Roles of Toll-like Receptor 3 in Intrauterine Transmission of Hepatitis B Virus in Mothers with High Viral Load

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Introduction

Chronic hepatitis B virus (HBV) infection leads to chronic hepatitis, liver failure, cirrhosis and hepatocellular carcinoma [1]. Mother-to-infant transmission (MTIT) is one of the main mechanisms of hepatitis B virus (HBV) transmission in pregnant mothers with chronic HBV infection [2,3]. Although long-term antiviral therapy and passive-active immunoprophylaxis are available, there are still a few newborns who acquire HBV infection [4].

There are three possible routes for MTIT [5]. Currently, MTIT of HBV primarily occurs by intrauterine transmission, accounting for 13-44% of HBV transmission [6]. A growing number of studies compared mothers with high HBV viral load (ranged from 10^4-10^6 copies/ml) or with HBeAg positivity to those with low HBV DNA levels or HBeAg negativity during gestation. They found that intrauterine transmission was more likely to occur during gestation in people with high viral load or HBeAg positivity, leading to transmitting HBV to their babies [7-11]. Reduction of HBV DNA levels below 10^6 copies/ml reduced the risk by approximately 30% [12]. According to evidence from the European Association for the Study of Liver (EASL), mothers with HBV DNA of >10^5-10^6 copies/ml require antiviral therapy to reduce the risk of MTIT [7,13,14], and the Chinese Guidelines for the Prevention and Treatment of Chronic Hepatitis B (version 2019) determined that mothers with HBV DNA of >2×10^5 IU/ml require antiviral therapy to prevent MTIT [15]. Many studies have shown that the potential mechanisms for intrauterine transmission include cellular transmission via peripheral blood mononuclear cells (PBMCs), placental infection, trans-placental leakage and germline transmission [5]. However, the precise molecular and immunological mechanisms about MTIT with HBV infection are not well understood.

Innate immune responses against invading pathogens at the placental barrier play a critical role to protect the fetus. Toll-like receptors (TLR) help trophoblast cells to recognize and induce a subsequent immune response, as a critical sensor of innate immunity [16]. TLRs identify different pathogen-related pattern molecules, mediate the activation of intracellular signaling pathways, and induce the production of cytokines [17]. Ten functional human TLRs have been characterized [18]. We found the expression of TLRs in immune cells and in the placental trophoblast that have no immune function [19]. Previous studies have reported that primary placental trophoblast cells are superior to non-trophoblast cells in resisting viral infection [20] and as macrophages, placental trophoblasts are not able to recognize pathogens and mediated the immune response; however, TLRs help them in this function [21],
and in the prevention of intrauterine HBV transmission, as HBV translocation can be blocked from infecting trophoblasts by TLR7 and TLR8 on trophoblastic cells \[22\]. However, the research on placental TLRs and the mechanisms of intrauterine transmission of HBV are still scarce.

Previous studies have demonstrated that decreased TLR3 expression is associated with attenuated immune responses against HBV and that TLR3 plays significant roles against HBV \[23,24\]. We hypothesized that TLR3 also contributes to the prevention of intrauterine transmission of HBV in mothers with high viral load.

To verify this hypothesis, the expression of TLR3 was measured in placental samples of mothers with different levels of HBV viral load and with HBeAg positivity or negativity and in an in vitro cell model exposed to HBV with different viral loads.

Methods

Subjects and sample collection

Pregnant women with CHB and healthy pregnant women were recruited from the Gynecology and Obstetrics Research Center of Beijing Youan Hospital, Capital Medical University, from January to December of 2016 during their delivery. Pregnant women who were infected with hepatitis A, C, D, and E viruses, Epstein-Barr virus, cytomegalovirus (CMV), human immunodeficiency virus (HIV) and other diseases were excluded. All infants born to HBV-positive mothers were provided the standard passive and active immunoprophylaxis strategy.

