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Chlamydia Heat Shock Protein 60 Induces Trophoblast Apoptosis through TLR4\(^{1,2}\)

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Intrauterine infection affects placental development and function, and subsequently may lead to complications such as preterm delivery, intrauterine growth retardation, and preeclampsia; however, the molecular mechanisms are not clearly known. TLRs mediate innate immune responses in placenta, and recently, TLR2-induced trophoblast apoptosis has been suggested to play a role in infection-induced preterm delivery. *Chlamydia trachomatis* is the etiological agent of the most prevalent sexually transmitted bacterial infection in the United States. In this study, we show that in vitro chlamydial heat shock protein 60 induces apoptosis in primary human trophoblasts, placental fibroblasts, and the JEG3 trophoblast cell line, and that TLR4 mediates this event. We observed a host cell type-dependent apoptotic response. In primary placental fibroblasts, chlamydial heat shock protein 60-induced apoptosis was caspase dependent, whereas in JEG3 trophoblast cell lines it was caspase independent. These data suggest that TLR4 stimulation induces apoptosis in placenta, and this could provide a novel mechanism of pathogenesis for poor fertility and pregnancy outcome in women with persistent chlamydia infection. *The Journal of Immunology*, 2006, 177: 1257–1263.

*Chlamydia trachomatis* is the most common reportable sexually transmitted bacterial species in the United States (1, 2). In pregnant women, cervical infection with *C. trachomatis* is independently associated with preterm premature rupture of the membranes, preterm labor, and low birth weight (reviewed in Ref. 3); however, the molecular mechanisms are not clearly known.

*Chlamydia* organisms are not acutely toxigenic, and in the majority of females, genital tract infection is asymptomatic and persistent. Recent evidence suggests that organism may persist for as long as 5 years in the genital tract (4). Interestingly, the organism has a distinct antigenic profile during persistent infection, where overall expression of all proteins decreases except for the 60-kDa heat shock protein (HSP60)\(^4\) (5), which is associated with the outer membrane complexes of *Chlamydia* and appears to be responsible for proinflammatory pathologic manifestations of human chlamydial disease in the reproductive tract (6–10).

TLRs have been identified as mediators of microbial Ag induced innate immune responses (11), regulators of adaptive immune responses (12), as well as inducers of apoptosis (13–19). TLR expression in the female reproductive tract has recently been examined. There is constitutive TLR1 to TLR6 mRNA and protein expression as well as MyD88 and CD14 mRNA expression in the fallopian tubes, uterine endometrium, cervix, and ectocervix (20, 21). TLR2 mRNA levels are highest in the fallopian tubes and cervical tissues, followed by endometrium and ectocervix. In contrast to TLR2, TLR4 expression declines progressively along the tract, with highest expression in the upper tissues (fallopian tubes and endometrium), followed by cervix and endocervix (20, 21). Fazeli et al. (21) have also shown the expression of a secretory form of TLR4 in the endocervical glands. TLR4 and TLR2 are also expressed in placenta cells (trophoblast, fibroblasts, Hofbauer-placental macrophages), and levels of TLR4 expression in placenta increase in the presence of infection and vascular insufficiency (22, 23).

TLRs are increasingly documented to play a role in female reproductive tract physiology. Stimulation of TLR2, TLR3, TLR4, and TLR5 with their ligands has been shown to induce proinflammatory cytokine release in uterine epithelial cells (24–26). In placenta, binding of TLR2 with its ligand Gram-positive bacterial cell wall component peptidoglycan induces trophoblast apoptosis (27). Both maternal and fetal polymorphisms of the TLR4 gene have been associated with spontaneous preterm labor and preterm birth in certain populations (reviewed in Ref. 28).

We have previously shown that TLR4 mediates innate immune responses to *C. trachomatis* HSP60 (cHSP60) and that stimulation with cHSP60 induces innate immune responses in endothelial cells (29). Here, we show that HSP60 treatment induces caspase-8, caspase-3, and caspase-9 activation in trophoblasts and leads to trophoblast apoptosis through TLR4. Because healthy trophoblast development is essential for healthy placenta and fetal development, these data provide a novel mechanism for *Chlamydia*-induced infertility—early pregnancy loss, preeclampsia, and preterm delivery.

