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ORIGINAL ARTICLE

An immunohistochemical study of Toll-like receptors 2 and 4 in placenta with and without infection

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Abstract

Objective: The placenta constitutes a physical and immunological barrier against infectious agents. Toll-like receptors (TLRs) are essential components for the induction of innate immunity responses in different human tissues including the placenta. We investigated the expressions of TLR2 and TLR4 in the decidua and amniotic cells in non-inflamed placenta and placenta with infection. *Materials and Methods:* There were a total 74 placentas (37 with infection and 37 without infection- 25 bacterial, 10 viral and 2 toxoplasma). TLR2 and TLR4 expressions were assessed using immunohistochemical technique. Positive cells were indicated by cytoplasmic staining and the percentage of positive in 100 cells was recorded and graded. The grades were 1+ (<25%), 2+ (25-75%) and 3+ (>75%). *Results:* We found significantly higher expression of TLR2 in the amniotic cells and decidua cells in infected placentas as compared to non-inflamed placentas among the preterm placenta. A higher number of cases have TLR4 expression in the amnion of preterm infected placenta than in term placenta. This, however, is not statistically significant. *Conclusion:* Our findings suggest that TLR2 plays a role in the innate immunity in bacterial and viral infection in the placenta, however, their role in protection against toxoplasma may be limited. This study further supports the observations that TLR2 expression was higher in placenta with infection which strengthened the role of TLR2 in the protection of preterm placenta against infection.

Key words: amnion, deciduas, chorioamnionitis, placenta, toll-like receptors

INTRODUCTION

Placental inflammatory disorders represent a spectrum of important pathological processes leading to fetal and neonatal morbidity and mortality. These processes can be divided into two broad subcategories, those caused by micro-organisms and by host immune responses to non-replicating antigens for example in case of villitis of unknown aetiology.[1] Mechanisms that protect the product of conception from infection are both structural and functional. The structural barriers include mechanical closure of the cervical canal by mucus plugs, the integrity of the chorioamnionic membrane and the syncytial nature of the cells surrounding the villi. The functional barriers include endocervical immune system, expression of antibacterial proteins in the secretions of endometrial glandular cells and amniotic fluid, and rapid mobilization of myeloid lineage cells to the maternal fetal interfaces of the placenta, which is derived from both the mother and the fetus.[2-4]

An understanding of the different patterns of placental inflammation requires careful consideration of possible routes of infection and the maternal fetal interface. Early in pregnancy the placenta and embryo are relatively insulated from infection as the amnion has not fused with the chorion. The gestational sac has not fused with the opposite side of the uterus. Even the intervillous space lacks an arterial blood supply because of endovascular trophoblasts, which plug the spiral arteries. These factors probably account for the infrequent transmission of congenital infections in the first trimester.[5]

Intervillous space perfusion begins at 11-12 weeks of gestation, hence organisms in the maternal circulation may gain access to the fetally perfused placental villi. Occasionally organisms that are able to cross the syncytiotrophoblast

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barrier are confronted by endogenous phagocytes (Hofbauer cells).[6] Organisms, which escaped phagocytosis by Hofbauer cells, can infect fetal endothelial cells and gain access to the fetal circulation.[7] At 18-20 weeks of gestation, the membranes come in contact with the uterine lining (decidua vera). Organisms can enter the decidua vera by contiguous spread from the cervix (premature dilation), fallopian tubes (chronic salphingitis) and bladder (acute cystitis), or they may enter the decidua haematogenously.[8]

Inflammation may occur in the placenta (placentitis, villitis), in the fetal membranes (chorioamnionitis), and in the umbilical cord (funisitis).[9] Chorioamnionitis is the commonest form of placental inflammation in humans. It has been reported in about 4% of otherwise uncomplicated term births. Its frequency is inversely proportional to gestational age, showing the strong association between chorioamnionitis and preterm birth (PTB).[10] Chorioamnionitis frequently precedes membrane rupture, causing premature rupture of membranes and preterm labor. Bacteria are the commonest cause of ascending infection. Other vaginal inhabitances such as herpes virus and Candida may be implicated.[11] The histological evidence of response to amniotic infection is neutrophilic infiltration in the decidua, chorion, amnion, and into amniotic fluid. Frequently, the inflammatory response is most intense at the site of membrane rupture.

