Effect of oestradiol on PAMP-mediated CCL20/MIP-3α production by mouse uterine epithelial cells in culture

Gisela Soboll,[1] Mardi A. Crane-Godreau,[2] Magdalena A. Lyimo[1] and Charles R. Wira[1]

Departments of [1]Physiology and [2]Microbiology and Immunology, Dartmouth Medical School, Lebanon, NH, USA

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Correspondence: Dr Charles R. Wira, Department of Physiology, Dartmouth Medical School, Lebanon, NH 03765, USA.
Email: Charles.R.Wira@Dartmouth.EDU
Senior author: Gisela Soboll,
email: husseygs@colostate.edu

Summary

The present study was undertaken to establish whether mouse uterine epithelial cells produce CCL20/macrophage inflammatory protein 3α (CCL20/MIP-3α) and to determine whether secretion is under hormonal control and influenced by pathogen-associated molecular patterns (PAMPs). In the absence of PAMPs, polarized uterine epithelial cells grown to confluence on cell culture inserts constitutively secreted CCL20/MIP-3α with preferential accumulation into the apical compartment. When epithelial cells were treated with the Toll-like receptor (TLR) agonists Pam_3Cys (TLR2/1), peptidoglycan (TLR2/6) or lipopolysaccharide (LPS; TLR4), CCL20/MIP-3α increased rapidly (4 hr) in both apical and basolateral secretions. Time–course studies indicated that responses to PAMPs added to the apical surface persisted for 12–72 hr. Stimulation with loxoribin (TLR7) and DNA CpG motif (TLR9) increased basolateral but not apical secretion of CCL20/MIP-3α. In contrast, the viral agonist Poly(I:C) (TLR3) had no effect on either apical or basolateral secretion. In other studies, we found that oestradiol added to the culture media decreased the constitutive release of CCL20/MIP-3α. Moreover, when added to the culture media along with LPS, oestradiol inhibited LPS-induced increases in CCL20/MIP-3α secretion into both the apical and basolateral compartments. In summary, these results indicate that CCL20/MIP-3α is produced in response to PAMPs. Since CCL20/MIP-3α is chemotactic for immature dendritic cells, B cells and memory T cells and has antimicrobial properties, these studies suggest that CCL20/MIP-3α production by epithelial cells, an important part of the innate immune defence in the female reproductive tract, is under hormonal control and is responsive to microbial challenge.

Keywords: CCL20/macrophage inflammatory protein-3α; epithelial; oestradiol; rodent; Toll-like receptor; uterus

Introduction

The innate immune system at mucosal surfaces is present to protect against microbial invasion.[1] As sentinels of innate immune defence, mucosal epithelial cells provide a physical barrier to potential pathogens; they also recognize potential pathogens, respond rapidly to pathogenic challenge and, when necessary, signal the adaptive immune system. Uterine epithelial cells express cell surface and internal receptors to microbes and sex hormones[2] which, when stimulated, alter the epithelial cell's secretion of cytokines and chemokines.[3,4] Production of cytokines and chemokines in turn leads to the recruitment and activation of immune effector cells of both the innate and adaptive immune systems. In addition to responding to potential pathogens and hormone

Abbreviations: ELISA, enzyme-linked immunosorbent assay; IgA, immunoglobulin A; LPS, lipopolysaccharide; MIP-3α, macrophage inflammatory protein 3α; PAMP, pathogen-associated molecular patterns; TER, transepithelial resistance; TLR, toll-like receptor; TNF-α, tumour necrosis factor α.

fluctuations, epithelial cell signalling in the female reproductive tract is structured so as to provide a level of tolerance to sperm, the blastocyst at implantation, and an immunologically distinct fetal–placental unit throughout pregnancy.[5]

Recent studies indicate that the upper female reproductive tract, including the uterus and Fallopian tubes, is periodically exposed to bacteria and viruses, including potential pathogenic organisms.[6] Following placement of radiolabelled sperm-sized macrospheres or radio-opaque dye in the vagina of women, each moved into the uterus and Fallopian tubes within between 1 min and 2 hr.[7,8] Epithelial cells that line the female reproductive tract form the first line of defence. Beyond their role as a barrier, epithelial cells play a significant role in sensing the lumen of the reproductive tract, responding to maintain an environment that on the one hand protects the female, while on the other, provides an environment compatible with reproduction.