**Materials and Methods**

**Cell lines and reagents**

JEG3, human syncytiotrophoblast cell line was obtained from American Type Tissue Culture Collection (ATCC). Cells were cultured in MEM (Invitrogen Life Technologies) supplemented with 10% FBS, 10 mM

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\(^4\) Abbreviations used in this paper: HSP60, 60-kDa heat shock protein; cHSP60, *Chlamydia trachomatis* HSP60; PI, propidium iodide.
HEPES, 1 mM sodium pyruvate, and 100 nM penicillin/streptomycin (Invitrogen Life Technologies).

THP-1 cells were obtained from ATCC. They were grown in RPMI 1640 medium with 2 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, and 1.0 mM sodium pyruvate and supplemented with 0.05 mM 2-ME (90%) and FBS (10%) (30). Highly purified, phenol-water extracted, and protein-free (<0.0008% protein) E. coli-derived P-40, and 1:100 Protease Inhibitor Set III (Calbiochem). Protein concentration was determined using a colorimetric assay (Bio-Rad DC protein assay). Lysis buffer containing 50 mM HEPES (pH 7.9), 250 mM NaCl, 20 mM β-glycerophosphate, 2 mM DTT, 1 mM sodium orthovanadate, 1% Nonidet P-40, and 1:100 Protease Inhibitor Set III (Calbiochem). Protein concentration was determined using a colorimetric assay (Bio-Rad DC protein assay). A total of 55 μg of protein was analyzed on a 10% Tris-HCl polyacrylamide gel (Bio-Rad). Membranes were blocked in 5% milk, 0.1% Tween 20 in TBS for 2–3 h at 4°C; incubated overnight at 4°C with anti-human TLR4 and anti-human TLR2 Ab (Santa Cruz Biotechnology) (1: 250) followed by a 1-h incubation at room temperature with anti-rabbit HRP (1:2000); developed by LumiGlo (Cell Signaling); and exposed to radiographic film.

Caspase activity assay

We assessed caspase activation by using the Caspase-Glo assay (Promega) according to the manufacturer’s directions. Briefly, 10 μg of whole-cell lysates were incubated at room temperature in the dark for 1 h with either the caspase-3, -8, or -9 substrate. Following incubation, luminescence was measured using a TD-20/20 luminometer (Turner Designs). The amount of luminescence detected as relative light units was proportional to caspase activity.

FACS analysis of apoptosis

Apoptotic cells that are accompanied by phosphatidylserine exposure to the outer membrane were analyzed by incubation of cells with FITC-conjugated annexin V (Roche Molecular Biochemicals). Labeling procedures followed those suggested by the manufacturer’s manual. Briefly, cells were resuspended in annexin labeling solution containing 10 mM HEPES (pH 7.4), 140 mM NaCl, 5 mM CaCl₂, and fluorescein-conjugated annexin V for 15 min. After being washed twice with PBS, cell pellets were resuspended in propidium iodide (PI) (2 μg/ml) containing PBS and analyzed by flow cytometry. At least 10,000 events were analyzed, and apoptosis was presented as percent positive cells stained with annexin V.

Statistical analysis

The experiments were set up in triplicate and were repeated on at least three separate occasions. Student’s t test was used to compare the means between medium and treatment groups. A value of p < 0.05 was reported as statistically significant.

Results

Ex vivo cHS60 stimulation of primary trophoblasts induces apoptosis

We examined the apoptotic effect of cHS60 on primary trophoblasts isolated from early second-trimester elective termination placentas. Cells were incubated with medium, protein kinase C inhibitor calphostin (as positive control for apoptosis) and various concentrations of cHS60 for 4 h. Cell death was assessed by staining with PI and FITC-labeled annexin V to detect necrotic and apoptotic cells, respectively, and by using flow cytometry.

We observed that treatment with cHS60 (5 μg/ml) resulted in a 5-fold increase (11.76–62.09%) in the number of apoptotic cells compared with medium-treated cells (Fig. 1). Because treatment with higher concentration of cHS60 (20 μg/ml) increased double-positive cells (PI and annexin positive) that could be late apoptotic or necrotic (0.59–3.37%) (Fig. 1), we elected to use the lower concentration of cHS60 (5 μg/ml) to study cHS60-induced apoptosis in the remainder of the experiments. These data suggest that, in pregnant women with persistent chlamydia infection, the release of extracellular cHS60 may lead to trophoblast cell apoptosis.

