Uterine natural killer cells: insights into their cellular and molecular biology from mouse modelling

B. Anne Croy¹, Hong He¹, Souad Esaedeg¹, Qingxia Wei², Daniel McCartney¹, Jianhong Zhang¹, Angela Borzychowski¹, Ali A. Ashkar³, Gordon P. Black¹, Sharon S. Evans⁴, Sirirak Chantakru⁵, Marianne van den Heuvel¹, Valdemar A. Paffaro, Jr⁶ and Aureo T. Yamada⁶

¹Department of Biomedical Sciences, Ontario Veterinary College, University of Guelph, Guelph, ON, N1G 2W1, Canada; ²Arthur and Sonia Labatt Brain Tumor Research Centre, Hospital for Sick Children, 555 University Avenue, Toronto, ON, M5G 1X8, Canada; ³Department of Pathology and Molecular Therapeutics, McMaster University, 1200 Main Street W., Hamilton, ON, L8N 3Z5, Canada; ⁴Department of Immunology, Roswell Park Cancer Institute, Elm at Carlton Streets, Buffalo, NY 14623, USA; ⁵Department of Anatomy, Kasetsart University, 50 Phahonyothin Road, Chatucak BangKhen, Bangkok, 10900, Thailand; and ⁶Department of Histology and Embryology, Institute for Biology, UNICAMP, Campinas, 13083-970, S.P. Brazil

In primates, including women, and in rodents, natural killer lymphocytes (NK cells) have a unique relationship with the decidualizing uterus. Implantation sites from genetically modified and transplanted mice have proven useful models for understanding potential mechanisms involved in the recruitment, activation and functions of human CD56brighuterine (u)NK cells. Key findings are reviewed in this article. In mice, uNK precursor cells are recruited from secondary lymphoid tissues and are activated coincident with their uterine arrival. uNK cells proliferate, produce cytokines (interferon gamma (IFN-γ) and interleukin 18 (IL-18) and IL-27), and terminally differentiate into granulated lymphocytes. Many uNK cells proliferate within the myometrium at each implantation site forming a structure, the mesometrial lymphoid aggregate of pregnancy (MLAp) that surrounds blood vessels servicing each placenta. Post-mitotic uNK cells are abundant within decidua basalis; frequently (> 25%) associating with spiral arteries, intramurally and intraluminally. From mid-gestation, numbers of uNK cells decline. Studies of implantation sites in mice lacking uNK cells, IFN-γ, components of IFN-γ-induction and -signalling pathways or IFN-γ-regulated genes indicate that uNK cell-derived IFN-γ is essential in triggering pregnancy-induced spiral artery modification. Decidual maintenance and uNK cell death are additional effects of uNK cell-derived IFN-γ. Thus, during the first half of gestation, uNK cells contribute to and sustain important changes in the maternal placental bed.

During uterine decidualization in women, non-human primates and rodents, lymphocytes of the natural killer (NK) lineage appear and become abundant as large, granulated cells (Fig. 1; Parr et al., 1987; Peel, 1989; Moffett-King, 2002). NK cells differ from T and B lymphocytes because they lack somatically rearranged antigen-sensing receptors (Natarajan et al., 2002). NK cells contribute to innate immunity, participating in early immune protection, before clonal expansion of B and T lymphocytes. Functions of NK cells are lysis and cytokine production, with individual cells having single or dual capacity. Lysis is directed against virally infected cells and tumour cells. Interferon-gamma (IFN-γ), which restricts viral infection, is a major cytokine product (Trinchieri, 1995). Uterine (u)NK cells are predominantly activated, cytokine-producing NK cells (Moffett-King, 2002). Fifteen years lapsed between NK cell identification in marrow, blood and spleen and recognition of endometrial granulocytes (human designation) and

Email: acroy@uoguelph.ca

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granulated metrial gland cells (murine designation) as NK cells.