Gestational age during the infection and causative organisms may determine the fetal outcome. Group B β-haemolytic streptococcus, Escherichia coli, and Haemophilus influenzae are the commonest causes of neonatal infection.[12] Infectious agents reaching the placenta through the haematogenous route include viruses, some bacteria, and parasites, such as cytomegalovirus, listeria, toxoplasmosis, syphilis, rubella, tuberculosis, cryptococcus, malaria as well as Psittacosis.[11]

The placenta constitutes a physical and immunological barrier against invading infectious agents and has been suggested to be a pregnancy specific component of the innate immunity system.[13] Infectious organisms in the placenta elicit primarily innate immune responses. Pattern recognition receptors (PRRs) are proposed not only to sense the presence of microorganisms but also to be central to innate immunity.[14] Toll like receptors (TLRs) are essential PRRs and are involved in the recognition of conserved molecules of microorganisms, so-called pathogen associated molecular patterns (PAMPS). Toll was first to discover that Drosophila lacks an adaptive immune response but is resistant to fungal infection through the action of Toll.[13]

TLRs are membrane proteins that recognize a variety of microbe-derived molecules and stimulate innate immune responses against the microbes. Ten human TLRs have now been identified. TLRs are expressed in many different cell types that participate in the innate immune responses, including macrophages, dentritic cells, neutrophils, NK cells, mucosal epithelial cells and endothelial cells. Some of the microbial products that stimulate TLRs includegram-negative bacterial lipopolysaccharides (LPS), gram-positive bacterial peptidoglycans, bacterial lipoproteins, bacterial flagella protein flagellin, and double stranded RNA (found in RNA viruses).[15] Most studies on TLR2 and TLR4 expressions in placenta inflammatory disorder were on the amniotic epithelium, deciduas, trophoblasts and hofbauer cells.[13-14,16-17]

TLR2 and TLR4 proteins are members of the Toll like receptor (TLR) family, which play fundamental roles in pathogen recognition, and activation of innate immunity. They recognize pathogen associated molecular patterns (PAMPs) that are expressed on infectious agents, and mediate the production of cytokines necessary for the development of effective immunity. The aim of this study was to assess the expression of TLR2 and TLR4 in amniotic cells and decidua cells in non-inflamed placenta and placenta with infection.

MATERIALS AND METHODS

Study Design

This is a retrospective study on cases diagnosed as normal placenta and placenta with infection, due to bacteria, virus, or toxoplasma, obtained from the histopathology files of the Department of Pathology, University Kebangsaan Malaysia Medical Centre (UKMMC) for the five years from 1st January 2001 to 31st December 2005. The study was approved by the ethics committee of our university. The paraffin blocks were retrieved, sectioned and stained by immunohistochemical technique using TLR2 and TLR4 antibodies. There were a total of 74 cases included in this study. Cases classified as bacterial infection are based on histological finding of acute chorioamnionitis, whereas viral and toxoplasma infections are based on maternal IgM positivity.

Antibodies and immunohistochemistry
The antibodies were goat polyclonal antibody against TLR2 (Gene Tex, GTX21655) and rabbit polyclonal antibody against TLR4 (USBiological-T8050-29D). The immunohistochemical procedure and preparation of controls were done according to the manufacturing company's standards and guidelines. All tissues were fixed in 10% formalin and embedded in paraffin. A representative block for each case was selected and 3 μm thick sections were made for immunohistochemistry using the standard labeled streptavidin –biotin method. Deparaffinised sections were placed in a pressure cooker for antigen retrieval using citrate buffer pH6 for 10 minutes. These were then incubated at room temperature and washed with distilled water. After washing, the sections were placed in hydrogen peroxide 3% for 6 minutes to block endogenous peroxide, then the sections were washed with water three times and finally washed by Tris-buffered saline (TBS) for 10 minutes to eliminate non-specific staining. The excess TBS was removed from the slides before incubation with primary antibody.

The sections were incubated with the primary antibodies (TLR2 [1:250] and TLR4 [1:250]) for 30 minutes at room temperature. The sections were washed with TBS and incubated with link antibody for 15 minutes each. Then the section were washed with TBS and incubated with labeled Streptavidin-biotin (LSAB) for 15 minutes at room temperature. The sections were again washed with TBS and incubated with diaminobenzidine (DAB) and substrate chromogen system, for 5 minutes at room temperature which resulted in brown coloured precipitates at the antigen site. The sections were then counterstained with haematoxylin for 1 minute and were mounted.