Chlamydia HSP60 stimulation of JEG3 trophoblast cells leads to apoptosis

We used the JEG3 trophoblast cell line to assess the molecular mechanisms involved in cHS60-induced trophoblast apoptosis.

FIGURE 1. Primary trophoblasts isolated from elective termination placentas were treated with various concentrations of cHS60 for 4 h. Apoptosis was assessed by PI and FITC-labeled annexin V staining and flow cytometry. The data shown are representative of three independent experiments.
To do this, we first demonstrated that the JEG3 human trophoblast cell line expressed 88-kDa protein corresponding to TLR4 and a 90-kDa protein corresponding to TLR2 (Fig. 2). Next, we examined the apoptotic effect of cHSP60 on JEG3 trophoblast cells by treating them with medium, calphostin, and cHSP60. Apoptosis was determined by staining with PI and FITC-labeled annexin V and performing flow cytometry. Treatment of the JEG3 trophoblast cell line with cHSP60 induced apoptosis (p < 0.05), but at a much reduced rate compared with primary trophoblasts (7.7–13.3%) (Fig. 3). These data suggest that cHSP60 induces apoptosis in both primary trophoblasts and JEG3 cells, and therefore JEG3 cell system can be used to study the molecular mechanisms of cHSP60-induced trophoblast apoptosis.

Anti-TLR4 Ab blocks cHSP60-induced apoptosis in JEG3 cells

TLR4 mediates cHSP60-induced immune responses in macrophages and endothelial cells (29). We assessed the role of TLR4 in cHSP60-induced trophoblast apoptosis by treating JEG3 cells with function-blocking anti-TLR4, anti-TLR2, and nonspecific IgG1 control Ab before stimulation with cHSP60, and measuring apoptosis by FITC-labeled annexin V staining and flow cytometry. Pretreatment with anti-TLR4 Ab blocked the cHSP60-induced apoptosis, whereas control Ab and anti-TLR2 Ab had no effect (Fig. 4). These data suggest that TLR4 mediates cHSP60-directed trophoblast apoptosis.

Stimulation with TNF-α induces apoptosis in trophoblasts (34). To assess whether cHSP60-induced apoptosis was secondary to TNF-α released, we treated JEG3 cells with anti-TNF-α Ab before stimulation with cHSP60, and assessed apoptosis by FITC-labeled annexin V staining and flow cytometry. We observed that pretreatment with anti-TNF-α Ab did not decrease cHSP60-induced apoptosis (Fig. 4). These data suggest that cHSP60-induced trophoblast apoptosis is not due to the autocrine effect of cHSP60-induced TNF-α.

TLR4 is the receptor for enteric Gram (-) bacterial LPS (35), and TLR4 signaling of HSP has recently been attributed to their LPS contamination (36, 37). To test the role of LPS contamination of cHSP60 in JEG3 apoptosis, we treated JEG3 cells with various concentrations of LPS that induce signaling in these cells (data not shown) and assessed apoptosis by using annexin V staining and flow cytometry. We observed that LPS treatment did not induce apoptosis in JEG3 cells (Fig. 4). These data suggest that cHSP60-induced JEG3 cell apoptosis is due to the direct effect of cHSP60 but not due to LPS contamination.

cHSP60 treatment activates caspase-3, caspase-8, and caspase-9 in JEG3 cells

In the majority of cases, apoptosis is mediated through activation of caspases. Therefore, we assessed the effect of cHSP60 stimulation on caspase-3, caspase-8, and caspase-9 activity. Stimulation with cHSP60 but not LPS induced caspase-8, caspase-3, and caspase-9 activation in JEG3 cells (Fig. 5, A–C, respectively). These data suggest that caspases may mediate cHSP60-induced apoptosis in JEG3 trophoblasts.