uNK cells share many, but not all, of their features with peripheral NK cells. For example in humans, most blood NK cells analysed by flow cytometry express the surface marker CD16, an immunoglobulin domain receptor, and have dim expression of CD56, an adhesion molecule. About 1% of circulating lymphocytes are CD16⁺, CD56bright NK cells and these co-express high amounts of the vascular addressin L-selectin (Campbell et al., 2001). In contrast, most human uNK cells express CD56 brightly but lack CD16 and L-selectin (Searle et al., 1999). Lymphotoxin β receptor (LTBR) is a murine example. LTBR is essential for peripheral NK cell differentiation and secondary lymphoid tissue formation (Fu and Chaplin, 1999). However, uNK cells differentiate and an architecturally correct mesometrial lymphoid aggregate of pregnancy (MLAp) forms in pregnant LTBR⁻/⁻ mice (Kather et al., 2003). Such findings limit the usefulness of studying NK cells not isolated from or functioning within the uterus and make the unique features of uNK cell recruitment, activation and differentiation key questions. It is of critical importance to recognize that these processes occur without pregnancy or fetal trophoblast tissue. In women, uNK cells differentiate in every menstrual cycle, 3–5 days after the LH surge (Bulmer et al., 1987; King, 2000). In rodents, induction of artificial deciduomata induces fully mature uNK cells (Peel, 1989).

The question of uNK cell function during pregnancy is also of tremendous interest. The cells are highly mobile and their high content of lytic molecules plus partnering antigen receptor display makes them potentially dangerous to trophoblast cells in implanting primate blastocysts and in developing placentae (primates and rodents). uNK cells could play essential physiological roles limiting normal trophoblast invasion or could destroy trophoblast, leading to pregnancy loss. Full definition of uNK cell antigen recognition on trophoblast and the intracellular signalling involved in uNK cell activation, differentiation and senescence would resolve such functional debates and provide clinically relevant.
Table 1. Summary of histological findings in uterine natural killer (uNK) cell-deficient mice with or without bone marrow transplantation

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Name</th>
<th>Manipulations</th>
<th>Histological findings</th>
<th>Strain</th>
<th>DB</th>
<th>SA</th>
<th>MLAp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>C57Bl/6J, 129J, Balb/cJ, CD1</td>
<td>–</td>
<td>+ Cellular</td>
<td>+ Cellular</td>
<td>+</td>
<td>Modified</td>
<td>Present</td>
</tr>
<tr>
<td>T ± B deficient</td>
<td>Nude, SCID</td>
<td>–</td>
<td>+ Cellular</td>
<td>+ Cellular</td>
<td>+</td>
<td>Modified</td>
<td>Present</td>
</tr>
<tr>
<td>NK/uNK, T ± B deficient</td>
<td>IL-2/15Rβγc−/−, IL-2/13Rγc−/− (yc), IL-15c−/−, Tg c−/−, RAG-2c−/−/yc−/−</td>
<td>–</td>
<td>– Hypocellular</td>
<td>Unmodified Absent</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bone marrow graft</td>
<td>into uNK deficient</td>
<td>RAG-2c−/−/yc−/− + SCID BM + C57Bl/6J BM + IL-15c−/− BM + βERKO BM + αERKO BM</td>
<td>+ Cellular</td>
<td>Modified</td>
<td>Present</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

BM: bone marrow; DB: decidua basalis; ERKO: oestrogen receptor knockout mouse; MLAp: mesometrial lymphoid aggregate of mouse pregnancy; RAG-2c−/−/yc−/−: double knockout mouse for recombinase activating gene-2/common cytokine chain gamma; SA: spiral artery of the decidua basalis; SCID: severe combined immunodeficient.

Information. Mice have proven excellent models for characterization of uNK cell biology by allowing manipulative studies that are not possible in pregnant women (Table 1). Strains of genetically mutant mice are available to address uNK cell regulation and studies are relatively short in duration (mouse pregnancy is 19–20 days). Furthermore, murine pregnancies can be interrupted and entire implantation sites studied. Inbred strains permit investigations of replicate pregnancies in time-course analyses that precisely define the changing cell and tissue relationships during gestation (Fig. 1). Murine uNK cell analyses are usually histological or molecular because no successful culture conditions or long-term cell lines are known. In the present study, at least six implantation sites derived from two or three pregnant females on at least two different days of gestation were serially sectioned. From each implant site eleven central sections were selected for computer-based image analysis in a manner ensuring that no duplicate counting of individual cells occurred. Control pregnancies were matched by genetic background as closely as possible, being sibling matings or matings of a congenic inbred partner strain matched for day of gestation. Study of human uNK cells occurs on more restricted times, largely those associated with biopsy or elective termination, and is genetically heterogeneous. In addition to histopathology, flow cytometry is widely used to study lymphocytes dissociated from human specimens and primary cloning has been achieved (Christmaset al., 1990). Application of expression microarrays and laser capture microdissection combined with quantitative analyses will provide information unique to human uNK cells and their environment, an environment distinct from that of the rodent anatomically, endocrinologically and in duration of gestation. The mouse will remain a key tool in critical assessment of new data from women because this model can move studies from correlative findings to genetically defined in vivo gestational data with potential for experimental interventions. uNK cells are infrequent or absent at term in both mice and women (Delgado et al., 1996; King, 2000), making term tissue irrelevant for defining uNK cell functions.

