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MINI REVIEW

Immunopathogenic consequences of *Chlamydia trachomatis* 60 kDa heat shock protein expression in the female reproductive tract

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Abstract *Chlamydia trachomatis* is an obligate intracellular bacterium that infects chiefly urogenital and ocular epithelial cells. In some infected women the microorganism migrates to the upper reproductive tract resulting in a chronic, but asymptomatic, infection. The immune response to this infection, production of interferon-γ and pro-inflammatory cytokines, results in interruption of chlamydial intracellular replication. However, the *Chlamydia* remains viable and enters into a persistent state. In this form, most chlamydial genes are inactive. An exception is the gene coding for the 60 kDa heat shock protein (hsp60), which is synthesized in increased amounts and is released into the extracellular milieu. The chronic release of chlamydial hsp60 induces a local pro-inflammatory immune response in fallopian tube epithelia and results in scar formation and tubal occlusion. In addition, long-term exposure of the maternal immune system to the chlamydial hsp60 eventually results in the release of tolerance and generation of an immune response that recognizes regions of the chlamydial hsp60 that are also present in the human hsp60. Production of cross-reacting antibodies and cell-mediated immunity to the human hsp60 is detrimental to subsequent pregnancy outcome and may also possibly increase susceptibility to atherosclerosis, autoimmune disorders, or malignancies.

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Introduction

Chlamydia trachomatis is a unique obligate intracellular bacterium. *C. trachomatis* serovars A through C infect mucosal epithelial cells in the conjunctivae and cause trachoma, the leading cause of infectious blindness worldwide. Serovars D through K infect mucosal epithelial cells in the urogenital tract and are the leading cause of sexually transmitted bacterial infections in the United States and Europe (Ward 1995). Serovars L1, L2, L2a, and L3 infect the genital epithelium as well as monocytes and cause a systemic disease called lymphogranuloma venereum (Mabey and Peeling 2002). A striking feature of genital chlamydial infections is their asymptomatic nature or lack of distinguishing symptoms. Only about one quarter of the 4 million genital chlamydial infections estimated to occur annually in the United States are diagnosed and treated (Workowski and Berman 2006). *Chlamydia* genital serovars can migrate from the lower to the upper genital tract and this infection is the leading cause of fallopian tube occlusion, infertility, ectopic pregnancy, and salpingitis (Morrison 1991). Within a host, *C. trachomatis* is able to evade immune defenses (see below); persistence in the reproductive tract for as long as 5 years has been reported (Dean et al. 2000).

Chlamydial life cycle

C. trachomatis strains are energy parasites in that they lack enzymes of the electron transport chain and thus, must

acquire nutrients and adenosine triphosphate from the host to promote its metabolism and replication. The life cycle of *C. trachomatis* is unique and biphasic. The infectious form of the organism, the elementary body (EB), exists extracellularly and is metabolically inert. The EB attaches to an epithelial cell surface and becomes incorporated into a phagosome (inclusion vacuole) that migrates to the distal region of the Golgi apparatus. Lysosome fusion is prevented and the *Chlamydia* remains within this protected environment. Within the inclusion vacuole, the EBs differentiate into a metabolically active, noninfectious replicative form, the reticulate body (RB). Using host metabolites, the RB divides by binary fission, expanding the volume of the phagosome. To ensure its intracellular survival, *C. trachomatis* inhibits the infected cell from undergoing apoptosis (Dean and Powell 2001; Greene et al. 2004). The mechanism is unclear but may involve the production by *Chlamydia* of factors that actively block host apoptotic pathways (Greene et al. 2004). Concomitantly, apoptosis is induced in cytotoxic T lymphocytes that can destroy infected cells (Jendro et al. 2000) by a mechanism involving secretion of tumor necrosis factor-α by infected macrophages (Jendro et al. 2004). The RBs differentiate back into EBs and are released by either cell lysis or exocytosis into the extracellular milieu where they infect neighboring cells (Peeling and Brunham 1996; Wyrick 2000).

Persistence

Another unique attribute of *C. trachomatis* biology is that this microorganism is able to persist in the host subsequent to induction of anti-chlamydial immune defenses. A chlamydial infection activates host immune responses and production of interferon-γ and pro-inflammatory cytokines are induced. These mediators may effectively combat an extracellular infection but their influence, especially interferon-γ, on intracellular RBs is complex. Interferon-γ, by inducing the tryptophan-degrading enzyme, indoleamine 2,3-dioxygenase, and thereby reducing intracellular levels of this essential amino acid, effectively blocks RB replication. Genital tract *C. trachomatis* strains do not have all the genes required for tryptophan biosynthesis (Caldwell et al. 2003). However, the RBs remain viable and convert into what is known as a persistent form (Beatty et al. 1994). Chlamydial persistence can also be induced by tumor necrosis factor α (Holtmann et al. 1990) and in vitro, by the addition of penicillin to chlamydial cultures (Kramer and Gordon, 1971). Persistence is defined as the continued viability of the organism despite its inability to replicate in vitro when placed in a suitable culture medium (Beatty et al. 1994). It is a reversible state and recovery of the normal replicative cycle occurs upon a return to favorable conditions, for example after clearance of the extracellular infection and cessation of interferon-γ production or removal of penicillin from the in vitro culture medium (Skilton et al. 2009).