A growing body of evidence indicates that to combat exposure to potential pathogens, epithelial cells express cell receptors to common pathogen-associated molecular patterns (PAMPs). Eleven toll-like receptors (TLRs) recognizing viral, bacterial and fungal structures have been identified in mammals. TLR2 builds heterodimers with TLR1 and TLR6, and recognizes a broad range of microbial products and components including Gram-positive bacteria, mycobacteria, peptidoglycan, zymosan or Pam_3Cys-Ser-$(Lys)_4$.[9–13] TLR3 recognizes double-stranded RNA,[14] TLR4 (in association with CD14 and MD-2) recognizes Gram-negative bacteria,[15] TLR5 recognizes bacterial flagellin,[16] TLR7 and TLR8 recognize single-stranded RNA and some synthetic molecules,[17,18] and TLR9 recognizes CpG containing DNA.[19] Recently, TLR11 was shown to recognize parasite-associated profilin-like molecules,[20,21] whereas TLR10 remains an orphan receptor.[22]

When epithelial cells are exposed to pathogens, the engagement of multiple receptors and adaptors results in unique downstream events and the stimulation of genes that lead to the secretion of antimicrobials and chemokines.[22,23] Antimicrobials function in host defence with antibacterial, antifungal, and antiviral properties.[24–28] More recently, chemokines including CCL20/macrophage inflammatory protein 3α (CCL20/MIP-3α) have been found to exhibit antimicrobial properties.[29] CCL20/MIP-3α is unique in that it binds exclusively to the CCR6 receptor in both humans and mice.[30–32] In addition to having antimicrobial properties similar to the β-defensins, CCL20/MIP-3α is involved in the recruitment of immature dendritic cells, B cells and memory T cells to a site of antigen exposure via its exclusive relationship with the CCR6 receptor.[33–36] Expression of CCL20/MIP-3α has been reported in epithelial cells of the female reproductive tract, the gastrointestinal tract and the respiratory tract and secretion of CCL20/MIP-3α occurs constitutively as well as being induced under inflammatory conditions.[37–40] In the rat, we found that polarized epithelial cells in culture constitutively secrete CCL20/MIP-3α and that exposure to TLR2 and TLR4 agonists significantly increases CCL20/MIP-3α secretion.[41,42]

Previously, oestradiol has been shown to play a critical role in regulating the immune system throughout the female reproductive tract. In response to oestradiol, levels of immunoglobulin A (IgA) and IgG increased in the uterine secretions of ovariectomized rats at a time when vaginal immunoglobulin levels decreased.[43,44] Oestradiol acts at multiple levels in the reproductive tract to alter epithelial cell and vascular permeability, the recruitment of immunoglobulin-producing cells to the uterus and vagina, and the expression of polymeric IgA receptor mRNA and protein, which, in turn, leads to the selective movement/transport of IgA and IgG into uterine and vaginal secretions.[44–48] Uterine epithelial cells also produce a spectrum of growth factors, cytokines and antibacterial factors both constitutively and in response to hormones.[49,50] As a part of these studies we found that, in response to oestradiol, antigen presentation by rat uterine epithelial cells is stimulated while antigen presentation by mouse uterine epithelial cells is inhibited.[51] Moreover, we found that, in both the rat and mouse, oestradiol inhibited uterine stromal cell and vaginal antigen presentation to memory T cells[51–54] (Wira and Rossoll, unpublished observation). More recently, we found that in polarized rat uterine epithelial cells in culture, constitutive as well as PAMP-induced CCL20/MIP-3α secretion is under oestradiol control.[55] Oestradiol inhibited the constitutive secretion of tumour necrosis factor-α (TNF-α) and CCL20/MIP3α into culture media. Interestingly, oestradiol pretreatment enhanced CCL20/MIP-3α secretion as a result of lipopolysaccharide (LPS) and Pam_3Cys administration.[55]

The goal of this study was to test the hypothesis that mouse uterine epithelial cells secrete CCL20/MIP-3α in response to PAMPs and that oestradiol regulates PAMP stimulation of CCL20/MIP-3α. Our objective in these studies was to (1) determine whether polarized mouse uterine epithelial cells constitutively secrete CCL20/MIP-3α; (2) define the role of PAMPs in regulating CCL20/MIP3α secretion; and (3) examine the role of oestradiol in regulating both the constitutive and the PAMP-induced secretion of CCL20/MIP-3α.

Materials and methods

Animals

Animals used in the experiments were sexually mature BALB/c mice, supplied by Charles River Laboratories (Kingston, NY). Animals were housed and fed according

to the guidelines of the Dartmouth College Institutional Animal Care and Use Committee, and all procedures were approved before the experiments started. For each experiment, animals were killed by CO_2 asphyxiation and uterine tissues from 10–15 adult animals were isolated and pooled for each experiment.