NK cell differentiation and establishing the uNK cell lineage

Origins

NK cells are generated from pluripotent bone marrow stem cells. Their differentiation in mice is highly dependent upon stromal factors including stem cell factor, interleukin 7 (IL-7) and IL-15 (Kennedy et al., 2000; Rosmaraki et al., 2001). Lineage-committed NK-precursor cells have limited self-renewal ability and are present in secondary lymphoid tissues (blood, spleen, lymph nodes (LN)) but not the uterus (Chantakru et al., 2002 and references therein). Transplants from NK-cell-sufficient mice to NK cell-deficient mice established that uNK precursors are present in fetal liver; fetal, neonatal and adult thymus; marrow, LN and spleen (Chantakru et al., 2002). Only spleen showed enhanced ability to generate uNK cells if harvested from pregnant donors (days 3–7 of gestation), whereas LN draining...
pregnant uteri were devoid of uNK precursors (Fig. 2). These observations strongly indicate that, before implantation, endocrine signals co-ordinately mobilize uNK precursors from spleen and induce mechanisms for trapping and retaining these circulating cells within the uterus (Chantakru et al., 2002).

**Ovarian hormone effects**

The present authors addressed functionally the postulated steroid hormone-regulated interactions between lymphocytes and endothelium by assaying human blood lymphocyte adhesion to high endothelial venules in cryostat sections of mouse subcutaneous LN pools (PLN). Lymphocytes from anonymous blood-bank donors were evaluated using PLN from virgin and pregnant mice. Endothelium from pregnant donors attracted more cells (Chantakru et al., 2002). Endothelium in PLN from ovariectomized mice given oestradiol or progesterone or both also gained adhesive function equalling pregnancy-induced gains. Antibody-blocking studies established that L-selectin and α4-integrin ligands mediated the functional changes. Adhesion assays were extended using mouse uterine tissues as substrate (virgin, pregnant or steroid hormone-treated) instead of PLN. Binding again occurred via L-selectin and α4-integrin-dependent mechanisms and numbers of cells adhering per mm² of uterus were increased if tissue came from pregnant or steroid hormone-treated (oestrogen, progesterone, or both) ovariectomized mice. Prelabeling the human lymphocytes with anti-CD56 revealed that gains in functional adhesion to decidua basalis (DB) occurred in both CD56⁺ and CD56⁻ cells but greatest enrichment occurred in CD56⁺ cells (大于70-fold compared with the starting blood-cell suspension; Chantakru et al., 2002). Gain in ability of endothelium to attract lymphocytes was not systemic, as no pregnancy- or steroid hormone-induced changes were observed in pancreatic endothelium (Chantakru, 2002; S. Chantakru, W-C Wang, B. A. Croy and S. S. Evans, unpublished).

Two studies were performed to determine whether hormones regulate lymphocytes independently but co-ordinately with endothelium. First, splenic lymphocyte suspensions from virgin, pregnant or ovariectomized mice treated with placebo, or oestradiol in the presence or absence of progesterone, were tested for adhesion to PLN from a single donor. Adhesion rates for splenocytes from virgin and ovariectomized, placebo-treated mice were similar and statistically lower than adhesion of splenocytes from pregnant or steroid hormone-treated donors, indicating that pregnancy, oestrogen and progesterone enhance the ability of murine lymphocytes to interact with endothelium (Chantakru, 2002; S. Chantakru, W-C Wang, B. A. Croy and S. S. Evans, unpublished). For the second study, blood samples were collected from women three times per week over their...
menstrual cycle and adhesion to constant mouse tissue was assessed. A dynamic, predictable pattern was found with significant gains in cells adhering to PLN and to decidualized uterus at the LH surge (M. van den Heuvel, S. Bashar, J. E. Lewis, X. Xie, J. Horrocks and B. A. Croy, unpublished). These data are consistent with a hypothesis of periovulatory mobilization of human uNK cell precursors to blood and their uterine appearance at LH+3–5 (King, 2000). Limited duration and importance of the luteal phase in mice may explain why different hormones appear to mediate the pregnancy-associated gains in lymphocyte–endothelial interactions in women and mice. Whether these dynamic changes reflect important steps in vivo in recruitment of uNK precursors to human uterine remains to be addressed experimentally. In an in vivo model of fever-range hyperthermia, doubling in vitro adhesion correlated with quadrupled lymphocyte trafficking to the targeted site (Evans et al., 2001).