Most *C. trachomatis* fallopian tube infections do not exhibit characteristic symptoms and samples of tubal exudates are typically culture-negative. However, by the use of gene amplification technology chlamydial DNA, RNA and/or antigens can often be identified (Holland et al. 1992; Campbell et al. 1993; Patton et al. 1994a, b; Gerard et al. 1998; Toth et al. 2000). The detection of chlamydial-specific RNA is especially indicative of the presence of viable organisms. Thus, in asymptomatic fallopian tube infections *C. trachomatis* exists in a persistent form.

During the persistent state, *C. trachomatis* genes coding for proteins that are involved in chlamydial replication and energy generation, as well as for the production of structural components, are not activated. In marked contrast, the gene coding for the chlamydial 60 kDa heat shock protein (hsp60) is up-regulated and hsp60 is released into the extracellular milieu (Beatty et al. 1993a, b). This is undoubtedly a response to the external stresses being placed on the microorganism.

Consequences of chlamydial persistence

The mechanism of persistence allows *C. trachomatis* to remain viable within host epithelial cells despite generation of an anti-chlamydial immune response. The consequences of this chronic infection appear to depend, at least to a large extent, on the continued generation and release of the chlamydial hsp60. In guinea pigs previously sensitized to *C. trachomatis*, chlamydial hsp60 elicited a severe mononuclear, hypersensitivity inflammatory response when applied to the conjunctivae (Morrison 1991). Subsequent investigations demonstrated a similar induction of a delayed type hypersensitivity reaction when recombinant chlamydial hsp60 was inoculated into the fallopian tubes of previously infected monkeys (Patton et al., 1994a, b; Lichtenwalner et al. 2004). In contrast, inoculation of other chlamydial components was without effect. These observations suggest that release of the chlamydial hsp60 from a stressed but quiescent form of *C. trachomatis* is capable of eliciting a potent localized pro-inflammatory immune response. Chronic or intermittent hsp60 release into the extracellular milieu results in prolonged exposure of the epithelium to inflammatory mediators. Cumulative tissue damage eventually results in scar formation and occlusion of the fallopian tubes.

In human disease, elevated antibody responses to the chlamydial hsp60 have been strongly correlated with the presence of pelvic inflammatory disease, ectopic pregnancy,

scarring trachoma, and tubal infertility (Neuer et al. 1997; Peeling et al. 1998; Tiitinen et al. 2006; Jakus et al. 2008; Mascellino et al. 2008; Ondondo et al. 2009). Similarly, cell-mediated immune reactivity to the chlamydial hsp60 is also demonstrable in women with salpingitis or tubal infertility (Witkin et al. 1993; Witkin et al. 1994a, b; Kinnunen et al. 2000; Tiitinen et al. 2006). Of potential major significance are the observations that the heat shock protein antibodies in women with chlamydial-related pathology also react with either the corresponding human hsp60 or with conserved heat shock protein epitopes that are expressed in the homologous chlamydial and human proteins (Domeika et al. 1998; Witkin et al. 1998; Sziller et al. 1998; Sziller et al. 2008). This is consistent with a mechanism whereby a long-term exposure to the chlamydial hsp60 eventually leads to a loss of tolerance and the generation of immunity to conserved amino acid sequences that are also present in the homologous human hsp60.

Chlamydia trachomatis-induced Hsp60 autoimmunity and pregnancy failure

Most women who are infertile due to fallopian tube occlusion have never been diagnosed as having a sexually transmitted disease and have never had symptoms consistent with this diagnosis (Cates et al. 1993). However, women with asymptomatic tubal infertility have a greatly increased prevalence of antibodies to *C. trachomatis* (Dabekausen et al. 1994) and chlamydial hsp60 (Tiitinen et al. 2006). Furthermore, there is no association between the extent or severity of tubal damage and the presence or absence of symptoms. This reinforces the likelihood that a chronic chlamydial infection induces localized tissue damage while not invoking clinical symptoms.

Women who are infertile due to occluded fallopian tubes now try to achieve a pregnancy by undergoing in vitro fertilization and embryo transfer, a process that bypasses the need for the ovum to be fertilized in the fallopian tube and pass through to the uterus. However, several studies have demonstrated that the human hsp60 is expressed during early embryo development and that an immune response that recognizes the human hsp60 is detrimental to successful pregnancy.