Isolation and preparation of epithelial cells

Uterine epithelial cell suspensions were prepared by enzymatic digestion and screen disruption as described previously.[49] Briefly, after removal, uteri were slit lengthwise and digested with 46 500 Units trypsin (Sigma, St Louis, MO) in 2·5% pancreatin (Gibco/Invitrogen, Grand Island, NY) for 1 hr at 4°, followed by a 60-min digestion at 22°. Digested tissues were then vortexed three times in Hanks' balanced salt solution (Gibco-BRL/Invitrogen) to release the epithelial sheets, before passing the cell suspension through a 20-μm Nylon mesh (Small Parts Inc., Miami Lakes, FL) for collection by centrifugation. Uterine epithelial cells were resuspended in Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 nutrient mixed 1 : 1 (Gibco/Invitrogen) plus 10% charcoal–dextran-stripped fetal bovine serum (Hyclone, Logan, UT), 20 mM HEPES, 100 μg/ml streptomycin, 100 U/ml penicillin, 2 mM L-glutamine (all from Gibco/Invitrogen) and seeded onto 0·4-μm pore size/10-mm diameter Nunc cell culture inserts (Nalgene Nunc International, Rochester, NY) coated with Matrigel (Collaborative Biomedical Products, Bedford, MA) at 300 μl/apical chamber of the cell culture insert. Inserts were placed into 24-well tissue culture plates (Nalgene Nunc International) containing 500 μl/well of culture media. When epithelial cells reached confluence, as indicated by transepithelial resistances (TERs $>1000\ \Omega$/well, the culture media was switched to Cellgro (Gibco/Invitrogen) plus 100 μg/ml streptomycin, 100 U/ml penicillin, and 2 mM L-glutamine, before stimulation with TLR agonists and or treatment with oestradiol or conditioned stromal media.

Treatment of epithelial cells with PAMPs

To determine whether CCL20/MIP-3α secretion by epithelial cells was affected by PAMPs, uterine epithelial cells were grown in Cellgro for 2 days before incubation with TLR agonists placed in the apical media. TLR agonists used in these studies included TLR1/TLR2: Pam_3Cys-Ser-$(Lys)_4$ (Pam_3Cys) (EMC Microcollections, Tuebingen, Germany), 10 μg/ml; TLR2/TLR6: Peptidoglycan from *Staphylococcus aureus (*Invivogen, San Diego, CA), 10 μg/ml; TLR3: Poly(I:C) (Invivogen), 25 μg/ml; TLR4: ultra-pure LPS from *Salmonella minnesota* (List Biological Laboratories, Inc, Campell, CA), 1 μg/ml; TLR7: loxoribine (Invivogen), 100 μM; TLR9: CpG oligonucleotide (Invivogen), 1 μM. Wells containing media alone were included in all experiments as controls. Following a 48-hr incubation, apical and basolateral supernatants were collected and centrifuged at 10 000 ***g*** for 5 min to remove cell debris before storage at −80°C until further analysis. In time–course studies, apical and basolateral media were collected at 4, 8 and 12 hr and immediately replaced with media containing fresh TLR agonists. TLR agonists were tested for LPS contamination using the *Limulus* assay (BioWhittaker, Inc., Walkersville, MD) according to the manufacturer's instructions before being used in the cell culture assays and contamination was found to be below the level of detection.

Hormone preparation and treatment

Oestradiol (Calbiochem, La Jolla, CA) was dissolved in 100% ethanol, evaporated to dryness, and re-suspended in Cellgro to the appropriate concentration. To control for the alcohol present in the steroid preparation, an equivalent amount of ethanol was evaporated in flasks used to prepare the control medium. In experiments involving the treatment of polarized epithelial cells with oestradiol, epithelial cells were grown to high TER ($>1000\ \Omega$/well), Cellgro was removed from the apical and basolateral compartments and replaced with fresh Cellgro alone or Cellgro containing oestradiol at the appropriate concentration. In experiments involving PAMPs plus oestradiol, the media in the apical compartment was replaced with media containing PAMPs alone or PAMPs with oestradiol and that in the basolateral compartment was replaced with fresh medium alone or medium containing oestradiol at the appropriate concentration.

CCL20/MIP-3α analysis

The amounts of CCL20/MIP-3α in apical and basolateral media were determined using a commercial enzyme-linked immunosorbent assay (ELISA) specific for mouse CCL20/MIP-3α (DuoSet ELISA development system; R & D Systems, Minneapolis, MN) according to the manufacturer's instructions. Media from a minimum of four cell culture inserts were analysed per treatment group.

Statistical analysis

Standard errors of the mean and mean values were calculated for each experiment by averaging the results of four to six culture inserts per treatment group. For statistical analysis analysis of variance (ANOVA) plus a Tukey–Kramer multiple comparisons test were used to compare apical and basolateral CCL20/MIP-3α levels, as well as TLR agonist induction of CCL20/MIP-3α versus media control and levels of CCL20/MIP-3α collected at

different time-points in the time–course experiment. Differences with *P*-values $< 0{\cdot}05$ were considered statistically significant.

Results

Preferential release of CCL20/MIP3α by mouse uterine epithelial cells

To study the secretion of CCL20/MIP-3α by polarized murine epithelial cells, isolated mouse uterine epithelial cells were grown to confluence on cell inserts (four to five inserts/treatment group) before analysis of apical and basolateral media collected at 24, 48 and 72 hr of incubation. Within 3–4 days of culture, as previously reported,[49] cells reached confluence and formed tight junctions as indicated by TER values between 1000 and 1500 Ω/well (background resistance 150 Ω/well) (data not shown). As seen in Fig. 1, CCL20/MIP-3α was secreted constitutively into the apical compartment when measured at 24, 48 and 72 hr in culture. In contrast, very little CCL20/MIP-3α was secreted by epithelial cells into the basolateral compartment irrespective of the length of incubation. CCL20/MIP-3α release into the apical media at all time-points measured was significantly greater than that seen in the basolateral media. These findings, which are representative of three separate experiments, indicate that CCL20/MIP-3α secretion by polarized mouse uterine epithelial cells is preferential to the apical surface.