IL-15

IL-15, a stromal cell and macrophage-derived cytokine, is critical for NK cell differentiation in human and murine lymphoid tissue (Carson et al., 1994; Kennedy et al., 2000). IL-15 acts on precursor and immature NK cells but not during NK progenitor cell differentiation. Human and murine endometria express IL-15 (Ye et al., 1996; Kitaya et al., 2000; Okada et al., 2000; Dunn et al., 2002). In mice, IL-15 mRNA is transcribed between day 6 and day 11 of gestation (Ye et al., 1996; A. A. Ashkar, G. P. Black, Q. Wei, J. R. Head and B. A. Croy, unpublished). In humans, IL-15 transcription is more abundant during the secretory than during the proliferative cycle phase and is sustained in early pregnancy with localization to endothelium and perivascular stromal cells of decidual spiral arteries (SA; Kitaya et al., 2000). These correlative time-course data strongly indicate that IL-15 participates in uNK cell differentiation, whereas the localization data indicate that IL-15 may contribute to chemotactic localization of uNK cells within the lumina and walls of the spiral arteries.

IL-15 shares two of its three receptor chains (β and γ) with IL-2, a cytokine not normally found in gestational uteri. Mice genetically ablated for either shared receptor or for IL-15 do not differentiate uNK cells (Guimond et al., 1998; Croy et al., 2003). Reconstitution and blocking bone marrow transplantation experiments were conducted to establish that IL-15 contributes to terminal uNK cell differentiation. The reconstitution experiment involved transplants between two strains that do not differentiate uNK cells. Ailymphoid mice (RAG-2−/−/common cytokine receptor chain γ (γc)−/−, a double knockout strain that makes IL-15) were grafted with bone marrow from IL-15+/− mice, then mated and studied histologically. uNK cell differentiation occurred at frequencies identical to those observed in normal congenic C57Bl/6J mice. For the blocking experiment, C57Bl/6J bone marrow was given to IL-15−/− mice. This bone marrow produces normal uNK cells when grafted into alymphoid mice (Ashkar et al., 2000), but was totally blocked from differentiation in uteri of IL-15−/− recipients (Croy et al., 2003; A. A. Ashkar, G. P. Black, Q. Wei, J. R. Head and B. A. Croy, unpublished). These experiments indicate that IL-15 is the crucial factor regulating uNK cell differentiation and provoke questions concerning the regulation of uterine IL-15 and whether its deficiency or over-abundance has clinical consequences in women, as suggested by Chegini et al. (2002) for women who experience recurrent spontaneous abortions. Interferon regulatory factor (IRF)-1, a key regulator of IL-15 in marrow, is expressed in the uterus in humans and mice (Jabbour et al., 1999; Kitaya et al., 2001; A. A. Ashkar, G. P. Black, Q. Wei, J. R. Head and B. A. Croy, unpublished) but does not appear to regulate murine uterine IL-15 (Ashkar and Croy, 1999; A. A. Ashkar, G. P. Black, Q. Wei, J. R. Head and B. A. Croy, unpublished). In humans, uterine IRF-1 appears to be regulated by prolactin (Jabbour et al., 1999), whereas uterine IL-15 expression appears to be regulated by progesterone (Okada et al., 2000; Kitaya et al., 2000) and prostaglandins (Dunn et al., 2002). Additional information on uterine specific regulation of IL-15 is needed.

Clearly, ovarian steroid hormones act on uterine stroma, including endothelium, as discussed above, in ways that promote differentiation of uNK cells, but whether the hormones act directly on the lymphocytes or their precursors is less clear. Availability of mice ablated for the oestrogen receptor (ER) α or β (αERKO and βERKO) (Couse and Korach, 1999) permitted functional assessment of ER utilization by uNK cells. Marrow transplanted from αERKO and βERKO to alymphoid mice fully reconstituted uNK cells, indicating no direct functional action in mouse uNK cells through oestrogen receptors. This conclusion was supported by RT–PCR analysis of highly purified (99%) uNK cells at days 12 and 13 of gestation, isolated rapidly from perfused, normal (C57Bl/6) mice using a newly reported protocol involving magnetic beads coated with the lectin Dolichos biflorus agglutinin (DBA) (Borzyszkowski et al., 2003; Paffaro et al., 2003). mRNA for ERα and ERβ were absent, whereas mRNA for other genes was detected (Borzyszkowski et al., 2003). Human uNK cells have recently been reported to be ERα negative, as in earlier studies, but to express ERβ (Henderson et al., 2003), indicating species difference or transcripts from rare contaminating cells. In both humans and mice, studies of the complex interactions between the endocrine and stromal environments on lymphocyte differentiation within the uterus will be profitable and of clinical importance for endometrial as well as reproductive health.
NK cell and uNK cell activation

