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TLR10 PLAYS A ROLE IN MEDIATING BACTERIAL PEPTIDOGLYCAN-INDUCED TROPHOBLAST APOPTOSIS

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Abstract

Problem—There is a strong correlation between intrauterine bacterial infection and preterm labor. While inflammation is a common mechanism, certain pathogens may trigger placental apoptosis. TLR2 activation by gram-positive bacterial peptidoglycan (PDG) induces first trimester trophoblast apoptosis and decreased IL-6 secretion. This is dependent upon the presence of TLR1 and the absence of TLR6, both TLR2 co-receptors. Since TLR10 is also a TLR2 co-receptor, the objective of this study was to determine its expression and function in the trophoblast.

Method of Study—First and third trimester human placental tissue and isolated trophoblast were evaluated for TLR10 expression. A first trimester human trophoblast cell line, stably transfected with a TLR10 dominant negative (TLR10-DN) or vector control was treated with or without PDG and analyzed for apoptosis and IL-6.

Results—TLR10 was expressed by trophoblasts during the first and third trimesters of pregnancy. PDG-induced trophoblast caspase-3 activity was inhibited by the presence of the TLR10-DN. The presence of the TLR10-DN had no effect on PDG- reduction in trophoblast IL-6 secretion.

Conclusion—This study demonstrates that trophoblast TLR10 plays a role in promoting apoptosis triggered by gram-positive bacterial components, and suggests that TLR10 may regulate the balance between trophoblast survival and cell death.

INTRODUCTION

There is a strong correlation between bacterial infection and adverse pregnancy outcomes, such as preterm labor. The mechanism by which a bacterial infection can lead to preterm birth is thought to involve innate immune Toll-like receptors (TLRs), which can trigger inflammation at the maternal-fetal interface¹. While inflammation is a frequent and common mechanism², pathogens also represent a potential trigger for placental apoptosis, and certain innate immune Toll-like receptors (TLRs) can mediate this response³. We previously demonstrated that TLR2 activation by gram-positive bacterial peptidoglycan (PDG) triggers human first trimester trophoblast cells to undergo apoptosis, concomitant with decreased constitutive IL-6 secretion^{3, 4}. Similarly, *in vivo* administration of PDG to pregnant mice early in pregnancy triggers excessive placental apoptosis in the absence of an inflammatory response⁴.

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TLR2 utilizes other TLRs as co-receptors to signal. Thus, TLR2 may either homodimerize with itself, or heterodimerize with either TLR1 or TLR6⁵. Human first trimester trophoblast cells express TLR2 and TLR1, but not TLR6³. The absence of TLR6 is what confers the cell's sensitivity to PDG-induced apoptosis and decreased IL-6 secretion, such that ectopic expression of this co-receptor prevents PDG-induced apoptosis and restores constitutive chemokine production⁴. Recently, human TLR10 has also been characterized as a TLR2 co-receptor and is able to interact with TLR2 and TLR1, as well as associating with itself^{5, 6}. Therefore, the objective of this study was to determine the expression and function of TLR10 in the trophoblast.

MATERIALS AND METHODS

Reagents and antibodies

Peptidoglycan (PDG) isolated from *Staphylococcus aureus* was purchased from InvivoGen (San Diego, CA). The rabbit polyclonal antibody to human TLR10 (#NB110-83980SS) was purchased from Novus Biologicals (Littleton, CO). The mouse anti-HA monoclonal antibody (#ab-hatag) was purchased from Invivogen. The rabbit polyclonal antibody for human beta-actin (β -actin) was purchased from Sigma Aldrich (St Louis, MO).

Trophoblast cell lines

The human first trimester trophoblast cell lines, 3A and HTR-8/SVneo (H8) were used in these studies^{3, 4, 7}. Cells were cultured in RPMI 1640 (Gibco), supplemented with 10% fetal bovine serum (Hyclone, South Logan, UT), 10mM Hepes, 0.1mM MEM non-essential amino acids, 1mM sodium pyruvate and 100nm penicillin/streptomycin (Gibco). Cells were maintained at 37°C/5% CO₂.

Isolation of primary trophoblast cells

Primary cultures were prepared from first trimester placentas (7 – 12 weeks gestation) that were obtained from elective terminations of normal pregnancies performed at Yale-New Haven Hospital (n=4). Placental tissue was also obtained from uncomplicated normal term pregnancies (37–41 weeks of gestation) collected after elective caesarean section without labor (n=5). The use of patient samples was approved under Yale University's Human Investigations Committee. First trimester and third trimester trophoblast cultures were isolated as previously described^{8, 9}.