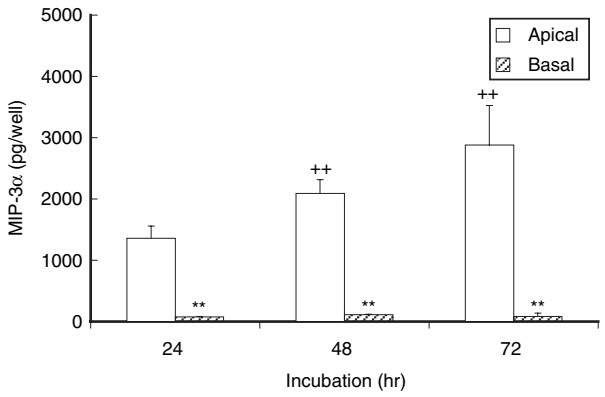

Figure 1. Preferential release of CCL20/MIP-3α by polarized mouse uterine epithelial cells in culture. Mouse uterine epithelial cells from 10 to 15 animals were isolated and grown to high transepithelial resistance on cell inserts as described in the Materials and methods. The 24, 48 and 72-hr accumulation of CCL20/MIP-3α in the apical chambers (white bars) and basolateral chambers (hatched bars) were analysed by ELISA. Mean ± SEM were derived from four to six cell inserts/treatment group. Data are representative of three separate experiments. Differences between apical and basolateral release were tested by ANOVA. Significance is indicated as $*P < 0{\cdot}05$ and $**P < 0{\cdot}01$.

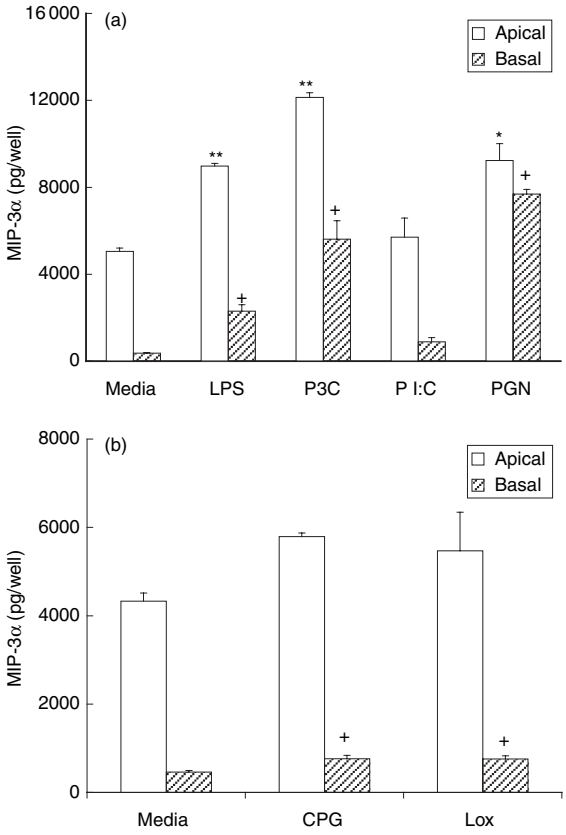

Figure 2. CCL20/MIP-3α release by polarized mouse uterine epithelial cells following stimulation with PAMPs. (a) PAMPs used were the TLR4 agonist LPS at 1 μg/ml, the TLR2 (1/6) agonist Pam_3Cys-Ser-$(Lys)_4$ (P_3C) at 10 μg/ml, the TLR3 agonist Poly(I:C) (P I:C) at 25 μg/ml and the TLR2 (1/6) agonist peptidoglycan (PGN) at 10 μg/ml. (b) PAMPs used were the TLR9 agonist CpG at 1 μM and the TLR7 agonist loxoribine at 100 μM. The 48-hr accumulations of CCL20/MIP-3α in the apical chambers (white bars), and basolateral chambers (hatched bars), were analyzed by ELISA. Means ± SEM were derived from a minimum of four cell inserts. Data are representative of three separate experiments. Differences between PAMP stimulation and controls were tested by ANOVA. Significant differences apically are indicated as $*P < 0{\cdot}05$ and $**P < 0{\cdot}01$ and basolaterally as $^{+}P < 0{\cdot}05$.

