell amplification control. Primers were NCAM-1 f-ACGTCCGGTTCATAGTCCTG; r-CACACACCAGGGTGACAGAC; giving a 219 bp product and NK1.1 (Klrb1c) f-TGACCCTGATTGGGATGAGT; r-TTGAATGAGCAGCAAAGTGG; giving a 224 bp product. Negative controls lacked cDNA.

Histological studies: For histology, mice were anaesthetized and perfused (200 mL 4% paraformaldehyde (PFA) in 0.2 M phosphate buffer saline (PBS)). Uteri were removed, immersed overnight in the same fixative (20°C), transferred to 30% sucrose in 0.1 M PBS for 72 h (20°C), then transacted between implant sites, mounted in OCT compound (Fisher Scientific, Ottawa, ON), frozen using Methylbutane cooled by dry ice and stored at -80°C until cut as 10 μm cryostat serial sections. Primary antibodies were rabbit anti-neurofilament 150 kD (Chemicon International, Temecula, CA; AB1981; 1/2000 in 3% BSA in 0.25% Tris buffer), a pan neuronal marker and 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 (Walsh and Kawaja, 1998) and no counterstains. UNK cells were stained in adjacent frozen sections using a standard PAS reaction followed by counterstaining with methylene blue.

RESULTS

To evaluate whether murine uNK cells are capable of NCAM-1 transcription, RT-PCR was employed. Transcription of NCAM-1 and NK1.1(Klrb1c), a positive control transcript for NK cell identification, was demonstrated (Fig. 1A).

Antibodies identified nerve fibers in all sections of virgin and pregnant uteri. Those localized by the pan 150 kD neurofilament marker (Fig. 1B-D) appeared to be the same fibers identified by the antibody to tyrosine hydroxylase (Fig. 1E and F). The nerves were embedded in the walls of uterine artery branches located in the mesometrium. As the vessels crossed the myometrium, reactive fibers were noticeably reduced within vessel cross-sections. No reactive nerve fibers were present in decidualized endometrium. PAS reactive uNK cells were present in decidua basalis and many associated with non-innervated segments of uterine artery branches (Fig. 1G). 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.