Lymphocyte activation requires signals additional to those involved in lymphocyte differentiation. IL-12 is a cytokine important for induction of IFN-γ synthesis in NK and T cells (Trinchieri, 1995). The action of IL-12 is enhanced by IL-18 (Nakanishi et al., 2001). IL-12 and IL-18 are found in normal human and mouse implantation sites (Devergne et al., 2001; Yoshino et al., 2001; Zourbas et al., 2001; Chaouat et al., 2002; Zhang et al., 2003). IL-18 shows a dynamic protein expression pattern by immunohistology, appearing first in decidual stromal cells at days 4–6 of gestation, then exclusively from day 8 to day 14 of gestation in uNK cells (Chaouat et al., 2002; Zhang et al., 2003). Pregnant mice deficient in both IL-12 and IL-18 (Takeda et al., 1998) have been compared with littermates deficient in only one or neither cytokine (Zhang et al., 2003). Females and males matched in genotype were mated. uNK cell differentiation was morphologically and numerically similar in the four strains. In the three cytokine-deficient strains, mid-gestation IFN-γ (after day 8 of gestation) was somewhat reduced and SA dilation was impaired but not as severely as in IFN-γ<sup>−/−</sup> implantation sites. This finding indicated that additional cytokines contribute to uNK cell activation. Two recently described cytokines, IL-23 and IL-27, are related to IL-12 and contribute to IFN-γ induction (Parham et al., 2002; Pfanz et al., 2002 and references therein). IL-23 and IL-27 expression is absent from virgin mesometrial uterus and mesometrial uterus at day 3 of gestation, but present in mesometrial decidua from day 4 of gestation (Zhang et al., 2003). Analyses of mRNA, from DBA lectin-purified uNK cells, indicate that stromal cells produce IL-23, whereas uNK cells produce IL-27. The time-course patterns for induction of these cytokines match well with putative roles for these cytokines in uterine IFN-γ regulation in normal mice because IFN-γ protein is absent mesometrially from virgin and pre-implantation uteri at day 3 of gestation but is detected from day 6 of gestation by ELISA. Peak mesometrial IFN-γ occurs at day 10 of gestation and then declines (Ashkar et al., 2000).

NK cells interact with other cells via surface receptors that are classified as activating or inhibitory, depending on whether lytic activity is displayed when the receptor is engaged in in vitro assays. Individual cells display multiple receptors to give target recognition specificity. The ligands initially defined for NK cell receptors were classic major histocompatibility complex (MHC) class I molecules or their structural relatives (Cerwenka and Lanier, 2001; Natarajan et al., 2002). Because the pathway resulting in terminal uNK cell differentiation in primates and rodents is triggered in the absence of conception, the role of non-self antigen recognition during uNK cell activation is unclear. Trophoblast has restricted transplantation antigen expression that, in outbred matings, will include paternally derived, non-self antigens (Moffett-King, 2002). The human uNK cell population expresses a full repertoire of immunoglobulin-like NK cell receptors, although in different proportions than expressed by blood NK cells (Hiby et al., 1997). The major NK cell receptors in mice are lectin-like LY49 gene family members. uNK cells from C57Bl/6 mice express all of the LY49 NK receptors typical of the strain (V. A. Paffaro, Jr, H. He and B. A. Croy, unpublished). This sharing of receptors between blood NK and uNK cells indicates that uNK cell activation will display many features in common with NK cell activation in other tissues and predicts that the unique properties of uNK cells are not defined by these receptors.

