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Estradiol regulates MICA expression in human endometrial cells

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Abstract

The human endometrium undergoes cyclical changes regulated by sex hormones. Evidence suggests sex hormones regulate NK cell recruitment into the uterus in large numbers. NKG2D is an activating receptor expressed on human NK cells, $\gamma\delta$ and CD8 T cells. NKG2D ligands are known to be sensors of cellular “stress”. In this study, we investigated whether sex hormones directly regulate expression of NKG2D ligands in the human uterus. Estradiol increased MICA expression on uterine epithelial cells; regulation was estrogen receptor-dependent. Real-time PCR analysis showed that NKG2D ligands MICA and MICB were expressed in the human endometrium. MICA protein was detected primarily on epithelial cells, and greater expression was observed in immunohistochemical analysis of tissues from patients in the secretory phase of the menstrual cycle. Thus, estrogens regulate expression of MICA. These data suggest hormonal regulation of innate immunity and NKG2D-mediated recognition in other tissues and diseases where estrogen may be involved.

Keywords

NKG2D; MICA; Hormones; Immune Regulation; Human female reproductive tract

INTRODUCTION

The human endometrium is the site of an immunological paradox. The immune system defends the female reproductive tract (FRT) against potential pathogens yet maintains tolerance to a semi-allogeneic fetus. Studies on immune cells in the FRT reveal altered NK cell numbers and activity in various clinical conditions involving reproductive organs and reproductive failure [1,2]. Uterine NK (uNK) cells can comprise up to 70% of the leukocyte population in the human endometrium; and the numbers of uNK cells fluctuate depending on the phase of the menstrual cycle, being highest in the secretory phase [3]. Studies have shown that sex hormones induce specific chemokines in nonpregnant human endometrium that can activate NK cell migration. This may account for the increased NK cell numbers in endometrium during the menstrual cycle [4]. UNK cells express a unique phenotype vis-à-vis blood NK cells; they express CD56,

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CD94, CD9, and CD69, but they lack CD57 and CD16. [5–7]. Specific combinations of cell surface molecules differentiate NK cells from different tissues within the FRT[8].

The cyclical change in uNK cell numbers mimics other changes in the endometrium that occur in response to estradiol and progesterone. In the first half of the menstrual cycle, which is known as the proliferative phase, estradiol concentrations are low and gradually increase. Post ovulation, in the secretory phase, estradiol concentrations are elevated, but decrease slowly while progesterone concentrations gradually increase. Hormones influence the immune response in various ways. Estrogens depress T cell dependent responses and enhance the humoral response, while progesterones are natural immunosuppressors [9]. In the endometrium, estradiol and progesterone regulate many aspects of the innate immune system [10]. Sex hormones modulate the ability of uterine epithelial cells to respond to pathogenic microbes [11]. Estrogen has also been reported to have pleiotropic effects on cytokine production by human endometrial macrophages, which express estradiol receptors (ER α , β) [12,13]. Henderson et al. reported that NK cells express ER β 1, but it is not known if this is a functional receptor [14]. Therefore, the action of sex hormones on NK cell function and/or recruitment is likely mediated through hormone action on cells in the surrounding tissue that do express estrogen receptors.

NK cell activity is regulated by the balance of activating signals and inhibitory signals it receives from its milieu via expressed receptors [15]. NKG2D is an activating receptor that can trigger activity against ‘self’ cells if they express sufficient amounts of cognate ligands. NKG2D is also expressed by $\gamma\delta$ T cells and CD8 T cells including those that lack the CD28 co-receptor [16]. In T cells, NKG2D acts as a costimulatory molecule [17].

