Herpes Simplex Virus Type 2 Infection during Pregnancy is Correlated with Elevated TLR9 and TNFα Expression in Cervical Cells

Svitich Oxana A., MD¹, Gankovskaya Ludmila V., MD², Lavrov Viacheslav F., MD³, Grigor'eva Oxana Iu., PhD¹, Karaulov Alexander V., MD³, Zverev Vitalii V., MD¹

Abstract—Activation of toll-like receptors (TLRs) by viruses induces secretion of pro-inflammatory cytokines that are suggested to be involved in preterm birth. In our study, we found that infection of cervical epithelial HeLa cells with herpes simplex virus type 2 (HSV-2) is accompanied by a dramatic elevation in TLR9 and TNFα gene expression in time- and dose-dependent manners. We also observed a significant elevation in TLR9, NF-κB and TNFα gene expression in cervical epithelial cells obtained from pregnant women with genital HSV-2 infection as compared to cells collected from healthy pregnant women. In addition, premature births were registered in 81% of pregnant women with genital HSV-2 infection, among which 38% exhibited a considerable increase in the cervical epithelial cell TLR9 gene expression. These data suggest involvement of TLR9, NF-κB and TNFα activation in HSV-2-mediated preterm labor.

Keywords — Toll-like receptor, herpes, innate immunity, TNF, pregnancy.

I. INTRODUCTION

HERPES virus infection is one of the most widespread human viral infections. It is also known that genital herpes virus infection is a main cause for pregnancy complications [1-3]. Indeed, primary infection or reactivation of herpes virus infection (HSV-2) during pregnancy often results in preterm labor, prenatal death or intrauterine transmission of HSV-2 to fetus [4]. Mucosal surfaces serve as the entry sites for the majority of infectious agents, including HSV-2, and provide the first line of anti-infection defense. However, the mechanisms underlying anti-HSV-2 mucosal immunity remain poorly understood. Previous studies have shown that the innate immunity is critically involved in early control of mucosal viral infections including HSV-2 [3, 5]. Innate immune system components, such as toll-like receptors (TLRs), expressed by epithelial cells of female reproductive tract, are able to recognize genomic signatures of viruses [6-11]. In particular, unmethylated CpG motifs of bacterial and viral nucleic acids bind to the endosomal TLR9 [12-14], leading to a strong innate immune response via activation of nuclear factor-κB (NF-κB)-dependent signaling pathway [15]. It is well established that NF-κB regulates the expression of various chemokines and pro-inflammatory cytokines, such as TNFα, IL-12 and IL-1 [11, 13, 15-19], which are presumably involved in preterm birth [20-22]. However, the relationship between the activation of TLR9-mediated signaling pathway in the cervix and preterm birth has not been addressed yet. Following this notion, we studied TLR9, TNFα and NF-κB gene expression in cervical epithelial cells in both healthy and genital HSV-2-infected pregnant women, and found an association between elevation in the expression of these genes in infected women and the occurrence of preterm birth.

II. MATERIALS AND METHODS

Cells and virus
We used epithelial cell line HeLa generated from an adenocarcinoma of the human cervix [23]. Herpes Simplex Virus type 2 (HSV-2, strain MS) was obtained from the National Virus Collection (London, UK).

Clinical groups
Cervical epithelial cells were obtained from pregnant women (n=16) with genital HSV-2 infection and normal pregnant women (n=10) in their third trimester of pregnancy (28-33 weeks of gestation), 24-34 years old. The pregnant women were the patients of the Department of Obstetrics and Gynecology in the Russian State Medical University. The university review board approved the protocol and statement of written informed consent, which was signed by each participant. Samples of vaginal epithelial cells from HSV-2 infected (seropositive) and non-infected participants were collected using swabs that were placed into vials containing 1 ml of polymerase chain reaction (PCR) transport medium and kept at

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¹I.Mechnikov Research Institute of Vaccines and Sera RAMS Moscow, Russian Federation; ²Pirogov Russian National Research Medical University, Moscow, Russian Federation; ³I.M. Sechenov First Moscow State Medical University, Moscow, Russian Federation.

*Correspondence to Svitich, Oxana A. (e-mail: switchos@yandex.ru).
Infection of cells

HeLa cells (6 x 10^5 cells/ml) were seeded in 24-well tissue culture plates (Costar, USA) in DMEM (Sigma, USA) supplemented with 10% fetal calf serum (HyClone, USA), glutamine (PanEco, Russia) and antibiotics (PanEco, Russia), and incubated at 37°C in a humidified atmosphere with 5% CO2 for 48 h and then infected with HSV-2 (2.5 and 3.5 TCID50/0.1 ml). After different periods of time (1, 3, 6, 12 and 24 h post infection), the cells were harvested for RNA isolation.