A second type of NK cell activation receptor in mice and humans is NKG2D. The Rae-1 family of five retinoic acid-induced oncodevelopmental antigens was recently found to engage murine NKG2D, whereas the homologous human ligands are products of the HLA-associated genes MICA and MICB (Cerwenka and Lanier, 2001). Interactions between murine NK cells and Rae-1 genes are normally studied in vitro using embryonic cell-line targets. In vivo expression of Rae-1 is reported only in mid-gestation developing mouse brain (Nomura et al., 1996). The present authors addressed transcription of Rae 1-α-β-γ-δ-ε- in uteri from C57Bl/6 and 129/J strains by RT–PCR. Non-pregnant uteri expressed no Rae-1 signal but strain appropriate Rae-1 expression was induced by day 6 of gestation in DB and sustained at day 10 of gestation in DB, MLAp and placenta. Immunohistochemistry using a pan Rae-1 polyclonal antibody showed that extra-fetal expression was most strongly localized to trophoblast (B. A. Croy and T. Seya, unpublished). These and other developmental antigens expressed by trophoblast may be of major importance in sustaining uNK cell activation when trophoblasts and NK cells begin to intermingle (in mice from about day 8 of gestation). Roles for oncodevelopmental antigens are not yet explored in humans.

Functions of uNK cells

uNK-deficient and IFN-γ signal disrupted mice

Several mouse strains have major (> 99%) or absolute deficits in NK cells. Five strains, each having different gene deletions, are uNK cell-deficient and have common histopathology (Table 1; Guimond et al., 1998; Ashkar et al., 2000). Three anomalies accompany absence of uNK cells: absence of myometrial MLAp development (that is, a decidual bed deficit), hypocellularity and oedema of DB and persistence of vascular smooth muscle in the SA with limited lumen dilation and vessel lengthening at mid-gestation. There is no overgrowth of trophoblast and no consistent quantifiable impairment in fetal or postnatal health (Greenwood et al., 2000). Issues such as maternal vascular or fetal gene expression, fetal hypoxia, brain development, ventricular hypertrophy,
maturity onset diabetes and others have not been addressed experimentally between uNK-deficient and uNK cell-sufficient gestations. These findings imply that the normal functions of uNK cells during pregnancy are to congregate lymphocytes myometrially at the portals of uterine arteries and veins, to interact with and provide growth support for stromal cells committed to decidual differentiation, and to trigger events that culminate in normal, pregnancy-induced SA modification. These interpretations were confirmed by normalizing implantation sites in NK/uNK cell-deficient mice by bone marrow transplants from severe combined immunodeficient (SCID) (T-B-) mice (Guimond et al., 1998). Effector mechanisms were addressed by transplanting NK/uNK cell-deficient females with bone marrow deleted for IFN-γ, IFN-γRs or their downstream signalling molecule Stat-1. Implantation sites in the recipients revealed that IFN-γ is not required to initiate uNK cell differentiation but low concentrations are required for full maturation and senescence of uNK cells. Terminal uNK cell maturation required about 1 μg per implantation site, which is adequately provided by other types of cell (Ashkar et al., 2000). uNK cell-derived IFN-γ provides higher tissue contents (> 6 μg per implantation site) and these contents support decidual integrity and SA modification. Daily infusions of mriFN-γ (100–3000 μg per treatment) into alymphoid females at days 6–11 of gestation were effective in induction of normal SA modification and decidual maintenance in the absence of uNK cells (Ashkar et al., 2000). Thus, pro-inflammatory cytokines act physiologically in normal pregnancy.

The α2-macroglobulin gene family, possible targets of uNK-cell derived IFN-γ

As the present study indicated that IFN-γ acts on gene expression in a complex tissue that includes lymphocytes, stromal, vascular smooth muscle, endothelial and decidual cells, a cDNA microarray (GEM®, Incyte, St Louis, MO) was undertaken. Maternal mesometria from C57Bl/6j mice at day 6 to day 10 of gestation were compared. Forty-three genes, documented among the hundreds regulated by IFN-γ, were present on the array and differentially expressed (B. A. Croy, unpublished). Among these IFN-γ-regulated genes were mouse α2-macroglobulin (MAM) and its receptor. A related EST777415, defined as α2-macroglobulin (α2M) precursor-like, was among the most differentially upregulated genes. The array results were extended by northern blot analyses. Non-decidualized mouse uterus (virgin and at day 3 of gestation) did not express EST777415 but expression was induced by day 6 of gestation, peaked at days 10 and 12 of gestation and then declined by day 14 of gestation (H. He, D. McCartney, Q. Wei, S. Esadeg, J. Zhang, A. Hayes, F. van Leuven and B. A. Croy, unpublished). Members of the α2M family are abundant plasma proteins that regulate the bioavailability of proteases and cytokines in tissue (Borth, 1994). The genes map closely in mice to the NK cell LY49 receptor gene complex on chromosome 6 (Ensembl, Mouse Genome Browser BLASTView).