Controls
Positive and negative controls were included with each batch. Normal human spleen tissue obtained at autopsy served as positive control, whereas in the negative control, the primary antibody was omitted.

Interpretation of results
TLR 2 and TLR 4 positivity were indicated by cytoplasmic staining. Two cell types were assessed: amniotic and decidua cells. The number of positive amniotic and decidua cells were graded based on the percentage of immunoreactive cells, as 1+ (<25%), 2+ (25-75%) and 3+ (>75%).

Statistical analysis
The results were analysed statistically by using Statistical Package for Social Sciences programme version 12.0 (SPSS Inc., Chicago, IL). Chi-square or Fischer exact tests were used for the categorical data analysis. Any p value of <0.05 was considered to be statistically significant.

RESULTS

There were a total of 74 samples, consisting of 37 placenta with infection and 37 non-inflamed placenta. Among the infection group, 25 were due to bacteria, 10 virus and 2 toxoplasma. Thirty (63.8%) of the placentas with infection were from preterm delivery (<37 weeks of gestation) and 17 (36.2%) of the non-inflamed placenta were from preterm delivery (Table 1).

TLR2 – decidual cells in preterm placenta
TLR2 expression in decidua cells (Figure 1) in preterm placenta (non-inflamed and infection) is shown in Table 1. Sixteen (53.3%) of preterm placentas with infection showed 3+ for TLR2 staining in the decidua cells compared to the preterm non-inflamed placenta which showed 3+ TLR2 staining in only 2 (11.8%) of the cases. This finding was statistically significant (P value = 0.006).

TLR2- amniotic cells in preterm placenta
TLR2 expression in amniotic cells (Figure 2) in preterm placenta (non-inflamed and infection) is shown in Table 1. Ten (33.3%) preterm placenta with infection showed 3+ for TLR2 staining in the amniotic cells as compared to 2 (11.8%) of the preterm non-inflamed placenta which showed 3+ TLR2 staining in the same cells. This finding was statistically significant (P value = 0.05).

TLR4 – decidual and amniotic cells in preterm placenta
TLR4 expression in decidua and the amniotic cells in the preterm placentas (non-inflamed and infection) are shown in Table 1. Their differences were statistically not significant (P value = 0.38 and 0.76 respectively).

TLR2 – decidua cells and amniotic cells in term placenta
The differences of TLR2 expression in decidua cells and amniotic cells of both non-inflamed and infection group were statistically not significant (decidua cells p value = 0.12 and amniotic cells

TABLE 1. TLR2 and TLR4 expressions in decidual and amniotic cells in preterm and term placentas

Grade		Preterm Placenta								Term Placenta							
		TLR2				TLR4				TLR2				TLR4			
		decidua		amnion		decidua		amnion		decidua		amnion		decidua		amnion	
		Non-inflamed	Infection	Non-inflamed	Infection	Non-inflamed	Infection	Non-inflamed	Infection	Non-inflamed	Infection	Non-inflamed	Infection	Non-inflamed	Infection	Non-inflamed	Infection
1+ <25%	Count	7	3	10	9	11	22	11	18	3	2	8	2	7	5	9	5
	% within each group	41.2%	10.0%	58.8%	30.0%	64.7%	73.3%	64.7%	60.0%	15.0%	28.6%	40.0%	28.6%	35.0%	71.4%	45.0%	71.4%
	% of total	14.9%	6.4%	21.3%	19.1%	23.4%	46.8%	23.4%	38.3%	11.1%	7.4%	29.6%	7.4%	25.9%	18.5%	33.3%	18.5%
2+ <25%	Count	8	11	5	11	5	8	4	6	9	3	4	3	12	2	9	2
	% within each group	47.1%	36.7%	29.4%	36.7%	29.4%	26.7%	23.5%	20.0%	45.0%	42.9%	20.0%	42.9%	60.0%	28.6%	45.0%	28.6%
	% of total	17.0%	23.4%	10.6%	23.4%	10.6%	17.0%	8.5%	12.8%	33.3%	11.1%	14.8%	11.1%	44.4%	7.4%	33.3%	7.4%
3+ >75%	Count	2	16	2	10	1	0	2	6	8	2	8	2	1	0	2	0
	% within each group	11.8%	53.3%	11.8%	33.3%	5.9%	0.0%	11.8%	20.0%	40.0%	28.6%	40.0%	28.6%	5.0%	0.0%	10.0%	0.0%
	% of total	4.3%	34.0%	4.3%	21.3%	2.1%	0.0%	4.3%	12.8%	29.6%	7.4%	29.6%	7.4%	3.7%	0.0%	7.4%	0.0%
Total	Count	17	30	17	30	17	30	17	30	20	7	20	7	20	7	20	7
	% within each group	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%
	% of total	36.2%	63.8%	36.2%	63.8%	36.2%	63.8%	36.2%	63.8%	74.1%	25.9%	74.1%	25.9%	74.1%	25.9%	74.1%	25.9%