Placental samples from 25 healthy and 144 HBsAg-positive pregnant mothers delivering at term were collected. They all had singleton pregnancies. Among all HBsAg-positive pregnant mothers, 87 were divided into a low load group (HBV loads <10^6, 35 cases), a medium loads group (10^6≤ HBV loads <10^7, 34 cases) and a high load group (HBV loads ≥10^7, 18 cases) according to their HBV viral load, 57 were divided into a HBeAg-negative group (30 cases) and a HBeAg-positive group (27 cases) according to their HBeAg expression. Furthermore, only 2 placent al samples were collected from HBV-positive pregnant women with their infants were infected via intrauterine transmission (infant-infected group). Placental samples were reserved immediately after delivery of the placenta. We dissected placental cotyledons at the middle zone. The samples were then washed three times with cold phosphate buffer solution (PBS). Placental samples were harvested and prepared for mRNA extraction. Both the mRNA and placenta samples were placed at -75ºC for subsequent analysis.

The definition of newborn infection: newborn babies were tested their venous blood after combined immunization (at the age of 7 months), HBs Ag and HBV DNA positive with or without HBeAg positive. The patients believed that newborn infection occurred.

The protocol of the current investigation was approved by the Ethics Committee of Beijing Youan Hospital, Capital Medical University, Beijing, China (approval ID: [2016]15). Informed consents were obtained from all the participants. Our procedures were in accordance with the ethical standards of the responsible committee on human experimentation and with the Helsinki Declaration of 1975, as revised in 1983.

Clinical data collection

Laboratory results of pregnant mother with HBV infection including biochemical tests and HBV DNA, were obtained from the electronic medical record system and hospital paper charts in Beijing Youan Hospital.

RNA preparation and quantitative RT-PCR analysis

Total RNA was extracted from placental tissues, its concentration was determined, and the sample was quantified.

The RNA was then reverse-transcribed into cDNA using a Prime Script First Strand cDNA Synthesis Kit (TaKaRaBio, Inc., Otsu, Japan), according to the manufacturer’s protocol. The reaction system was 20µl, and the PCR amplification was performed using a quantitative PCR instrument (ABI Prism 7500; Applied Biosystems Inc. Waltham, MA, USA). The reaction was processed with an initial 2 min denaturation step at 50°C, followed by 95°C for 5 min, 95°C for 15 secs, and 60°C for 30 secs, for 40 cycles, and then 55°C for 4 secs for 41 cycles. The mRNA levels were calculated using the 2^−\&Delta;\Delta^Ct method \[25\]. The hypoxanthine phosphoribosyl transferase (HPRT) gene was selected as an endogenous control.

Western blot analysis

Protein was extracted from placental tissues in RIPA buffer with phosphatase and protease inhibitors. Equivalent total protein amounts were determined using the BCA assay. Proteins were separated via SDS-12% polyacrylamide gel electrophoresis (PAGE) and were transferred to a PVDF membrane (Bio-Rad, CA). We used antibodies against TLR3 and β-actin (Cell Signaling Technology Inc, CA) (1:1000). Membranes were probed overnight at 4°C. Secondary antibody was then incubated (1:2000, Cell Signaling Technology Inc, CA). The proteins were then visualized using horseradish Supersignal West Pico Chemiluminescent substrates (Thermo Fisher Scientific, Rockford, IL).

Immunofluorescence staining

Frozen section specimens of placenta were fixed with cold methanol. A buffer containing 0.1% Triton X100 induced membrane rupture. The sections were washed with PBS and blocked by 10% goat serum at 37ºC. A mouse monoclonal antibody was incubated with the specimens overnight at 4 ºC. Secondary antibody was then incubated (1:2000, Cell Signaling Technology Inc, CA). The proteins were then visualized using horseradish Supersignal West Pico Chemiluminescent substrates (Thermo Fisher Scientific, Rockford, IL).

Cell culture and HBV exposure test

HBV carriers samples (normal liver function) with different levels of HBV DNA and those from healthy volunteers were collected. Serum samples filtered with a 0.22µm ion device (Costar Co., Ltd.) to eliminate any bacteria. Then, samples were inactivated and stored aseptically at 56°C for 30 min.