Potential roles of MAM and its related, expressed family member murinoglobulin-1 (MUG-1) in implantation sites were addressed by undertaking histological analyses using MAM™/MUG-1™ mice (Umans et al., 1999). Placental development was highly unusual; the labyrinth was reduced whereas giant cells were in excess (Fig. 3). In addition, intramural trophoblast invasion of the SA was aggressive and by mid-gestation reached the myometrial circular smooth muscle layer, a position normally achieved in very late gestation. uNK cells were numerous and SA, although cuffed by trophoblast, were dilated. This indicated that members of the α2M gene family regulate the rate at which trophoblast invades and led the authors to characterize EST777415, which remains expressed in decidua of MAM™/MUG-1™ (Esadeg, 2002).

A full-length cDNA, now designated α2M of mouse pregnancy (A2Mp) was obtained from EST777415 (Genbank accession no. AY185125). The predicted amino acid sequence is typical of members of the α2M family with bait, thiol ester and receptor-binding domains. The bait region is most distinctive. The predicted A2Mp amino acid sequence is closest to rat α2M, an acute phase protein (90%), and 71% and 63% matched to the non-acute phase reactants human α2M and pregnancy zone protein, a molecule of undefined function, which is highly upregulated during human gestation. Homology to the four other α2M mouse family members is at 50% (H. He, D. McCartney, Q. Wei, S. Esadeg, J. Zhang, A. Hayes, F. van Leuven and B. A. Croy, unpublished). Unlike other members of the mouse gene family, A2Mp is not detected in liver by RT–PCR or by in situ hybridization. Rather, testes and ovaries constitutively transcribe A2Mp; uterus transcribes A2Mp only with decidualization and the gene is transcribed in lactating mammary gland. In situ hybridizations (Fig. 4) localized A2Mp to primary spermatocytes and Sertoli cells in testes and to granulosa cells of secondary and pre-ovulatory ovarian follicles, in patterns distinct from those reported for MAM (Zhu et al., 1994; Dajee et al., 1998). Expression in 24 h postpartum, lactating mammary tissue was localized to alveolar epithelium (H. He, D. McCartney, Q. Wei, S. Esadeg, J. Zhang, A. Hayes, F. van Leuven and B. A. Croy, unpublished). Uterine localization was dynamic. At day 6 of gestation, A2Mp was expressed by anti-mesometrial decidua. Signal then migrated mesometrially and by days 8–14 of gestation was in vascular smooth muscle of SA and unmodified fibroblasts interfaced between mesometrial myometrium and decidua (full description of these experiments will be published elsewhere). One interpretation of these findings is that A2Mp binds molecules needed for cells
Fig. 3. Photomicrographs of implantation sites at day 10 of gestation in mice with different genetic mutations. (a) An implantation site in IL-12°/IL-18°. These mice have normal uterine natural killer (uNK) cell differentiation and mesometrial lymphoid aggregate of pregnancy (MLAp) (\( ) \) and placental (P) development but lower than normal levels of interferon gamma (IFN-\( \gamma \)) at mid-gestation and only limited modification of the spiral arteries (SA). Intravascular (black arrowhead) and perivascular (white arrowhead) localization of uNK cells is seen in the decidua basalis of these mice (insert). (b) An implantation site in IFN-\( \gamma \)° with over-development of uNK cells and the MLAp (\( ) \). uNK cells are unusually localized in decidua basalis close to the placenta (P), leaving a band of decidua relatively void of uNK cells (\( * \)). Upper insert shows this region at day 10 of gestation with healthy decidual cells (white arrow) clustered with darker, *Dolichos biflorus* agglutinin (DBA)-lectin stained uNK cells (arrowhead). Lower insert is of the same region at day 12 of gestation, when the decidua basalis has become necrotic (Ashkar et al., 2000). Decidual cells have been destroyed and amorphous material fills the spaces between cells. Small mononuclear cells and fibroblasts (black arrow) replace the decidual cell population. uNK cells (arrowhead) appear less granulated at this time point (day 12 of gestation) than in normal mice. No spiral artery modification occurs in IFN-\( \gamma \)° pregnancy. (c,d) Implantation sites in MAM°/MUG-1° with relative overgrowth of trophoblast particularly along the spiral artery (P: placenta); (d) is a higher magnification (\( \times 1000 \)) of the boxed area in (c) on a serial section stained with anti-cytokeratin antibody and illustrates intramural trophoblast cells. Erythrocytes (RBC) are present in the spiral artery lumen and endothelial cells (E) were always present between trophoblasts (arrow) and circulating cells in this segment of the vessel. Scale bars represent (a,b) 200 \( \mu \)m, (c) 400 \( \mu \)m and (d) 20 \( \mu \)m. (a,b) Stained with DBA lectin and haematoxylin; (c) stained with haematoxylin and eosin; and (d) counter-stained with haematoxylin.

in reproductive tissues to transition from quiescence to active differentiation.