Isolation of RNA and RT-PCR Analysis

Total RNA was isolated from HeLa cells and cervical epithelial cells by using a RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer’s instructions. For cDNA synthesis, 2 µl of total RNA was combined with random primers (Syntol, Russia) and reverse primers for the target genes (T9rev, NFrev, TNrev shown in Table 1). The mix was heated to 75°C for 3 min and then quick-chilled on ice; 3 µl of 10X Reverse Transcriptase Buffer (SibEnzime, Russia), 2 µl of 2.5 mM dNTP (SibEnzime, Russia) and 10 µl of M-Multi Reverse Transcriptase (SibEnzime, Russia) were added to the mix (final volume of 30 µl). Afterwards, the mix was kept at 37°C for 60 min and at 95°C for 10 min.

For quantitative analysis, real-time PCR was performed, using a SYBR Green Kit for qRT-PCR (Syntol, Russia), according to the manufacturer’s instructions. The primers and probes for the real-time PCR were synthesized by Syntol (Russia) and are listed in Table 1.

The PCR reactions were carried out at 50°C for 2 min, 95°C for 2 min, followed by 35–40 cycles at the primer-specific annealing temperature (shown in Table 1) for 50s and 95°C for 20s. The mRNA expression levels of the target genes were normalized with the expression of the housekeeping gene β-actin.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primers</th>
<th>Zonds</th>
<th>Annealing temperature</th>
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<tbody>
<tr>
<td>TLR9</td>
<td>T9for</td>
<td>ROX-ggc-tga-agt-cca-gtg-tcc-gt</td>
<td>64°C</td>
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<tr>
<td></td>
<td>T9rev</td>
<td>g-g-BHQ2</td>
<td></td>
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<tr>
<td>NFκB</td>
<td>NFfor</td>
<td>R6G-tac-cgt-tgt-ga-cac-tcct-gta-cag-ct</td>
<td>64°C</td>
</tr>
<tr>
<td></td>
<td>NFrev</td>
<td>-aag-BHQ2</td>
<td></td>
</tr>
<tr>
<td>TNFa</td>
<td>TNfor</td>
<td>ROX-gcg-cac-cac-gtg-gaa-cac-tct-gag-tcg</td>
<td>66°C</td>
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<tr>
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<td>TNrev</td>
<td>t-g-BHQ2</td>
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Figure 1. HSV-2-induced TLR9 (A) and TNFα (B) gene expression in the cervical epithelial cell line HeLa. Hela cells were cultured for 48 h and then were infected or not (control) with HSV-2 (2.5 and 3.5 TCID₅₀/0.1ml). At indicated time points after infection, the cells were harvested and TLR9 and TNFα gene expression was analyzed by qRT-PCR. For normalization of the results, the house-keeping gene β-actin expression has been evaluated. The results are presented as the mean ± SEM (for TLR9 in log scale). * p < 0.05; ** p < 0.01.

Figure 2. TLR9 (A), NF-κB (B) and TNFα (C) gene expression in the cervical epithelial cells of pregnant women with either healthy or with genital HSV-2 infection. Cervical epithelial cells were obtained from healthy pregnant women (n=10) and pregnant women with genital HSV-2 infection (n=16). TLR9, NF-κB and TNFα gene expression was analyzed by qRT-PCR. As a normalization control, the house-keeping β-actin gene expression was measured. The results are presented as the mean ± SEM. * p < 0.05; ** p < 0.01.

HSV-2-infected pregnant women as compared with the control group (17 x 10³ ± 2.1 x 10³ vs 3.9 x 10³ ± 0.31 x 10³ transcripts, respectively) (Fig. 2B). Moreover, there was a 3-fold increase in TNFα gene expression in the cervical cells of genital HSV-2-infected pregnant women as compared with the control group (17 x 10⁴ ± 2.4 x 10⁴ vs 5.4 x 10⁴ ± 0.87 x 10⁴ transcripts, respectively) (Fig. 2C). Importantly, clinical observations demonstrated premature births in 81% of pregnant women with genital HSV-2 infection, among which 38% revealed a considerable increase in TLR9 gene expression (4.7 x 10⁶ ± 5.2 x 10⁵). We also show that the levels in the serum of patients with herpes viral infection rise 6.2 times. Importantly, clinical observations demonstrated premature births in 81% of pregnant women with genital HSV-2 infection, among which 38% revealed a considerable increase in TLR9 gene expression (4.7 x 10⁶ ± 5.2 x 10⁵). We also show that the levels in the serum of patients with herpes viral infection rise 6.2 times.