JEG-3 cells were grown in DMEM/F12 (1:1) medium supplemented with 10% fetal bovine serum (FBS) and antibiotics at 37 ºC, at 5% CO2. Cells were seeded at 2×10^5 cells/mL and cultured over 24 hours, after which the medium was changed. For the different viral loads treatment, once cells reached a confluence of 50-60%, serum samples from healthy volunteers and those of HBV carriers with different viral loads (10^2, 10^5, 10^6, and 10^9) were added for 48 hours’ incubation. For the different treatment times, serum from HBV carriers with different viral load (2.4×10^4)
was incubated for 3, 6, 12 and 24 hours. We washed the treated cells three times with PBS and collected them. We repeated the experiments at least three times.

Statistical analysis
Results are expressed as the mean ± standard deviation for continuous variables and as number and percentage for categorical variables. Kruskal-Wallis analysis of variance (ANOVA) compared the differences between the groups. SPSS 13.0 was used for all analyses. A 2-sided P <0.05 was considered significant.

Results
Clinical data
The clinical characteristics of the different HBV viral load mother groups are shown in Table 1. There were significant differences in alanine aminotransferase (ALT) and aspartate aminotransferase (AST) expression among the normal, low viral load, medium viral load and high viral load groups. The clinical characteristics of the different HBeAg expression mother groups are shown in Table 2. There was a significant difference in AST expression among the normal, HBeAg-negative and HBeAg-positive groups.

Table 1. Clinical data of different HBV viral load groups

<table>
<thead>
<tr>
<th></th>
<th>Normal group (n=25)</th>
<th>Low virus load (HBV loads ≤10^3, n=35)</th>
<th>medium viral load (10^3&lt; HBV loads ≤10^6, n=34)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal age(y)</td>
<td>30.4±5.9</td>
<td>31.2±3.9</td>
<td>30.2±4.3</td>
<td>0.75</td>
</tr>
<tr>
<td>Gestational weeks (w)</td>
<td>39.0±1.2</td>
<td>38.9±1.3</td>
<td>39.0±1.3</td>
<td>0.973</td>
</tr>
<tr>
<td>Neonatal birth weight(g)</td>
<td>3357.1±418.8</td>
<td>3304.6±576.</td>
<td>3304.1±392.7</td>
<td>0.975</td>
</tr>
<tr>
<td>Alanine aminotransferase (ALT, U/L)</td>
<td>18.1±2.2</td>
<td>20.1±8.7</td>
<td>32.8±11.1</td>
<td>0.013</td>
</tr>
<tr>
<td>Aspartate aminotransferase (AST, U/L)</td>
<td>19.5±2.8</td>
<td>23.6±9.9</td>
<td>34.6±12.7</td>
<td>0.022</td>
</tr>
<tr>
<td>Alkaline phosphatase (ALP, U/L)</td>
<td>110.38±50.1</td>
<td>125.1±63.7</td>
<td>136.9±53.5</td>
<td>0.237</td>
</tr>
<tr>
<td>Total bilirubin (TBil,µmol/L)</td>
<td>8.2±3.1</td>
<td>9.6±5.9</td>
<td>10.7±6.8</td>
<td>0.331</td>
</tr>
<tr>
<td>Direct bilirubin (DBil,µmol/L)</td>
<td>3.2±1.3</td>
<td>3.6±2.6</td>
<td>3.8±3.5</td>
<td>0.77</td>
</tr>
<tr>
<td>HBV DNA (log_{10} copies/mL)</td>
<td>-</td>
<td>2.47±0.7</td>
<td>5.53±2.3</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 2. Clinical data of different HBeAg expression groups