The present authors reasoned that if the A2Mp gene-derived product was the molecule of key importance induced by uNK cell-derived IFN-\( \gamma \), administration of A2Mp to pregnant IFN-\( \gamma \)° mice would induce SA modification. As no gene product is yet available but homology is significant to human \( \alpha_2 \)M, human plasma-derived \( \alpha_2 \)M or PBS was infused from days 6–11 of gestation into pregnant IFN-\( \gamma \)° or alymphoid mice. In both stains, native human \( \alpha_2 \)M induced full SA modification whereas PBS induced no modification (H. He, D. McCartney, Q. Wei, S. Esadeg, J. Zhang, A. Hayes, F. van Leuven and B. A. Croy, unpublished). Thus, human \( \alpha_2 \)M must have regulated proteases, cytokines or other molecules that signal vascular dilation and elongation during gestation. When human \( \alpha_2 \)M, activated by methylamine to destroy its protease binding sites, was infused into pregnant alymphoid mice, SA dilation was again achieved. This experiment indicates
that cytokines rather than proteases are the key molecules involved in SA destabilization. If A2Mp is the key molecule induced by uNK cell-derived IFN-γ and if A2Mp, through its bound molecules, effects SA dilation and elongation, it is not necessary to postulate additional vasoactive uNK cell products. However, these are known. In mice, inducible nitric oxide synthase, the enzyme generating the powerful vasodilator NO, is predominantly found in uNK cells of normal implantation sites (Hunt et al., 1997). In contrast, implantation sites in uNK cell-deficient mice display expression of this enzyme in trophoblast (Hunt et al., 1997). Vascular endothelial cell growth factor (VEGF), a molecule bound by α3M, is produced by murine and human uNK cells (Wang et al., 2000; Li et al., 2001). In humans, placenta growth factor, a molecule upregulating the bioavailability of VEGF, is reported to be expressed exclusively in uNK cells (Li et al., 2001). Collectively these studies indicate that murine and human uNK cells contribute in various ways to the maternal vascular changes that occur in support of pregnancy.

Conclusions

Rodent studies strongly indicate that uNK cells are key contributors to maternal uterine changes induced by and in support of pregnancy. These studies indicate that many regulatory steps are involved, each of which could have a clinical correlate culminating in syndromes such as pre-eclampsia or intrauterine growth retardation. Although it is appealing to suggest that uNK cell-deficient mice may model pre-eclampsia, the present authors’ opinion is that they model only vascular aspects. Serial urinalysis of mated NK/uNK cell-deficient IL-15−/− and C57Bl/6 mice for protein (Lowry technique) failed to show proteinurea in uNK cell-deficient mice above that seen in pregnant normal mice (Fig. 5). Other strains, such as BMP5, which shows pregnancy-induced glomerulosclerosis, proteinurea and hypertension (Davison et al., 2002), or thrombomodulin−/−, which displays trophoblast death and fibrinoid deposition (Isermann et al., 2003), are important and investigations of pre-eclampsia may be best served by combining these or other strains as murine
Fig. 5. Analysis of mean urinary protein (Lowry assay) collected daily from individual uterine natural killer (uNK) cell-deficient IL-15+/− mice (dashed line; n = 6) and C57Bl/6j mice (solid line; n = 4) from one oestrous cycle (−4–0) and pregnancy (0–18). Every day, individual mice were placed in clean cages having a base composed of sterile 96-well plates. The mice were allowed to roam freely and void. Wells containing urine but no faeces were collected and pooled when urine volumes of 50–200 μL appeared to be available. The uNK cell-deficient mice were less proteinuric than their gestation-day matched normal congenic controls.

... study models. Although there are many differences between implantation sites in rodents and women, this review illustrates the dynamic and detailed information that can be collected from serial time-course studies of murine pregnancy to provide valuable concepts in understanding the biology of human CD56(+)-bright uterine cells. The resolving power of mouse genetics, combined with transplantation, in addressing questions of regulatory mechanisms in mammalian pregnancy in vivo are illustrated. The authors hope that others will develop and explore further variations of these approaches.

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