Human NKG2D binds to MHC class I chain-related protein A and B (MICA and MICB) as well as to molecules belonging to the UL-16 binding protein (ULBP) family [15]. NKG2D ligands share the characteristic that protein expression on the cell surface is almost undetectable in normal tissues, but expression can be induced by transformation, infection, or cellular stress, such as DNA damage [18,19]. The NKG2D receptor/ligand system can be considered a sensor system specifically for the recognition of “stressed” cells including tumors. In mucosal systems, such as the respiratory tract, cell surface expression of NKG2D ligands was detected under conditions of oxidative stress or infection [20,21]. Under physiological conditions, low surface expression of NKG2D ligands on gastric epithelium is hypothesized to be an effect of the microflora in the gut [18]. NKG2D ligand expression may play a local regulatory role, even without inflammatory signals, as it may affect the activity of various NKG2D expressing NK and T cells in the FRT under physiological conditions. A recent study by Strid et al. showed that induced expression of NKG2D ligands on skin keratinocytes led to local immune reorganization of innate immune cells in the absence of other signals [22]. The focus of this study was to determine if NKG2D ligands are expressed in the female reproductive tract, and how they are regulated by sex hormones. The data presented in this study indicate that the estradiol regulates uterine MICA expression in endometrial epithelial cells. Thus, these data and previous reports show that estradiol has the potential to regulate both the number of uNK cells recruited to the human endometrium and the activation of those uNK cells present in the endometrium.

MATERIALS AND METHODS

Cell Lines

ECC1 (human uterine adenocarcinoma) cells were grown in phenol red free Dulbecco’s modified Eagle medium (DMEM) with a high glucose concentration (4.5 g/liter) supplemented with penicillin (100 U/ml), streptomycin (100 μ g/ml), Hepes (10 mM) and 10% charcoal dextran treated fetal bovine serum (FBS; Hyclone, Logan, Utah). The other cell lines, HeLa

(human cervical carcinoma), P815 (murine mastocytoma) and B3Z (murine T cell hybridoma) were cultured in RPMI 1640 supplemented with 2-mercaptoethanol (50 μ M), penicillin (100 U/ml), streptomycin (100 μ g/ml), Hepes (10 mM), sodium pyruvate (1 mM), non-essential amino acids (0.1mM) and 10% FBS.

Treatment of ECC1 cells with hormones

ECC1 cells (2×10^5 /mL) were plated in 6 well flat bottomed plates (Corning Inc., Corning, NY) containing wells treated with estradiol (Calbiochem, San Diego, CA), ethanol, and/or ICI182,780 (a specific estrogen receptor antagonist, Tocris, Ellisville, MO) at 100 fold greater concentration than estradiol [13]. The hormones and inhibitor were dissolved in 100% ethanol, and the ethanol was allowed to evaporate before media was added. Plates were incubated at 37°C for 48 hours in 5% CO₂. Cells were harvested by treating with 1mM EDTA for 15 minutes. Cells were washed with PBS and analyzed for NKG2D ligand expression by flow cytometry.

Isolation of cells from human endometrium

Endometrial tissue specimens were obtained from women undergoing hysterectomy for various gynecological disorders. All studies were done with approval of the Dartmouth Institutional Review Board. The tissues used were distal to any pathology. Samples were staged by a pathologist as secretory, proliferative or atrophic. We obtained tissues from 72 patients with an average age of 44 $\pm$ 6 years. Tissue was snap-frozen for isolation of RNA, proteins, or embedded in OCT for tissue sectioning. Single cell suspensions were prepared using the protocol as in Eriksson et al.[23]. Cells were strained through 100 μ m filters, and then 40 μ m filters to capture epithelial cell sheets. The backwash from 40 μ m filters was pooled and a viable cell count determined. Cells were used directly for experiments or seeded in flat bottomed 96 well plates at 5×10^4 cells/well in DMEM medium.

Quantitative Real Time PCR

Total RNA was isolated and duplicate samples were analyzed on an ABI7700 thermal cycler (Applied Biosystems) as previously described[4]. The cycle time (Ct) was used as an indicator of the amount of gene product present in the sample, and the relative expression compared to β -actin was determined.

Western Blotting

Protein was isolated from endometrial tissue samples by grinding in M-PER (Pierce Biotechnology Inc., Rockford, IL). Protein content was quantified using a BCA protein assay (Pierce). Proteins were resolved and transferred as described [12]. Membranes were probed with goat anti-human MICA antibodies (R&D systems, Minneapolis, MN) followed by streptavidin-HRP (BD Pharmingen, San Diego, CA). Anti- α -Tubulin (DM1A, Sigma) was chosen as a loading control since the band size did not overlap the range of MICA bands. SuperSignal chemiluminescence substrate (Pierce) was used for visualization.