Next, we assessed the role of caspases in cHSP60-induced trophoblast apoptosis by treating JEG3 cells with various concentrations of pancaspase inhibitor (Z-VAD-FMK) before treatment with cHSP60 and assessing apoptosis by FITC-labeled annexin V staining and flow cytometry. We observed that, at the highest concentration of Z-VAD-FMK, inhibition of caspases only partially blocked the cHSP60-induced apoptosis in JEG3 cells (Fig. 6).

In parallel, we isolated primary fibroblasts from minced, PBS-washed second-trimester elective termination placentas cultured in DMEM medium with 10% FBS and antibiotics. Fibroblasts were then used in apoptosis experiments. Initial experiments suggested that Z-VAD-FMK was toxic to primary fibroblasts at the concentrations used in trophoblasts. Therefore, a lower concentration of Z-VAD-FMK (100 μM) was used in fibroblast experiments. Similar to trophoblasts, cHSP60 treatment led to apoptosis in fibroblasts and pretreatment with Z-VAD-FMK blocked the cHSP60-induced apoptosis (Fig. 7). These data suggest that caspase-dependent apoptotic pathways may mediate cHSP60-induced trophoblast and fibroblast apoptosis.

Discussion

Chlamydia is an obligate intracellular bacteria and has three major species: C. trachomatis, C. pneumonia, and C. psittaci. C. trachomatis is the etiological agent of the most prevalent sexually transmitted bacterial disease in the United States. There are roughly 3 million cases annually, most occurring in men and women < 25 years of age (38) with direct and indirect costs (mainly costs for complications) of ~$2.4 billion a year (39, 40).

Chlamydia are clearly known to be induce an immune response, and apoptosis in the neighboring uninfected cells (41). It is proposed that Chlamydia infection protects the host cell against apoptosis to provide survival advantage to this obligate intracellular...
organism (42–48). Recently, Zhong and Kihlstrom and coworkers (48, 49) have shown that, although chlamydia infection does not induce cell death in infected cells, there is significant apoptosis in the nearby uninfected adherent cells as measured by immunofluorescence. Eley et al. (50, 51) have shown that coincubation of human sperm with C. trachomatis leads to premature sperm death and that this is due primarily to chlamydial LPS. Dumrese et al. (52) have shown that C. pneumoniae infection of human aortic smooth muscle cells results in cell death with both apoptotic and necrotic characteristics. In addition, in RAW cells, infection with live C. pneumoniae or exposure to heat-killed or UV-inactivated C. pneumoniae led to aponecrosis, which was mediated via caspase-3-independent mechanisms (53). In a recent elegant review of Chlamydia pathogenesis, Byrne and Ojcius (54) state that “Chlamydia can elicit both the induction of host cell death, or apoptosis, under some circumstances and actively inhibit apoptosis under others. This subtle pathogenic mechanism highlights the manner in which these highly successful pathogens take control of infected cells to promote their own survival—even under the most adverse circumstances.” In this study, we show that stimulation with an effector of Chlamydia, cHSP60, induces apoptosis in primary trophoblasts and JEG3 trophoblast cell line.

In our experiments, cHSP60-induced apoptosis was much lower in JEG3 cell line than in primary trophoblasts, suggesting that primary trophoblasts are more sensitive to apoptosis than the trophoblast cell line. This difference agrees with the observation that primary smooth muscle cells die through “aponecrosis” during C. pneumoniae infection (52), and that primary fibroblasts are more prone to die through apoptosis during C. trachomatis infection than epithelial cell line (55).

Zhong and coworkers (49) suggested that the overall rate of apoptosis (<15%) in chlamydia-infected cultures is low and would not have any biological significance. It has been suggested that the techniques used can account for some of the differences in interpretation regarding cell death during chlamydia infection (41). Besides, a 6– to 7-day-old blastocyst has on average 100–150 cells, from which the embryo and placenta develop (56). Our data suggest that cHSP60-induced trophoblast apoptosis at this early stage may have a significant impact on placental and fetal development, and consequently pregnancy outcome. Indeed, trophoblast apoptosis is increased in pregnancies complicated by preeclampsia and intrauterine growth restriction and are often associated with insufficient trophoblast invasion (57–59).