<table>
<thead>
<tr>
<th></th>
<th>Normal group (n=25)</th>
<th>HBeAg positive (n=30)</th>
<th>HBeAg negative (n=27)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal age(y)</td>
<td>30.4±5.9</td>
<td>30.9±3.91</td>
<td>29.9±4.46</td>
<td>0.213</td>
</tr>
<tr>
<td>Gestational weeks (w)</td>
<td>39.0±1.2</td>
<td>38.7±1.3</td>
<td>39.2±1.2</td>
<td>0.249</td>
</tr>
<tr>
<td>Neonatal birth weight(g)</td>
<td>3357.1±418.8</td>
<td>3264.4±544.8</td>
<td>3320.2±427.3</td>
<td>0.538</td>
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<tr>
<td>Alanine aminotransferase (ALT, U/L)</td>
<td>18.1±2.2</td>
<td>18.8±18.92</td>
<td>24.0±20.36</td>
<td>0.158</td>
</tr>
<tr>
<td>Aspartate aminotransferase (AST, U/L)</td>
<td>19.5±2.8</td>
<td>24.3±1.85</td>
<td>30.1±13.76</td>
<td>0.027</td>
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<tr>
<td>Alkaline phosphatase (ALP, U/L)</td>
<td>110.38±50.1</td>
<td>131.8±56.56</td>
<td>136.0±55.26</td>
<td>0.689</td>
</tr>
<tr>
<td>Total bilirubin (TBil,µmol/L)</td>
<td>8.2±3.1</td>
<td>11.0±7.34</td>
<td>9.1±2.8</td>
<td>0.089</td>
</tr>
<tr>
<td>Direct bilirubin (DBil,µmol/L)</td>
<td>3.2±1.3</td>
<td>3.5±1.4</td>
<td>3.8±2.9</td>
<td>0.127</td>
</tr>
<tr>
<td>HBV DNA (log_{10} copies/mL)</td>
<td>-</td>
<td>5.86±2.5</td>
<td>4.67±3.5</td>
<td>-</td>
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</tbody>
</table>
expressed differently in placentas between healthy and CHB women. Compared with healthy pregnant women, TLR2 and TLR9 were decreased, while TLR3 and TLR7 were increased in the placentas of CHB women. Therefore, the expression levels of TLRs are differently regulated in the placentas of pregnant women with CHB.

Expression levels of TLR3 in the placentas of pregnant women with different viral loads
Although a clear correlation between the high levels of maternal serum HBV and MTIT has been established in clinical practice, the underlying role of TLR3 in HBV intrauterine infection remains elusive. In this paper, we focus on exploring the relationship between TLR3 expression in placentas and HBV intrauterine infection. Herein, the results showed that the mRNA and protein levels of TLR3 were significantly upregulated in the medium load viral group (10^6 ≤ HBV loads < 10^9) and the high load viral group (HBV loads ≥ 10^9), while they were not significantly changed in the low load viral group compared with the normal control group (Fig. 2A, B). Interestingly, compared to the medium load viral group, the levels of TLR3 were markedly immunofluorescence measurement confirmed the expression tendency of TLR3 in the placentas of pregnant women with different viral loads (Fig. 2C).

Expression levels of TLR3 in the placentas of pregnant women with different HBeAg expression
Previous studies have shown that HBeAg-positivity in pregnant women is an important high-risk factor for vertical transmission of HBV. Previous studies have shown that HBeAg-positivity in pregnant women with different HBeAg expression is demonstrated that an “immune barrier” protects HBV from invading the fetus from the mother, which is formed by hepatitis B immunoglobulin in the placenta and autophagy plays a mechanism of antiviral defense at the maternal-fetal interface. As is already known, TLRs play an important role in the chronic persistent infection of HBV; however, their roles in the mechanisms of HBV intrauterine infection are not clear. The results have shown that TLR7 and TLR8 on trophoblastic cells inhibit HBV translocation across trophoblasts, indicating an important role in the prevention of intrauterine HBV transmission: expression of TLR7 was significantly higher in HBV-positive women whose infants were noninfected (noninfected group) than HBV-positive women whose infants were infected (infected group), and TLR8 expression was significantly higher in the noninfected group than in the control. HBV can inhibit the secretion of TLR9 in HBV-positive women to some extent, but HBeAg can stimulate the secretion of TLR9, and with the increased severity of intrauterine transmission of HBV, the level of TLR9 expression in HBV-positive women is increased. Our results further demonstrated that, among all TLRs, the expression of TLR2, TLR3, TLR7 and TLR9 showed significant difference in the placenta between normal women and HBV-positive women; however, the rest of TLRs showed no significant difference. Moreover, the levels of TLR3, and TLR9 were increased in the placentas of HBV-positive women. Therefore, the research on the mechanism of intrauterine infection of HBV and the involvement of TLRs in regulating placental immunity is worthy of further study and exploration.