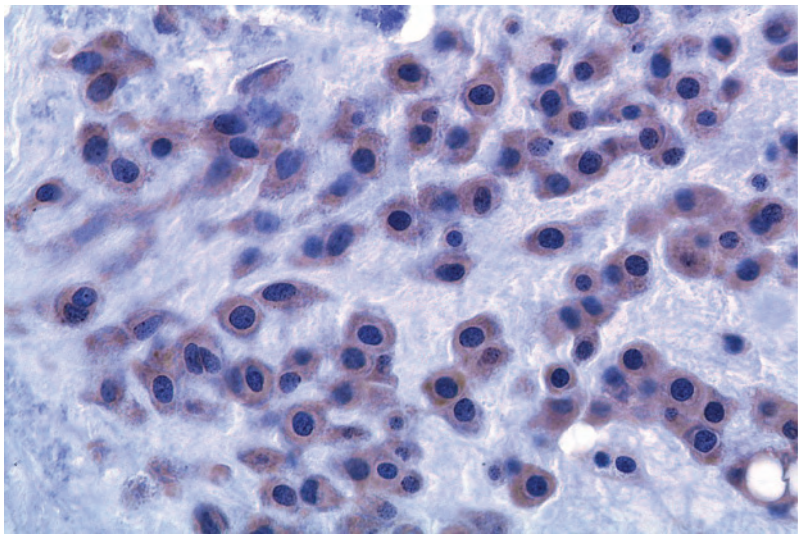

FIG. 1: TLR2 immunoreactivity in the cytoplasm of decidua cells (x200)

p value = 0.22). However, there were more cases in the non-inflamed placenta group (8/20) with TLR2 expression of 3+ in deciduas cells and amniotic cells compared to the infection group (2/7). 2+ TLR2 expression in decidua and amnion cells were also compared between preterm and term of non-inflamed placenta and placenta with infection. Their results, however, were not statistically significant (p value = 0.14 and 0.36 respectively).

TLR4 – decidual cells and amniotic cells in term placenta

The differences of TLR4 expression in decidua cells and amniotic cells in non-inflamed placenta group and placenta with infection group were statistically not significant (decidua cells p value = 0.06 and amniotic cells p value = 0.40). None of the cases in the infection group were positive in the 3+ category in both the decidua and amniotic cells. However, 3 of the cases in the non-inflamed placenta group were positive (Table 1).

TLR2 – Placenta with infection (bacterial, viral and toxoplasma)

The percentage of 3+ TLR2 expression in placenta with bacterial, viral and toxoplasma infections were 66.7%, 33.3% and 0%, respectively. All the toxoplasma cases were in