Effect of by TLR agonists on CCL20/MIP-3α secretion by uterine epithelial cells

Having previously demonstrated that epithelial cells express mRNA for TLRs 1–6 and occasionally express mRNA for TLRs 7–9,[4] our aim was to determine whether treatment with PAMPs would affect CCL20/MIP-3α secretion by polarized epithelial cells. Following growth to confluence on inserts and the formation of tight junctions as evidenced by high TER, apical media were replaced with media containing selected PAMPs that are known to act through specific TLRs. Following incubation with PAMPs for 48 hr, apical and basolateral media were

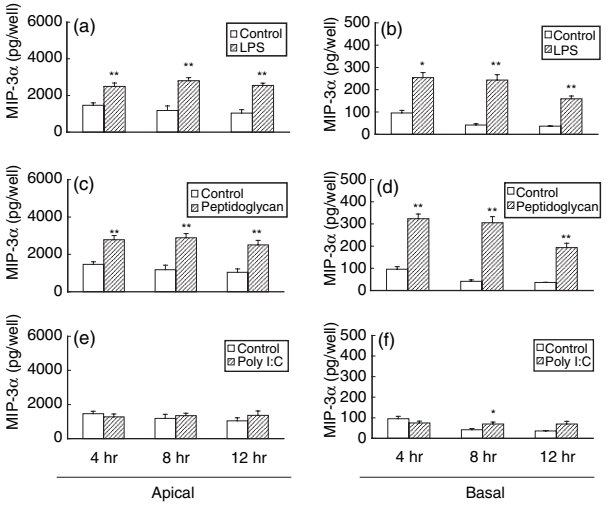

Figure 3. Time–course of CCL20/MIP-3α release by polarized mouse uterine epithelial cells. Apical and basal supernatants were collected at 4, 8 and 12 hr during stimulation with LPS (a,b), peptidoglycan (c,d) and Poly(I:C) (e,f). Controls are indicated by white bars and hatched bars indicate stimulation with PAMPs. Mean values ± SEM were derived from a minimum of four cell inserts. Significant differences between controls and PAMP treatment are indicated as $^{*}P < 0{\cdot}05$ and $^{**}P < 0{\cdot}01$.

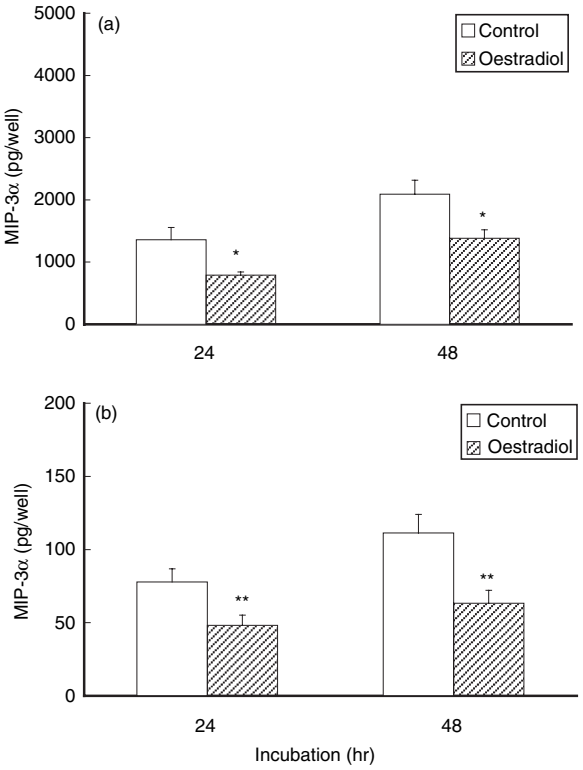

Figure 4. Influence of oestradiol on constitutive CCL20/MIP-3α release by polarized mouse uterine epithelial cells. Polarized uterine epithelial cells were incubated with oestradiol at 10^{-7} M. Open bars represent controls and hatched bars represent cells incubated with oestradiol. Apical (a) and basal (b) supernatants were collected at 24 and 48 hr. Means ± SEM were derived from five cell inserts and data are representative of three separate experiments. Significant differences between Poly(I:C) treatment and controls is indicated as $^{*}P < 0{\cdot}05$ and $^{**}P < 0{\cdot}01$.

collected and analysed for CCL20/MIP-3α. As shown in Fig. 2(a), treatment of epithelial cells with LPS (TLR4), Pam_3Cys (TLR1/TLR2) or Peptidoglycan (TLR2/TLR6) increased both apical and basolateral (bi-directional) secretion of CCL20/MIP-3α. In contrast, the TLR3 agonist Poly(I:C) had no effect on epithelial cell secretion of CCL20/MIP-3α. As seen in Fig. 2(b), loxoribin (TLR7) and CPG (TLR9) induced an increase in basolateral release of CCL20/MIP-3α.