Discussion
Maternal HBV DNA levels and maternal HBeAg status are the most important factors affecting mother-to-child transmission of HBV, and HBV anti-viral treatment during pregnancy can significantly reduce the maternal viral load and the intrauterine infection rate. The placenta shields the embryo from the spread of pathogens, including HBV. TLR3 is one of the major signaling molecules in the Toll-like receptor family. In this paper, the relationships between TLR3 with HBV-DNA loads or maternal HBeAg status in the placenta of pregnant women were further explored. Our results demonstrated that TLR3 is highly expressed in the placentas of HBV-infected mothers compared with the placentas of normal mothers; among placentas of mothers with different viral loads, the expression level of TLR3 in placentas of mothers with 10^6 viral load was the highest, and TLR3 expression showed a downward trend in the placentas of mothers with >10^9 HBV loads. Compared with HBeAg-negative mothers and infected-infants’ mothers, TLR3 expression levels in the placentas of HBeAg-positive mothers and uninfected-infants’ mothers were higher, respectively. Taken together, TLR3 may play a critical role in evolutionary adaptation to enhance the protection of the developing fetus against HBV intrauterine transmission.
Fig. 2. Expression levels of TLR3 in placenta of pregnant women with different viral loads.
A. Gene expression of TLR3 was measured by quantitative real-time PCR.
B. Protein expression of TLR3 and β-actin was measured by western blot. A representative blot from three placental tissues of normal group and different viral loads groups is shown.
C. The protein levels of TLR3 were measured with immunofluorescent assays in placental tissue of normal subjects and different viral loads groups (200×).

Fig. 3. Expression levels of TLR3 in placenta of pregnant women with different HBeAg expression.
A. Gene expression of TLR3 was measured by quantitative real-time PCR.
B. Protein expression of TLR3 and β-actin was measured by western blot. A representative blot from three placental tissue of normal group, HBeAg negative group and HBeAg positive group is shown.
C. The protein levels of TLR3 were measured with immunofluorescent assays in placental tissue of normal group, HBeAg negative group and HBeAg positive group (200×).
Fig. 4. Expression levels of TLR3 in of pregnant women with infant-uninfected and infant-infected group, and in JEG-3 cell exposed to HBV.
A. Protein expression of TLR3 and β-actin was measured by western blot. A representative blot from three placental tissue of normal group, four samples of infant-uninfected group and two samples of infant-infected group is shown.
B. The protein levels of TLR3 were measured with immunofluorescent assays in placental tissue of normal group, infant-uninfected group and infant-infected group (200×).
C. Gene expression of TLR3 was measured by quantitative real-time PCR in JEG-3 cell exposed to different HBV loads for 24 hours.
D. Gene expression of TLR3 was measured by quantitative real-time PCR in JEG-3 cell exposed to different HBV loads for 24 hours. A representative blot from three independent experiments is shown.
E. Protein expression of TLR3 and β-actin was measured by western blot in JEG-3 cell exposed to different HBV loads for 24 hours. A representative blot from three independent experiments is shown.
F. Protein expression of TLR3 and β-actin was measured by western blot in JEG-3 cell exposed to 10^8 HBV loads for different times. A representative blot from three independent experiments is shown.