Transfection of trophoblast cells with TLR10-DN

The 3A trophoblast cell line was transfected with either the pZERO-HA plasmid containing a dominant-negative form of the human TLR10 gene (TLR10-DN) that lacks the signaling TIR domain, or the p-ZERO vector control (Invivogen). Stable cell lines were generated by selecting with puromycin (50 μ g/ml), and transfection was confirmed by performing Western blot analysis for the HA tag.

Immunohistochemistry

The cellular localization of TLR10 expression in first trimester and term placenta was performed as previously described⁸. Briefly, 5 μ m sections of placenta were incubated with either the anti-TLR10 polyclonal antibody at 1:1000 or non-specific rabbit IgG. Specific staining was detected using a biotinylated goat anti-rabbit antibody at 1:500 dilution (Vector Laboratories; Burlingame, CA) with DAB substrate (Vector Laboratories). Tissue sections were counterstained with haematoxylin (Sigma) and visualized by light microscopy.

Western blot analysis

For analysis of proteins, Western blot analysis was performed as previously described⁹. For TLR10 analysis, membranes were incubated overnight at 4°C with the anti-TLR10 polyclonal antibody at 1:2000 in PBS-Tween/1% goat serum. Following this incubation, membranes were washed and then incubated with the goat anti-rabbit IgG secondary antibody conjugated to peroxidase (Vector Labs; Burlingame, CA) diluted 1:10,000 in PBS-Tween/1% goat serum. For detection of the HA tag, membranes were incubated overnight at 4°C with the anti-HA mAb at 1:1000 in PBS-Tween/1% milk and then incubated with the horse anti-mouse IgG secondary antibody conjugated to peroxidase (Vector Labs; Burlingame, CA) diluted 1:10,000 in PBS-Tween/1% milk. β -actin was used as loading control. Images were captured using the Gel Logic 100 and analyzed using Kodak MI software (Eastman Kodak, Rochester, NY).

Apoptosis and IL-6 studies

Stable trophoblast cell lines expressing either the vector control or the TLR10-DN were treated with or without PDG (80 μ g/ml) in OptiMEM (Invitrogen; Carlsbad, CA). Following a culture of 48 hours, cell lysates and cell-free supernatants were collected as previously described⁴. For apoptosis, the levels of caspase-3 activity in cell lysates was measured using the caspase-glo assay (Promega, Madison, WI)⁴. Concentrations of IL-6 in supernatants were quantified by ELISA (R & D Systems, Minneapolis, MN).

Statistical analysis

Experiments were performed three times in triplicate. Data are expressed as mean $\pm$ standard deviation (S.D.). Statistical significance ($p < 0.05$) was determined using the paired student's *t*-test.

RESULTS

Immunohistochemistry revealed that TLR10 protein was expressed in first and third trimester placenta and was localized to the villous and extravillous trophoblast cells (Figure 1A). This was confirmed by Western blot analysis. As shown in Figure 1B, the human first trimester trophoblast cells line, H8 and 3A, as well as isolated primary human first and third trimester trophoblast cells expressed TLR10 protein (94kDa). Densitometry showed TLR10 expression levels were not significantly different between first and third trimester trophoblast cells (Figure 1B).

Having established TLR10 expression by the trophoblast, we next investigated its function in first trimester cells. For this, the trophoblast cell line (3A) was stably transfected with either a TLR10-DN or a vector control. Since apoptosis is often mediated through activation of the caspase pathway, and we have previously demonstrated the PDG-induced trophoblast apoptosis and decreased cell viability is directly associated with elevated caspase-3 activity^{3, 4}, we used this as a marker of apoptosis. As shown in Figure 2, PDG induced a significant increase in caspase-3 activity, when compared to untreated cells and this response was completely inhibited by the presence of the TLR10-DN (Figure 2A). In contrast, PDG significantly reduced trophoblast cell secretion of IL-6 similarly in both the TLR10-DN and vector control cells (Figure 2B).