Immunohistochemistry

Frozen sections (8 μ m) were fixed in cold acetone and washed with PBS. Sequential blocking was performed with 0.03% hydrogen peroxide, 10% goat sera and 1X Cohn's Fraction (Sigma-Aldrich, St. Louis, MO) in PBS, followed by use of the Avidin/Biotin Blocking Kit (VECTOR, Burlingame, CA). Sections were stained with anti-human MICA antibodies (BAF1300, R&D systems) or isotype matched IgG, and developed using VECTASTAIN® Elite ABC Kit (VECTOR). Sections were counterstained with hematoxylin, and mounted with Permount (Fisher, Pittsburgh, PA). Sections were analyzed in a blinded manner by three individuals, and scored based on intensity of expression using a scale of 1 (low) to 4 (high).

Flow cytometry

A FACSCalibur (BD Biosciences) was used for flow cytometric analysis. ECC1 cells were stained with human soluble NKG2D-mIgG1 fusion protein or anti-human MICA antibodies (159227, R&D systems), followed by staining with PE-labeled rat anti-mouse IgG1 antibodies (A85-1, BD Pharmingen).

Chimeric B3Z Assay

B3Z cells are a CD8⁺ T cell hybridoma that expresses *lacZ* upon engagement of the T cell receptor (TCR), which binds to H-2K^b and SIINFEKL peptide. B3Z cells contain a construct which employs three tandem copies of a 30-bp binding site for Nuclear Factor of activated T cells (NF-AT) linked to a minimal IL-2 promoter that drives expression of *lacZ*. The IL-2 promoter was used as IL-2 expression is triggered once TCR signaling is initiated via CD3 ζ [24]. These B3Z cells are hereafter referred to as wildtype (wtB3Z), and these cells do not recognize NKG2D ligands. Transfected B3Z cells, termed chimeric B3Z (ch) cells express a chimeric NKG2D receptor, consisting of the human NKG2D receptor ligated to the human CD3 ζ chain cytoplasmic region. Thus, chB3Z cells also express *lacZ* on engagement of the human NKG2D receptor [25]. Fresh and cultured endometrial epithelial cells were incubated overnight with wtB3Z cells or chB3Z cells (1:1). LacZ activity was determined by using a β -Galactosidase assay kit (Sigma). Blocking experiments utilized goat anti-human MICA antibodies (15 μ g/mL) or control goat IgG sera. For ligand blockade, freshly isolated epithelial cells were allowed to sit for an hour at 37°C, followed by incubation with antibodies for 30 min at RT. Samples were assayed in triplicates. B3Z cells were added, and cells cultured for 4 hours at 37°C. LacZ activity was determined.

Statistics

Differences between groups were analyzed using a paired or unpaired Student's *t* test or Mann Whitney test, as appropriate. *P* < 0.05 was considered significant.

RESULTS

MICA is upregulated on ECC1 cells exposed to estradiol

The expression of NKG2D ligands on cells can lead to the activation of immune cells, including the activation of NK cells that comprise up to 70% of the leukocytes in the human FRT. To determine how sex hormones may regulate immune cells in the FRT by sex hormones, we investigated if estradiol regulated MICA expression in a uterine epithelial cell line ECC-1. These cells which are known to express ER α were exposed to physiologically relevant estradiol concentrations (5 $\times$ 10⁻⁸ M) for 48 hours, and surface expression of NKG2D ligands were analyzed by flow cytometry using sNKG2D-Ig [25,26]. As shown in Figure 1, a 64% increase in ligand expression was detected in ECC1 cells treated with estradiol compared with ethanol controls. Furthermore, uterine NK cell clones killed ECC1 cells in an NKG2D-dependent manner (data not shown). Preliminary studies with progesterone indicated no effect on MICA expression (data not shown). To show that this protein induction was estradiol receptor-dependent, ligand expression in the presence of a specific ER antagonist, ICI182780, was determined. In ECC1 cells cultured with estradiol and a 100 fold excess of ICI182780, the ability of estradiol to upregulate NKG2D ligand expression was eliminated (Fig 1A, B). Analysis of MICA expression showed that MICA was being upregulated in response to estradiol (Fig 1C). Thus, MICA expression is upregulated on ECC1 cells exposed to estradiol in an ER-dependent manner.