The placental barrier is trophoblast cells, which are in direct contact with maternal blood. Trophoblast cells may play an important role in the defense against HBV intrauterine infection. It has been shown that trophoblast cells in placental explants can produce inflammatory cytokines, such as IL-6, IL-8, and nitric oxide, in a full-term pregnancy. The above cytokines have potent antibacterial properties, after TLR is engaged by zymosan or LPS (32,33). Subsequent studies have shown that TLR3 expression is downregulated in the livers of patients with chronic hepatitis B compared with normal controls (34). Some reports have shown that TLR3 is underexpressed in monocyte-derived dendritic cells from hepatitis B patients (35). Our study showed that, compared with normal controls, the mRNA and protein expression levels of TLR3 increased first and then decreased with the increase of virus gradient; the mRNA and protein expression levels of TLR3 in the placenta of HBeAg-negative pregnant women increased slightly, while TLR3 expression in HBeAg-positive pregnant women was significantly elevated in the placenta. The above results indicate that placental trophoblast cells in CHB pregnant women with low HBV loads may induce an innate immune response through TLR3 and resist HBV infection and invasion of trophoblast cells, but for pregnant patients with CHB with high HBV loads, the persistence of HBV in the maternal blood causes functional depletion of placental TLR3, further causing low expression of TLR3. Moreover, our results also showed that, for CHB pregnant women with different expression levels of HBeAg, antigen secretion is positively correlated with TLR3 expression in placenta, which is the same trend in the liver of CHB pregnant women. Other studies have shown that HBeAg is not involved in the replication and assembly of HBV (36); however, it is also believed that HBeAg is an immune tolerance factor that is related to specific T cell tolerance, and that can cause immune tolerance in the body and allow HBV persistent infection to escape immune clearance. In our experiments, with the increase of HBeAg, the expression of TLR3 increased correspondingly, indicating that the HBV virus and the organism play a game, between immune tolerance and immune escape, resulting in persistent infection and intrauterine infection. The specific mechanism needs further research.

Above all, we hypothesize that high viral HBV load (≥10^6) may regulate the expression of placental TLR3 by HBeAg (TLR3 depletion) and interfere with the recognition and presentation of HBV antigen by placental immune cells, leading to HBV immunization, and HBV escape, which leads to infection and persistent infection of maternal placenta HBV. Ultimately, there was an increased chance of intrauterine infection.

However, there are some flaws in this article. The placenta sample of pregnant women with infected infants was too small to adequately explain the phenomenon. The high success rate of newborn passive-active immunoprophylaxis made sample accumulation difficult and rare. The long period of pregnancy and infection is complex and multifactorial. Because of the above reasons, our experiments in vitro preliminarily tested the physiological phenomenon after HBV infection of the mother’s cells in the placenta during gestation.
Conclusions

We observed the expression of TLRs was differently regulated in the placentas of pregnant women with CHB; the mRNA and protein levels of TLR3 were significantly upregulated in the medium load viral group ($10^6 \leq \text{HBV loads} < 10^8$) and the high load viral group (HBV loads $\geq 10^8$), while there was no significant change in the low load viral group compared with the normal control group. The mRNA and protein levels of TLR3 were lower in the placentas of pregnant women with infected infants compared with pregnant women with uninfected infants. The expression of TLR3 was upregulated in $10^6$ and $10^7$ IU/ml HBV exposed for 24 hours, and was downregulated in $10^5$ IU/ml HBV exposed for 24 hours in Jeg-3 cells exposed to HBV in vitro. Therefore, we think that TLR3 contributes to HBV intrauterine infection for mothers with a high viral load and, importantly, prevents mother-to-infant transmission.

Abbreviations

ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; ALP, alkaline phosphatase; TBil, total bilirubin; DBil, direct bilirubin; HBV, Hepatitis B virus; MTIT, Mother-to-infant transmission; EASL, European Association for the Study of Liver; PBMCs, peripheral blood mononuclear cells; TLR, toll-like receptors; PAMPs, pathogen-associated molecular patterns; CMV, cytomegalovirus; HIV, human immunodeficiency virus; PBS, phosphate buffer solution; HPRT, hypoxanthine phosphoribosyl transferase; PAGE, polyacrylamide gel electrophoresis; FBS, fetal bovine serum.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Beijing Youan Hospital, Capital Medical University. Written informed consent was obtained from all participants.

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Conflict of interest

The authors declare that they have no competing interests.

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Authors’ contributions

FR and ZPD designed the experiments. HG and LX performed the experiments and wrote the manuscript. XYZ supervised the pathological observations. ZHF and HZ prepared the samples and collected the data. YT performed statistical analyses. All authors have read and approved the submission of the manuscript.

References