To more fully define the onset of epithelial cell responsiveness to PAMPs, we examined the pattern of CCL20/MIP-3α release at 4-hr time intervals after PAMP addition to the apical media. Following incubation in the presence and absence of PAMPs (control), apical and basolateral media were collected and analysed for CCL20/MIP-3α. Figure 3 shows the amounts of CCL20/MIP-3α in apical (Fig. 3a,c,e) and basolateral (Fig. 3b,d,f) media at 4, 8 and 12 hr of incubation. In this study, apical and basolateral media were replaced at 4-hr intervals. In all cases, PAMPs were present in the apical media throughout the course of the experiment. As seen in Fig. 3(a,b), LPS increased the secretion of CCL20/MIP-3α into both the apical and basolateral compartment within 4 hr of incubation. This increase persisted at 8 and 12 hr, suggesting that secretion in the presence of LPS was continuous. When peptidoglycan was added to the apical chambers of polarized cells, CCL20/MIP-3α secretion increased and persisted over the length of this experiment (4–12 hr) (Fig. 3c,d). In contrast, as seen in Fig. 3(e,f), Poly(I:C) had no effect on CCL20/MIP-3α secretion.

Effect of oestradiol on CCL20/MIP-3α production by uterine epithelial cells

Previously it has been shown that the stage of the menstrual cycle influences the expression of antimicrobials in the human female reproductive tract.[56–59] To determine whether oestradiol acts directly on epithelial cells to regulate cytokine secretion, mouse uterine epithelial cells were grown to confluence on inserts before incubation in the presence or absence of oestradiol (1×10^{-7} M). As seen in Fig. 4(a), oestradiol significantly reduced the release of CCL20/MIP-3α into apical media after 24 and 48 hr of incubation. As shown in Fig. 4(b), oestradiol also inhibited the basolateral release of CCL20/MIP-3α into apical media at 24 or 48 hr. An oestrogen effect on constitutive apical release of CCL20/MIP-3α was seen in all three experiments.

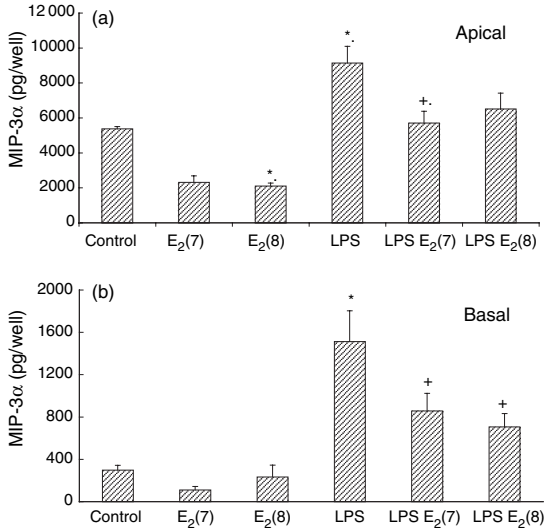

Figure 5. Influence of oestradiol on CCL20/MIP-3α induction by LPS. Polarized mouse uterine epithelial cells were incubated with media alone (controls), oestradiol (E_2) at 10^{-7} M, oestradiol at 10^{-8} M, LPS at 1 μg/ml, LPS plus oestradiol at 10^{-7} M, or LPS (1 μg/ml) plus oestradiol at 10^{-8} M for 48 hr of the TLR4 agonist LPS. (a) 48-hr accumulation of CCL20/MIP-3α per well in the apical compartment. (b) 48-hr accumulation of CCL20/MIP-3α per well in the basolateral compartment. Means ± SEM were derived from a minimum of four cell inserts and data are representative of three separate experiments. Statistical significance of LPS or oestradiol treatment compared to control is indicated as $^{*}P < 0{\cdot}05$ and $^{**}P < 0{\cdot}01$. Statistical significance of LPS plus oestradiol treatment compared to LPS treatment alone is indicated as $^{+}P < 0{\cdot}05$.

To more fully define the effect of oestradiol on constitutive and PAMP-induced release of CCL20/MIP-3α, polarized uterine epithelial cells were incubated for 48 hr in the presence of 10^{-8} and 10^{-7} M oestradiol. As shown in Fig. 5(a,b), constitutive release of CCL20/MIP-3α into the apical compartment was reduced at both concentrations (10^{-8} and 10^{-7} M) and in the basolateral compartment at 10^{-7} M. To determine whether oestradiol affects PAMP-induced CCL20/MIP-3α release, epithelial cells were incubated with LPS and oestradiol, both were added to epithelial cells at time zero. As seen in Fig. 5(a,b), irrespective of the concentration added to the incubation media, oestradiol inhibited the LPS-induced increases in CCL20/MIP-3α secretion into the apical and basolateral compartments. These findings, which are representative of four of four separate experiments, indicate that both constitutive and LPS-induced secretion of CCL20/MIP-3α by polarized mouse uterine epithelial cells is under hormonal control.