MicA mRNA is expressed in the human uterine endometrium (EM)

To determine if hormonal regulation occurred in primary tissue, we analyzed the expression of all known NKG2D ligands in human EM. Total RNA was extracted from 26 human endometrial tissue specimens and cDNA was analyzed for *mica*, *b* and *ulbp1,2,3,4* by real-time PCR analysis (Fig 2). Because the endometrium changes throughout the menstrual cycle [27], tissue samples were selected from different phases of the menstrual cycle. Only *mica* was highly expressed in the endometrium hence subsequent studies focused on the regulation of *mica*. A few endometrial samples expressed some *micB* and very low amounts of *ulbp3* and *ulbp 4 mRNA*. We observed constitutive mRNA expression of *mica* in EM derived from the 26 different patients. The cervical epithelial cell line HeLa was used as a positive control (Fig 2A). The mean amount of *mica* transcribed was 3-fold higher in secretory phase EM compared to proliferative phase EM. The difference was found to be statistically significant. These data suggest that *mica* expression may be regulated by sex hormones in the human EM.

Expression of MICA protein in the endometrium

To detect protein expression, we examined MICA in the EM by immunoblot analysis (Fig 3). Blots were also probed with anti-tubulin Abs to control for total protein loading. In accordance with the RNA data, each of the tissues analyzed expressed MICA protein. Untransfected P815 cells served as negative control, while HeLa cells known to express MICA of around 62 Kd were a positive control [28]. MICA has eight potential N-glycosylation sites and various sizes for this protein have been reported, ranging from 43 Kd (mature peptide) to 75 Kd (highly glycosylated) [18,28,29]. We detected MICA in uterine EM at approximately 45 Kd. In three out of six EM tissues taken in the secretory phase, a second band corresponding to 63 Kd was also observed.

MICA in the endometrium is localized to the epithelial cells

To determine which EM cells expressed MICA, immunohistochemical staining was conducted (Fig 4). MICA was localized to epithelial cells in all of the endometrial tissues analyzed (n=16). Epithelial cells were identified on the basis of their morphology as denoted by H&E staining. Expression of MICA was found in the glandular and luminal epithelial cells. Due to higher background staining on luminal epithelial cells, the amount of MICA expression on these luminal cells was difficult to determine. The intensity of MICA expression differed depending on the phase of the menstrual cycle, based on a blinded analysis of the stained sections (Fig 4G). Of the five tissues analyzed from the proliferative phase, only one showed intensity of staining similar to the secretory phase tissues. These data indicate that increases in sex hormones may play a role in upregulating MICA protein expression in EM epithelial cells.

MICA is expressed on the cell surface of epithelial cells

A reporter cell assay was used to detect if MICA was expressed on the cell surface of endometrial epithelial cells. In this assay, primary EM epithelial cells were co-cultured with B3Z reporter cells (wtB3Z) or chimeric NKG2D B3Z cells (chB3Z). The chB3Z cells express LacZ when cultured with target cells that express NKG2D ligands at the cell surface because of CD3 ζ triggering through their chimeric NKG2D receptor [25]. The wtB3Z cells do not recognize NKG2D ligands and serve as a negative control. This assay is very sensitive and only dependent upon the cell surface expression of human NKG2D ligands. As shown in Figure 5A, primary epithelial cells expressed NKG2D ligands on the cell surface, as demonstrated by increased LacZ⁺ B3Z cells. WtB3Z cells or chB3Z cells incubated with a mouse cell line (P815) did not express LacZ activity.

To determine if MICA was being recognized by the chB3Z cells, anti-MICA antibodies were used to block the interaction (Fig 5B). Most of the cell activity was removed when anti-MICA

Abs were used, so MICA accounted for most of the activity. It was noted that freshly isolated epithelial cells expressed much lower amounts of ligands compared to cultured epithelial cells. The enzyme digestion process used to isolate endometrial cells did not cleave ligands from the epithelial cell surface as enzyme treatment of P815-MICA cells showed no reduction in ligand expression when compared to untreated P815-MICA cells (data not shown). Hence, human endometrial epithelial cells express MICA on the cell surface that can be recognized by NKG2D expressing cells.