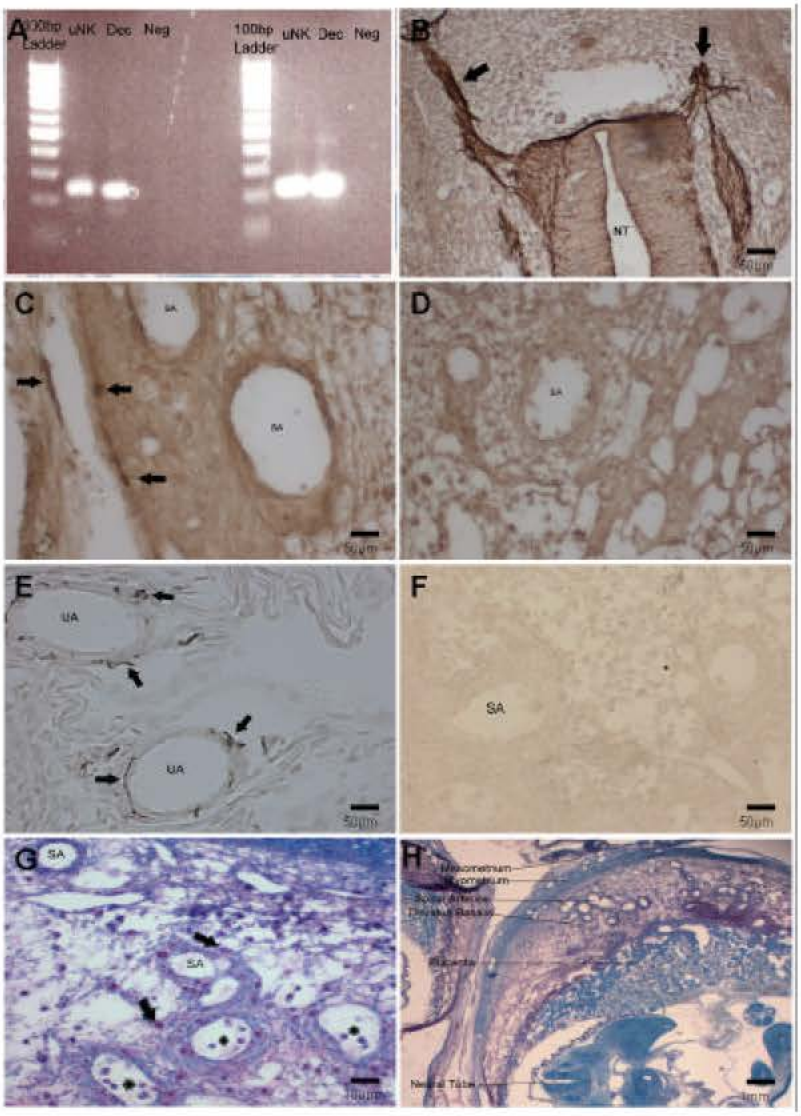

Fig. 1: (A) illustrates the electrophoresis of amplicons from gd12 B6 uNK cells and decidua basalis (Dec) probed for expression of /Ncam-1* */(Left panel) and /NK1.1/ (/Klrb1c/ Right panel). Neg indicates the negative water amplified control. Photomicrographs (B-H) represent sagittal cryostat sections of gd10 B6 implantation sites. (B, C, D) illustrate sections stained with anti-neurofilament 150 kD; (E) and (F) illustrate different regions of one sagittal section stained with a second neurofilament marker, anti-tyrosine hydroxylase while (G) and (H) are stained histochemically with Periodic Acid Schiff's (PAS) reagent. (B) illustrates the neural tube (NT) of the developing fetal central nervous system and peripheral nerve development (arrow). This tissue was used as the positive immune specificity control. (C) illustrates the mesentery (left edge), and myometrium at the MLAp, where cross sections of spiral arteries (SA) are seen. Immunoreactive nerve fibers (arrows) are present in walls of vessels in the mesentery and at the ablumenal surface of the myometrium. An edge of the decidua basalis is to the right side of this image. (D) shows the central decidua basalis where coils of the spiral arteries (SA) are also found, cut in cross section. No anti-neurofilament 150 kD reactivity was present in decidua. (E) 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). (F) shows the central decidua basalis of the same tissue section where no immunoreactivity to anti-tyrosine hydroxylase was found. The spiral artery (SA) in (F) is a branch from the immunoreactive uterine artery imaged in (E). (G) illustrates the numerous PAS-reactive uNK cells (example illustrated by arrow) in the central decidua basalis, the region imaged in (D) and (F). 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. (H) illustrates the mesometrial aspect of a sagittal section of a gd12 implantation site at low magnification for orientation. The neural tube of the fetus is indicated as is the placenta, the central decidua basalis, the spiral arteries, myometrium and mesometrium (broad ligament or uterine mesentery). (PAS counterstained with methylene blue)

DISCUSSION

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

Uterus arises embryologically from paramesonephric ducts and is described as receiving mixed sympathetic and parasympathetic innervation (Dyce *et al*., 1996). In adult rats and mice, dynamic, ovarian hormone-related changes are reported in location, distribution and density of nerve bundles in during the estrous cycle, pregnancy and parturition. Most uterine nerve bundles are sympathetic and associated with arteries. In rats, the highest nerve fiber density is reported in the mesometrial triangle of the caudal uterus (Houdeau *et al*., 2003; Haase *et al*., 1997; Zoubina and Smith, 2000, 2001). None of these authors comment on the distribution of lymphocytes, particularly the abundant uNK cells that require special stains for easy identification. We used two markers of nerve bundles to study normal murine implant sites and localized the fibers to arterial walls within the mesentery and myometrium, as previously reported. Nerve fibers were absent from decidua basalis and 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 neurotransmitter regulated. An alternate interpretation would be that uNK cells are repelled by signals from the nerve fibers reaching the myometrium. This appears unlikely as it would require long-range neurotransmitter function extending over more than 100 μm.

Mouse uNK cell progenitors are thought to home from blood (Chantakru *et al*., 2002). These cells would flow through innervated mesometrial arteries and, if marginating, could experience concentration-dependent neurotransmitter regulation that might contribute egress from the vessel into the uterus. The sympathetic mediator norepinephrine blocks target cell lysis by human NK and IL-2 stimulated lymphocytes but promotes migration (Lang *et al*., 2003). Immature, proliferative uNK cells are clustered in the MLAp, a transient structure in the uterine wall separating 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, while common in decidua basalis, are extremely rare in MLAp (Chantakru *et al*., 2001), making it possible that pro-, pre- or immature stages of uNK cells, like the decidual stages, do not experience neurotransmitter regulation. Alternatively, current techniques may not adequately identify the earliest cell stages committed to uNK cell differentiation and intravascular neurotransmitter action on these cells may promote their terminal differentiation and activation. It is also possible that uNK cells effect 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 and of repulsive positioning 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 are guided and regulated by the release of vascular endothelial cell growth factor (VEGF) from nerves (Carmeliet and Tessier-Lavigne, 2005). Because neuron marker-expessing uNK cells are abundant producers of VEGF (Wang *et al*., 2003; Tayade *et al*., 2006; Li *et al*., 2001; Hanna *et al*., 2006) in an environment devoid of nerves, uNK cells may assume some of the functions provided in other tissues by nerves. This could include guidance for arterial growth and branching (Tayade *et al*., 2006). UNK cells are mobile and express receptors that recognize trophoblast-expressed molecules. Thus, uNK cells would be able to provide rapid and flexible guidance of maternal vascular development towards each implantation site. Speed and flexibility in the pattern of endometrial angiogenesis are particularly important for support of each placenta in litter bearing species. Absence of nerve fibers from implantation sites suggests that the mechanisms promoting angiogenesis in other organs are not sufficient for reproductive and/or evolutionary processes.

ACKNOWLEDGMENTS

We thank Dr. M. Kawaja for helpful discussions and Ms. V. Norkum for technical assistance. This work was supported by NSERC and Zanjan University of Medical Sciences.

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