DISCUSSION

The way in which an infection of gestational tissues, such as the placenta, can lead to pregnancy complications like preterm birth, is thought to involve innate immune inflammatory responses towards the pathogen². Experimental models have demonstrated

that delivery of bacteria or bacterial components to a variety of animals triggers preterm labor, and that TLRs may play a role^{1, 10}. Moreover, clinical and experimental studies have extensively correlated placental infection and inflammation with preterm birth^{1, 10}. However, infection-associated apoptosis of the placenta has also gained more recent attention as an alternative mechanism of placental pathology and preterm labor^{4, 11}. Administration of either peptidoglycan isolated from *Staphylococcus aureus* or heat-killed group B Streptococcus, both Gram-positive organisms, induces preterm birth and placental apoptosis in mice^{4, 11, 12}. Furthermore, bacterial PDG, a TLR2 agonist, induces apoptosis in human first trimester trophoblast cells^{3, 4}. In a previous study we found that PDG-induced apoptosis in first trimester trophoblast was dependent upon the expression of TLR2 and its co-receptor, TLR1^{3, 4}. This apoptotic response was also associated with reduced chemokine secretion and NF κ B activity⁴. Moreover, this PDG-induced response could be prevented by the presence of another TLR2 co-receptor, TLR6, which these cells normally lack^{3, 4}. In this current study we sought to determine the role that TLR10 plays in human first trimester trophoblast response to PDG.

Although TLR10 was identified 11 years ago¹³, little is still known about its expression and function. However when first characterized, expression in placental tissue of unknown gestational age was reported¹³. Subsequently, TLR10 mRNA has been reported in term placental tissue with expression at similar levels with or without labor¹⁴. In our current study, we report for the first time that first and third trimester placental tissue express TLR10 protein, and that this is localized to the trophoblast. Moreover, unlike TLR6, which is only expressed by the trophoblast late in gestation (unpublished data), TLR10 expression levels do not appear to be gestationally regulated.

Phylogenetically, TLR10 is most closely related to TLR1 and TLR6¹³. Similarly to TLR1 and TLR6, TLR10 functions as a co-receptor for TLR2^{5, 6}. Indeed, in addition to self-associating, TLR10 can physically interact with TLR2 or TLR1^{5, 6}. Since we found TLR10 to be expressed by the first trimester trophoblast, we postulated it may play a role in the cell's response to PDG: induction of apoptosis and inhibition of IL-6 secretion^{3, 4}. In this current study we show using a dominant negative, that PDG-induced trophoblast apoptosis, in addition to TLR1 and TLR2⁴, is also dependent upon the function of TLR10. However, TLR10 does not regulate the inhibition in IL-6 secretion seen in response to PDG⁴. These findings suggest that interactions of TLR2, TLR1 and TLR10 all result in trophoblast apoptosis in the presence of PDG, and that perhaps the TLR2/TLR1 combination, rather than TLR2/TLR10, plays a role in regulating IL-6 production. Hasan *et al.*, first reported TLR10 to be functional and able to trigger NF κ B activity via MyD88. However, in this study they used a constitutively active version of the protein⁵. In contrast, Guan *et al.*, recently demonstrated that instead, TLR10 fails to activate the typical signals associated with MyD88, such as NF κ B⁶. This observation supports our findings and may explain why in our model, TLR10 promotes apoptosis rather than inflammation.

In summary, this study demonstrates for the first time that TLR10 is expressed by the trophoblast across gestation and that in early pregnancy TLR10 plays a role in promoting PDG-induced trophoblast apoptosis, but does not regulate PDG modulation of trophoblast IL-6 secretion. Together, these findings suggest that TLR10 may regulate trophoblast TLR2-mediated apoptosis, and thus, the balance between trophoblast survival and cell death.

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Figure 1. TLR10 protein expression in placental tissue and trophoblast cells

(A) TLR10 expression in placental villous and decidual tissue using immunohistochemistry. (i) First trimester placental villi (Insert shows the negative control); (ii) First trimester placental villi and decidua; (iii) Term placental villi; (iv) Term placental villi and decidua (Insert shows cytokeratin 7 staining of villous and extravillous trophoblast). Arrow heads show TLR10 positive staining for (i & iii) cytotrophoblast and syncytiotrophoblast and (ii & iv) extravillous trophoblast cells. (B) Western blot analysis of TLR10 protein expression in: human first trimester trophoblast cells lines, H8 and 3A; human first trimester primary trophoblast cells (from 4 different first trimester placenta); and human third trimester primary trophoblast cells (from 5 different term placenta). Bar chart shows quantification of TLR10 expression as determined by densitometry and normalized to β -actin. There was no significant (ns) difference in the levels of first and third trimester trophoblast TLR10.

Figure 2. Regulation of trophoblast caspase-3 activity and IL-6 secretion by TLR10

Trophoblast cell lines stably transfected with either a TLR10-DN or vector control were incubated with either no treatment (NT) or PDG at 80 μg/ml for 48 hours, after which: A) cell lysates were analyzed for caspase-3 activity, and B) culture supernatants were measured for IL-6. * $P < 0.01$ relative to the NT control unless otherwise indicated.