DISCUSSION

In this study, we demonstrated the hormonal regulation of MICA in the human endometrium. The human secretory endometrial tissues studied showed greater *micA* RNA expression compared with the mean of the proliferative endometrial tissues. These data indicate that expression of *micA* RNA is enhanced during the secretory phase of the menstrual cycle, coincident with enhanced circulating estradiol and progesterone. Initial studies that evaluated NKG2D ligand regulation by progesterone in primary endometrial cells and ECC1 cells have not shown any alterations due to progesterone (our unpublished data). Although progesterone is of great importance in the secretory phase, it may not be responsible for increases in MICA expression. Estradiol increases during the late proliferative phase and remains elevated until the mid-secretory phase, which may account for the range of gene expression observed in the two phases. The amounts of hormone also differ within the early and late stages of each phase in the menstrual cycle. Tissue lysates are composed of heterogeneous cell populations so the relative amounts of a cell type expressing the ligands may differ from tissue to tissue even within a particular phase. One possible mechanism by which estradiol enhances MICA gene expression is through transcriptional regulation. The consensus Estrogen Receptor Element (ERE) for mammals is 5'-GGTCAnnnTGACC-3' [30]. The MICA promoter contains an ERE sequence at the -46 position relative to the transcription start site (+1), suggesting that estradiol may regulate MICA expression by direct binding to DNA, and recruiting the initiation complex to the start site.

MICA function in human endometrial epithelial cells has not yet been established. Unlike the lower parts of the FRT (vagina, ectocervix), the upper parts of the FRT are exposed to much lower amounts of microorganisms [31]. The existence of fenestrations in human and rat intestinal basement membrane suggests a mechanism allowing for cellular interactions between epithelial cells and immune cells in the stroma [32,33]. In the endometrium similar fenestrations may allow NK cells to interact with epithelial cells. Immunohistochemical analyses has shown CD56+ cells scattered in the endometrial stroma near the glandular epithelial cells [34]. The number of endometrial NK cells significantly varies with the menstrual cycle. It is significantly higher in the secretory phase than in the proliferative phase [3,8,35]. Thus as the menstrual cycle progresses, increasing NK cells would be present and may interact with epithelial cells within the EM microenvironment.

Immunoblot analysis revealed MICA proteins of different sizes, which may be due to alternative glycosylation. Our observation that multiple isoforms were present in uterine tissue may be a reflection of the broad diversity and relative amounts of cell types present in whole endometrial tissue lysates. Experiments utilizing the chB3Z cells established cell surface expression of NKG2D ligands on human uterine epithelial cells and showed that a functional interaction occurred between receptor and ligand. Analogous to gut epithelial cells, which express low amounts of ligands on cell surface, freshly isolated endometrial epithelial cells also express low amounts of NKG2D ligands on the cell surface. Because expression of NKG2D ligands has been considered a "stress" response, it would be expected that a healthy, non-pregnant individual would express very low amounts of uterine NKG2D ligands. However, cellular stress induced during tissue culture may lead to an increased expression of

NKG2D ligands. Alternately, cell culture may lead to proliferation of cells such that more cells may express NKG2D ligands. There is a linear relationship between the number of ligand expressing epithelial cells and the activation of chB3Z cells (data not shown). Further increases in epithelial cell numbers did not result in an increased number of LacZ⁺ chB3Z cells.

There is always concern that analysis of human tissues may reflect the underlying disease rather than the normal physiology. In this study, the tissues utilized were distal to any sites of pathology and the reasons for hysterectomy were varied and included risk reduction. Expression of MICA did not correlate with any particular diagnosis. Because all tissues responded similarly, we believe that these findings largely represent underlying physiology of human EM.

MICA is expressed in intestinal epithelium, stressed human airway epithelium and tumors of epithelial origin. Studies in human endometrial epithelial cells have shown the presence of a luminal epithelium and a glandular epithelium with differential expression of integrins during the menstrual cycle [36]. Differential expression of estrogen receptors on human mammary gland: basal (and/or myoepithelial) cells and luminal epithelial cells has been reported; luminal cells express them while basal cells do not [37]. These two cell types are distinguished immunohistochemically by specific keratin markers. Our immunohistochemical studies showed high expression of MICA on glandular epithelium within the human endometrium. There may be differential expression of MICA on different epithelial cell types, although our data does not show clear differential expression between glandular and luminal epithelial cells. MICA on uterine epithelial cells can have a functional interaction with NKG2D on uterine NK clones (data not shown). NKG2D activation may play a local regulatory role, even without inflammatory challenge. A recent study by Strid et al. showed that expression of NKG2D ligands on skin keratinocytes led to local immune reorganization of innate immune cells [22].