Discussion

The results of this study demonstrate that polarized mouse uterine epithelial cells grown on cell inserts preferentially secrete CCL20/MIP-3α into the apical compartment. Exposure of epithelial cells to oestradiol in culture significantly decreases CCL20/MIP-3α secretion into both the apical and basolateral compartment. These studies show that uterine epithelial cell responses to TLR agonists vary with the PAMP used. Stimulation with Pam_3Cys (TLR2/1), peptidoglycan (TLR2/6), and to a lesser extent LPS (TLR4), increased CCL20/MIP-3α in both apical and basolateral secretions within 4 hr. In addition, we found that loxoribin (TLR7) and CPG (TLR9) increased basolateral secretion of CCL20/MIP-3α. When oestradiol was added to the epithelial cell culture along with LPS, LPS-induced increases in CCL20/MIP-3α release were inhibited, indicating that the constitutive and LPS-induced secretion of CCL20/MIP-3α is under hormonal control.

Epithelial cells at mucosal surfaces including the respiratory and gastrointestinal tracts are known to constitutively secrete CCL20/MIP-3α.[32,37–40] More recent studies from our laboratory have shown that CCL20/MIP-3α is constitutively produced in rat uterine epithelial cells and that secretion increases in response to live and heat-killed bacteria and selected PAMPs.[41,42] In the present study, we extend these findings by demonstrating that polarized mouse uterine epithelial cells also produce CCL20/MIP-3α both constitutively and in response to PAMPs. The presence of CCL20/MIP-3α is an important part of the immune readiness as it plays a role in the recruitment and retention of immune cells in the reproductive tract. Others have shown that in mice lacking the CCL20/MIP-3α receptor CCR6, dendritic cells expressing CD11c and CD11b are absent from the subepithelial dome of Peyer's patches and that humoral immune responses to orally administered antigen and to the enteropathic virus rotavirus are impaired.[60] Further studies are needed to demonstrate the central role of this chemokine/cytokine throughout the female reproductive tract.

Interestingly, our studies indicate that polarized mouse uterine epithelial cells preferentially release CCL20/MIP-3α is into the apical compartment. This is in contrast to our rat studies which demonstrated that CCL20/MIP-3α is preferentially released into the basolateral compartment.[41,42] This divergence of secretion polarity is also observed with epithelial cells from human mucosae. Whereas CCL20/MIP-3α was released preferentially into the basolateral compartment by colonic epithelial cells, respiratory epithelial cells were shown to preferentially secrete CCL20/MIP-3α into the apical compartment.[38,39] Just why preferential release varies from site to site and between species remains to be determined. One explanation may reside in the differences in antigen loads seen at different mucosal surfaces. In the reproductive tract, whereas semen is directly deposited into the uterus of the mouse at the time of mating, rat semen is placed in the vagina. In the mouse, uterine antigenic load in the form of sperm, commensal bacteria and sexually transmitted

pathogens, is significantly greater than that seen in the rat uterus.[61] Given the known bactericidal activity of CCL20/MIP-3α and its chemotactic effects on immune cells[35,36] it is likely that the reproductive tract has evolved to minimize the bacterial load in the uterus by secreting CCL20/MIP-3α which acts both as an endogenous microbicide and a chemotactic molecule to recruit immune cells to subepithelial regions of the uterus. It seems likely that CCL20, along with other antimicrobial molecules secreted into the lumen of the uterus, helps to maintain the environment that is necessary for successful procreation in mammals. Whether this occurs without destroying commensal organisms and sperm remains to be determined.

Our studies indicate that mouse uterine epithelial cells are responsive to a number of PAMPs. Recognized as ligands for TLRs, PAMPs added to the apical surface of polarized epithelial cells significantly increased CCL20/MIP-3α secretion both apically and basolaterally within 4 hr of exposure to LPS, Pam_3Cys and peptidoglycan. Moreover, we found that basolateral release of CCL20/MIP-3α occurred with CPG or loxoribin, whereas the addition of the viral PAMP agonist Poly(I:C) had no effect. These studies suggest that epithelial cells are able to respond rapidly by secreting a potent bactericidal agent that has the capacity to destroy or contain potential pathogens. In previous studies, we found that rat uterine epithelial cells respond in a similar fashion to LPS and Pam_3Cys.[42] Since LPS is known to stimulate CCL20/MIP-3α secretion by primary human alveolar and gut epithelial cells, it appears likely that CCL20/MIP-3α secretion in response to pathogens is TLR-mediated and a shared characteristic of mucosal epithelial cells in general.[37–40,62,63] Whether PAMPs act directly on uterine epithelial cells or exert effects that are mediated through the secretion of proinflammatory cytokines by epithelial cells remains to be determined. Others have reported that CCL20/MIP-3α secretion is enhanced by the proinflammatory cytokines interleukin-1β and TNF-α.[64,65] Our finding that TNF-α as well as a number of additional proinflammatory cytokines/chemokines are produced by polarized mouse epithelial cells following peptidoglycan, Pam_3Cys, LPS and Poly(I:C) treatment suggests that PAMP-induced secretion of CCL20/MIP-3α may be either direct and/or indirect (Soboll and Wira, unpublished observation).