Chlamydia has several cell wall and outer membrane components that are immunogenic and may serve as TLR ligands. Chlamydial LPS (59) as well as cHSP (29, 60, 61) are ligands for...
TLR4. Recent papers describe TLR4-independent cytokine production from inflammatory cells exposed to live chlamydial elementary bodies (62–64), and a dominant role for TLR2 in the recognition process of C. pneumoniae (65), and C. trachomatis (66). In this study, we used recombinant cHSP60 to assess the effect of persistent chlamydial infection on placenta. HSP are a class of highly conserved proteins that have important functions in cellular metabolism and aid cells in dealing with adverse environmental stimuli. There are several families of HSP classified by the approximate m.w. of their constituents (67). Members of the HSP60 family are found both in prokaryotes and eukaryotes; in eukaryotes their presence appears mainly restricted to the mitochondria and chloroplast organelles, although under stress conditions extracellular HSP60 has also been identified. In bacteria, HSP60 is located in the cytoplasm (68); however, in chlamydia, large amounts of HSP60 can be obtained merely by washing the organism with isotonic solutions (69), which suggests that much of cHSP60 is associated with outer membrane, and can be exposed easily to induce immune activation of the neighboring host cells, and models of persistent chlamydial infection have demonstrated the release of cHSP60 into the extracellular milieu. Indeed, several studies have revealed a correlation between cHSP60 responses and the immunopathologic manifestations of human chlamydial disease (6). In our hands, inhibition of caspases with pancaspase inhibitor, Z-VAD-FMK, blocked the cHSP60-induced apoptosis in trophoblast cells. We repeated the same experiments in primary human placental fibroblasts and showed that pretreatment with pancaspase inhibitor, Z-VAD-FMK, blocked the cHSP60-induced apoptosis. These data support the findings of Yarai et al. (53) in RAW 264.7 cells and suggest that caspase-dependent and -independent pathways may play roles in cHSP60-induced apoptosis in trophoblasts.

Apoptosis is classically initiated through extracellular ligands that bind to cell surface receptors. One of the best characterized receptors is a member of the TNF family, Fas. Binding of Fas with its ligand, Fas ligand, recruits an intracytoplasmic protein adaptor (the Fas-associated death domain). This binding eventually leads to activation of caspase-8, which then activates caspase-3 and indirectly activates caspase-9 and leads to apoptosis characterized by nuclear fragmentation (71–74). TLR4 and TLR2 adapter molecules, MyD88 and Toll/IL-1R domain-containing adaptor inducing IFN-β (TRIF), have death domains, and interact with Fas-associated death domain to activate caspase-8 (14, 75). In this study, we show that TLR4 stimulation with cHSP60 induces apoptosis in primary trophoblasts and the JEG3 trophoblast cell line through caspase-8, caspase-3, and caspase-9. Inhibition of caspases blocked TLR2-pentidoglycan-induced trophoblast apoptosis (27). In our hands, inhibition of caspases with pancaspase inhibitor partially blocked the block TLR4-cHSP60-induced apoptosis in JEG3 trophoblast cells. We repeated the same experiments in primary human placental fibroblasts and showed that pretreatment with pancaspase inhibitor, Z-VAD-FMK, blocked the cHSP60-induced apoptosis. These data support the findings of Yarai et al. (53) in RAW 264.7 cells and suggest that caspase-dependent and -independent pathways may play roles in cHSP60-induced apoptosis in trophoblasts.

Infection plays an important role in early pregnancy loss (76) and preterm delivery and its sequelae (1). It has been estimated that ~50% of all preterm deliveries and 80% of early preterm births (<32 wk) may be associated with intrauterine infection (2). Currently, the mechanisms of microbial Ag-induced preterm labor remain unknown. Based on our data, we propose that microbial Ag-induced trophoblast apoptosis may lead to “placental insufficiency” and play a role in early pregnancy loss in a group of susceptible women as well as development of preeclampsia, intrauterine growth restriction, and preterm delivery.

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Disclosures
The authors have no financial conflict of interest.

References


CORRECTIONS


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In Figure 2, the FACS profile showing expression level of CD11c was duplicated by mistake in panel A. The error does not affect the conclusions of the paper. The corrected figure is shown below.

![Corrections Image](image-url)

In Figure 6A inset, the blot representing PCR of HSF-1 knockout genotype is mislabeled. The corrected figure is shown below.