In summary, this study shows that MICA is expressed in the endometrium at higher amounts on epithelial cells in the secretory phase and that estradiol upregulated MICA expression on the surface of uterine epithelial cells. The regulation of MICA may lead to altered function of immune cells that express NKG2D, which includes NK cells, $\gamma\delta$ T cells, NKT cells, and CD8 + T cells. It is known that sex hormones regulate specific chemokines that can increase NK cell recruitment to the endometrium[4]. These new data show that estradiol has further affects on local innate immunity by regulating the expression of ligands for NKG2D, a key NK cell activation receptor. These findings highlight the complex interplay between sex hormones and immunity, and they suggest that sex hormone induction of MICA may have a role in regulation of immune activity in other tissues or diseases where sex hormones and activation of NK cells and innate-like T cells are involved in disease pathogenesis.

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Figure 1. NKG2D ligands are upregulated on ECC1 cells by estradiol

(A) ECC-1 cells were preincubated with estradiol (E2), vehicle (EtOH), with or without inhibitor (Inh) for 48h before analysis of NKG2D ligand expression using soluble NKG2D-Ig receptor. All treatments were done in triplicate. Bar graphs indicate values normalized to the vehicle control (EtOH = 100) $\pm$ SD. (B) Histogram denotes representative fluorescence intensities from one experiment. (C) Treated ECC1 cells were also analyzed for MICA expression. A paired Student's *t* test was used for statistical analyses. A ** indicates *p* value < 0.001 and a * *p* value < 0.05.

Figure 2. Expression of NKG2D ligands in human endometrial tissue

(A) Quantitative Real Time PCR data for *micA* expression in EM samples separated on the basis of phases of the menstrual cycle, proliferative (n=14) or secretory (n=14). Positive control was RNA from HeLa cells. No template control (water) showed no signal. (B) Real Time PCR was used to determine the relative amounts of other NKG2D ligands (*micB*, *ulbp1*, *ulbp2*, *ulbp3* and *ulbp4*) in a set of patients from the proliferative or secretory phase (n=6–7). Values were normalized against β -actin. Mean values shown. Data are shown as mean values $\pm$ SEM.

* P value < 0.05

Figure 3. Expression of MICA protein in human endometrial tissue

Immunoblot analysis of whole EM tissue lysates with anti-MICA or anti- α -tubulin antibodies. Positive control was protein lysate from HeLa cells. Protein lysate from P815 cells served as negative control. The data are representative of three experiments and eighteen EM tissues.

Figure 4. MICA in endometrial tissue is expressed more in the secretory phase than in the proliferative phase EM

H&E staining of two representative EM tissues at 200x magnification (A,D). Specific staining for MICA (B,E) with respective isotype controls (C,F) at 200x magnification. Each set of serially sectioned tissues (A–C and D–F) shown are representative of five individual EM samples from each menstrual stage. Stained tissues were scored on the basis of intensity in a blinded manner by three independent observers. Dot plot indicating mean scores is shown (G).

* P value <0.05.

Figure 5. NKG2D ligands are expressed on the surface of epithelial cells

(A) Primary fresh epithelial cells (fresh EM) and cultured epithelial cells (cul EM at day 21) from the same patient were co-cultured with chimeric (chB3Z) or wildtype B3Z (wtB3Z) cells. Number of LacZ positive cells in 5 fields/sample was enumerated under 100x magnification. Means $\pm$ SD are shown. Data are representative of 2 to 4 independent experiments. (B) Primary fresh epithelial cells were blocked with anti-MICA antibodies and then cocultured with chB3Z or wtB3Z cells. Number of LacZ positive cells in 5 fields/well were counted under 100x magnification. The values of the isotype controls were set to 100%. Means $\pm$ SD are shown. Data represent three patient samples. ** P value < 0.01