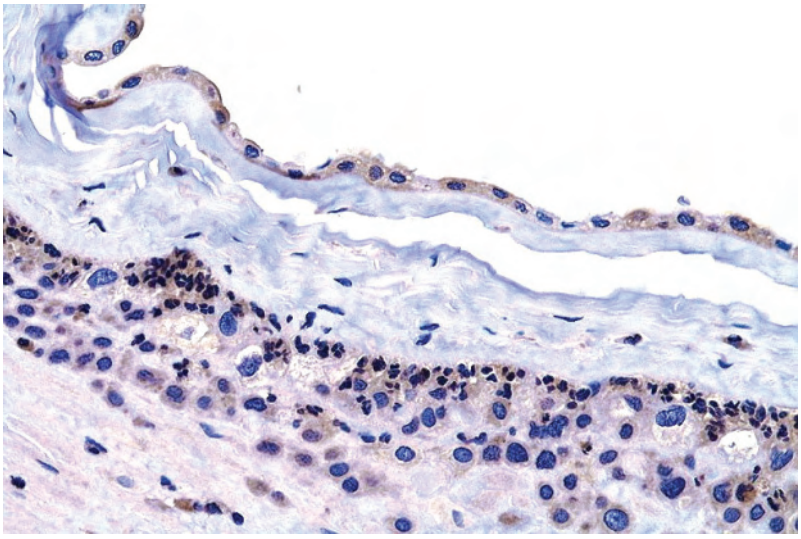

FIG. 2: TLR2 immunoreactivity in the cytoplasm of amniotic cells (x100)

the 2+ category (Table 2).

DISCUSSION

TLR proteins expression has been described in tissues like intestinal epithelium[18] and in human bladder urothelium,[19] indicating the mucosal linings have the capacity to respond to micro-organisms. At least 2 TLRs are known to express in the human placenta which have been described in previous studies.[13-17,20] Activation of these pathways will elicit antimicrobial factors such as cytokines, reactive oxygen and nitrogen intermediates. In the pregnant uterus, the broadening and intensification of the inflammatory response may even cross-activate pathways leading to premature labour.[19]

In this study, thirty (81.1%) placenta with infection had preterm delivery, further supporting the adverse relationship between placental infection and preterm delivery. We also found that the expression of TLR2 in amniotic and deciduas cells is up-regulated in the presence of infection in preterm delivery. In contrast, the expression of TLR2 was higher in non-inflamed placenta in term delivery, indicating its role is more in preterm placenta than in term placenta.

Kim *et al.*[14] also found increased expression of TLR2 and TLR4 in the amniotic cells during parturition and chorioamnionitis. Kumazaki *et al.*[16] was focusing on extra villous trophoblast and villous hofbauer cells in the placentas and they found enhancement of TLR4 immunoreactivity in the villous Hofbauer cells of preterm chorioamnionitis as compared to those placentas

Table 2 Percentage of TLR2 expression in decidua cells in different types of infection in placenta

		Percentage of TLR2 expression			
Grade		Type of infection			
		Bacteria	Viral	Toxoplasma	Total
1+ <25%	Count	2	3	0	5
	% within percentage of TLR2 expression	40.0%	60.0%	0.0%	100.0%
	% within type of infection	8.0%	30.0%	0.0%	13.5%
2+ 25-75%	Count	11	1	2	14
	% within percentage of TLR2 expression	78.6%	7.1%	14.3%	100.0%
	% within type of infection	44.0%	10.0%	100.0%	37.8%
3+ >75%	Count	12	6	0	18
	% within percentage of TLR2 expression	66.7%	33.3%	0.0%	100.0%
	% within type of infection	48.0%	60.0%	0.0%	48.6%
Total	Count	25	10	2	37
	% within percentage of TLR2 expression	67.6%	27.0%	5.4%	100.0%
	% within type of infection	100.0%	100.0%	100.0%	100.0%

without chorioamnionitis. Holmlund *et al*.[13] showed strong reactivity of TLR2 and TLR4 in the trophoblasts in term placentas. However, we found no significant difference in the expression of TLR4 in deciduas and amniotic cells between the non-inflamed placenta and placenta with infection.

In contrast to all other studies, Rindsjo *et al*.[17] demonstrated lower expression of TLR2 in placenta with chorioamnionitis compared to term placenta without chorioamnionitis, but they found higher expression in placenta from live born children when compared to still born children and higher expression in the placenta from second trimester than in third trimester.[17]

Our study also showed that percentage of cases with 3+ expression of TLR2 was higher among infection due to bacteria and viral as compared to toxoplasma. In toxoplasma infected placenta, the expression of TLR was only 2+. These findings suggest that TLR2 plays a role in innate immunity in bacterial and viral infection in the placenta. However, their role in protection against toxoplasma may be limited.

In conclusion, our data further support the observations that TLR2 expression was higher in placenta with infection which strengthened the role of TLR2 in the protection of preterm placenta and pregnancy against infection. However, we found that TLR4 does not play a vital role in protection of pregnancy.

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