IV. DISCUSSION

Despite advances in the management of pregnancy complications, including premature birth, their incidence remains high and the underlying mechanisms are still poorly understood. Recent animal and clinical observations suggest involvement of the reproductive tract mucosal immunity and in particular TLRs activated by local bacterial and viral infections (Patni S, Flynn P, Wynen LP, et al. An introduction to Toll-like receptors and their possible role in initiation of labour. BJOG 2007; 114:1326-1334).

For example, TLR3 agonist administration induces impairment of uterine vascular remodeling, leading to fetal losses in CBA × DBA/2 mice (Zhang J, Wei H, Wu D, Tian Z. Toll-like receptor 3 agonist induces impairment of uterine vascular remodeling and fetal losses in CBA × DBA/2 mice. J Reprod Immunol. 2007;74:61–67). Upon engagement of TLRs, the intracellular signaling adapter protein myeloid differentiation factor 88 (MyD88) is recruited, leading to a subsequent kinase cascade, which triggers the activation of NFκB pathway. A resultant production of pro-inflammatory cytokines may cause therefore...
a fetal loss (Akira S. Toll-like receptor signaling. J Biol Chem. 2003;278:38105–38108; Thaxton JE, Nevers TA, Sharma S. TLR-mediated preterm birth in response to pathogenic agents. Infect Dis Obstet Gynecol 2010; 2010:378472). Since genital epithelial cells are the first line of mucosal defense against bacterial and viral infections, we exploited the ability of these cells to express the innate immunity TLR9, NF-κB and TNFα genes and observed a significant elevation in TLR9 and TNFα gene expression in HeLa cells cultured in the presence of HSV-2. Interestingly, as compared to TNFα, TLR9 gene expression was found to be more pronounced and its elevation started earlier, confirming previously described sequence of triggered mucosal innate immunity events (Lee AJ and Ashkar AA. Herpes simplex virus-2 in the genital mucosa: insights into the mucosal host response and vaccine development. Curr Opin Infect Dis 2012; 25(1):92-9). Study of pregnant women revealed a significant elevation in TLR9, NF-κB and TNFα gene expression in women genitally infected with HSV-2 as compared to uninfected subjects, thus supporting also the involvement of the transcriptional factor NF-κB in the mucosal innate immunity activation following genital HSV-2 infection. This nuclear factor is present in unstimulated cells in its inactive form and after stimulation it translocates into the nucleus, after which it binds promoters of target genes [18], leading to transcription of pro-inflammatory cytokine genes, such as TNFα. Importantly, this cytokine along with its pro-inflammatory and cytotoxic activity against infected cells has been shown to play a role in pathology of different pregnancy complications [20-22], and its elevated levels are correlated with miscarriage and preterm labor [25]. In support, together with elevated levels of TLR9, NF-κB and TNFα gene expression in genital epithelial cells obtained from HSV-2 infected pregnant women, we also found that 81% of these women revealed premature births, among which 38% of the women demonstrated a considerable increase of TLR9 gene expression. In line with previous observations, we suggest a possible causal relationship between TLR9, NF-κB and TNFα activation following HSV-2 infection and preterm birth. Moreover, the results of the present study are consistent with our previous findings, demonstrating elevated TLR2 gene expression in cervical cells in pregnant women with genital bacterial infection as compared to healthy pregnant women. In addition, more than 70% of infected pregnant women revealed premature birth [26]. Collectively, our data suggest that the redundant expression of TLRs in genital epithelial cells in pregnant women with herpes virus infection or other types of genital infections results in increased production of pro-inflammatory cytokines, such as TNFα, thus leading to various pregnancy complications including preterm birth. Obviously, further studies on TLRs and their signaling pathways are necessary to elucidate the role of innate immunity in the development of pregnancy complications. Moreover, the capability of genital epithelial cells to be directly activated by both bacterial and viral TLR ligands to induce an innate anti-viral state [27] may help to develop new targets for the therapy of bacterial and viral infections during pregnancy.

V. CONCLUSIONS

Premature births were registered in 81% of pregnant women with genital HSV-2 infection, among which 38% exhibited a considerable increase in the cervical epithelial cell TLR9 gene expression. These data suggest involvement of TLR9, NF-κB and TNFα activation in HSV-2-mediated preterm labor.

REFERENCES