Our findings demonstrate that oestradiol inhibits the constitutive secretion of CCL20/MIP-3α by mouse uterine epithelial cells. This finding is complimentary to our previous studies with rat epithelial cells. Under comparable culture conditions, we found that oestradiol, when added at concentrations that saturate the oestradiol receptor, inhibits rat uterine epithelial cell CCL20/MIP-3α secretion.[55] As discussed previously, one interpretation of these findings is that oestradiol produced during the oestrous cycle, suppresses local constitutive CCL20/MIP-3α secretion to enhance the chances for successful fertilization.[55] Unexpectedly, in the present study, we found that oestradiol inhibits PAMP-induced secretion by epithelial cells from mice. This effect is opposite to that seen with rat epithelial cells incubated with oestradiol along with PAMPs.[55] Whereas mouse uterine epithelial cell PAMP-induced secretion of CCL20/MIP-3α was inhibited by the presence of oestradiol, production of CCL20/MIP-3α by rat epithelial cells was stimulated. Since oestradiol levels in blood are known to be elevated at the time of ovulation and mating in rats and mice[66] our findings indicate that oestrogen actions in the reproductive tract are species-specific and must be analysed on a case-by-case basis. To the best of our knowledge, this is the first demonstration that oestradiol inhibits both constitutive and PAMP-induced secretion of CCL20/MIP-3α by mouse uterine epithelial cells.

The present study demonstrates that oestradiol regulation of CCL20/MIP-3α is complex and may be associated with genomic and/or non-genomic effects of oestradiol. Oestradiol is known to influence genes when the liganded oestrogen receptor α binds to an Sp1 promoter sequence,[67,68] which has been demonstrated in the promoter region of the CCL20/MIP-3α gene.[69] This offers an explanation for a mechanism whereby oestradiol could influence the transcription of CCL20/MIP-3α. In other studies, we have found that, whereas treatment of polarized epithelial cells with oestradiol decreases TER, this inhibition is reversed when oestradiol is added to the culture media along with ICI 182,780, an oestradiol receptor antagonist.[70] These studies demonstrated that epithelial tight junctions are directly regulated by oestradiol and are oestradiol-receptor-mediated. In contrast, neither oestradiol nor ICI 182,780 had any effect on epithelial cell secretion of TNF-α.[71] However, when mouse epithelial cells were incubated along with underlying stromal cells, ICI 182,780 blocked the inhibitory effects of oestradiol on epithelial cells secretion of TNF-α. Non-genomic effects of oestradiol in the endometrium have also been reported. For example, treatment of endometrial cells in culture with oestradiol results in Ca^{2+} influx within 10 min, and treatment of ovariectomized rats with oestradiol results in changes in the morphology of uterine epithelial cells within 1 min.[72–74] Some of the non-genomic effects of oestradiol are reported not to be antagonized by ICI 182,780.[74] Further studies are needed to define the mechanism(s) whereby oestradiol exerts its effects on CCL20/MIP-3α production by uterine epithelial cells.

From an endocrine standpoint, the differences between mouse and rat uterine epithelial cell responsiveness to oestradiol are unexpected but not without precedence. For example, in studies examining the effect of oestradiol on uterine epithelial cell antigen presentation of ovalbumin to memory T cells, we found that oestradiol stimulates antigen presentation by rat uterine epithelial cells.[51] In contrast, we found that under the same endocrine

conditions, antigen presentation by mouse uterine epithelial cells is inhibited by oestradiol[54] (Wira and Rossoll, unpublished observation). In other studies, oestradiol stimulates rat uterine epithelial secretion of the polymeric immunoglobulin receptor (pIgR) and the expression of pIgR mRNA by mouse uterine epithelial cells[75,76] (Wira and Rossoll, unpublished observation). When considered along with our CCL20/MIP-3α findings, these results demonstrate the complexity of the female reproductive tract and its responsiveness to endocrine balance in the face of reproductive and pathogenic challenges. What is clear is that immune protection is precisely regulated to ensure reproductive health and fetal survival. As discussed elsewhere[77] protection occurs not through a single mechanism, but through the integrated actions of epithelial cells acting in concert with underlying immune cells in the uterine stroma. Oestradiol regulates uterine epithelial cell functions in a myriad of ways that includes barrier function, immunoglobulin transport, cytokine/chemokine secretion and the production of microbicides such as CCL20/MIP-3α.[55] Overall, these findings suggest that in response to oestradiol, some levels of protection are suppressed at times when others are enhanced to ensure successful fertilization, implantation and pregnancy. Moreover, when considered in the context of different species, endocrine responses are unique and dependent on the reproductive constraints that have evolved in a given species.

In summary, this study indicates that the secretion of CCL20/MIP-3α by uterine epithelial cells is an important part of innate immune defence in the female reproductive tract. Responsiveness to pathogenic stimuli is PAMP-dependent. Furthermore, constitutive secretion as well as LPS-induced production of CCL20/MIP-3α are tightly regulated by oestradiol. It remains to be investigated whether the oestradiol regulation is direct or indirect and whether the hormonal control is geared to successful fertilization and procreation.

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