Hsp60 expression in early mouse embryos (Bensuade and Morange, 1981) and on the surface of epithelial cells in the human decidua during early pregnancy (Mincheva-Nilsson et al. 1994; Neuer et al. 1996) has been demonstrated. A murine hybridoma specific for hsp60 was shown to react with human trophoblast, suggesting a cell surface location for hsp60 on these cells (Heybourne et al. 1994). A monoclonal antibody generated against the human hsp60 blocked the in vitro development of mouse embryos (Neuer et al. 1998). Thus, prolonged exposure to the chlamydial hsp60 as a consequence of a persistent asymptomatic upper genital tract infection may result in sensitization to conserved hsp60 epitopes that are also expressed in the human hsp60. Subsequent expression of the human hsp60 during early pregnancy, by the embryo and/or maternal decidua, will lead to reactivation of hsp60-sensitized lymphocytes. Pregnancy outcome may subsequently be compromised by the direct impairment of fetal or maternal cell viability by anti-hsp60 antibodies and/or induction of a pro-inflammatory response by hsp60-sensitized lymphocytes. An additional potential mechanism of hsp60-related early stage pregnancy loss has recently been identified. Chlamydial hsp60 binds to Toll-like receptor 4 on trophoblasts and induces apoptosis (Equils et al. 2006).

To more directly determine if antibodies to the chlamydial hsp60 interfered with fertility, 216 women who were undergoing a cycle of in vitro fertilization were tested for cervical IgA antibodies to recombinant *C. trachomatis* hsp60 at the time of embryo transfer. There was a strong positive correlation between a failure to become pregnant, or achieving only a transient biochemical pregnancy, and detection of anti-hsp60 IgA (Witkin et al., 1994a, b). Subsequently, the presence of antibodies to a synthetic peptide corresponding to an hsp60 epitope conserved between the chlamydial and human hsp60 was also associated with in vitro fertilization failure (Witkin et al. 1996). Additional evidence that immunity to the chlamydial hsp60 interferes with pregnancy comes from a study of women with a prior ectopic pregnancy who subsequently tried to become pregnant over a 5-year time period. The presence of antibodies to a synthetic peptide corresponding to a conserved epitope present in both the *C. trachomatis* and human hsp60 was associated with a lower spontaneous conception rate and increased incidence of adverse pregnancy outcome compared to women negative for this antibody (Sziller et al. 2008).

Immunity to hsp60 and pregnancy outcome

A cell-mediated immune response to the human hsp60, but not to the hsp60 of *Escherichia coli*, has also been associated with spontaneous abortion (Kligman et al. 1998). Indirect evidence for the involvement of antibodies to hsp60 in adverse pregnancy outcome comes from an examination of placentas from women who delivered preterm or at term. While hsp60 was identified in all placentas, only women who delivered preterm had IgG antibody associated with their placental hsp60 (Ziegert et al. 1999). Thus, immune sensitization to hsp60 as a consequence of a previous genital tract infection by *C. trachomatis* as well as possibly by other microorganisms

may result in induction of anti-hsp60 antibody production in response to placental hsp60 expression. Antibody binding to human hsp60 in placenta may activate a pro-inflammatory immune response thereby triggering the sequence of events that induce myometrial contractions and preterm labor and delivery. Further indirect evidence of the adverse influence of anti-hsp60 on placental function comes from a recent study in which IgM antibody to human hsp60, but not antibody to hsp70, obtained by cordocentesis from the fetal circulation, was found to be associated with development of a small for gestational age fetus (Belhia et al. 2010). In addition, quantitation of fetal anti hsp60 IgM levels was more sensitive in predicting the clinical outcome of unexplained cases of small for gestational age fetuses than the current standard evaluation by Doppler ultrasound.

Hsp60 species in *C. trachomatis*

In addition to the presence of an hsp60 protein in *C. trachomatis* (gene symbol Ct110) that is homologous to the *E. coli* GroEL, two other hsp60 genes have also been identified in *C. trachomatis* (gene symbols Ct604, Ct755). The CT110 product is identified as chsp60-1, the Ct604 product is chsp60-2, and the Ct755 product is chsp60-3 (Gerard et al. 2004; Ondondo et al. 2009). Ct110 and Ct604 are about 25% identical, Ct110 and Ct755 are about 30% identical, and Ct604 and Ct755 are only about 20% identical. All three genes appear to be expressed constitutively throughout the chlamydial life cycle and are present in elementary bodies. However, only CT110 is induced when infected HeLa cells are subjected to heat shock (Karunakaran et al. 2003). A recent study has shown that Ct755 is expressed only during an active productive infection, Ct604 is expressed during the establishment and maintenance of a persistent infection while Ct110 is the most immunogenic of the three chsps and has been shown to be expressed during active infection as well as during a persistent synovial infection in *Chlamydia*-associated arthritis (Gerard et al. 2004). The chsp60-1 protein has been shown to contain 17 regions where the amino acid sequence is similar or identical to that present in the human hsp60 and may be involved in induction of a cross-reactive hsp60-specific autoimmune response (Campanella et al. 2008). Similar to the situation in *Mycobacterium tuberculosis* where more than one hsp60-related gene is present and where each gene appears to have an independent function (Goyal et al. 2006), the multiple chlamydial hsp60 genes may also have autonomous or possibly interrelated roles in organism survival. An involvement of chsp60-2 and chsp60-3 in immune-mediated mechanisms remains to be determined.

A summary of the properties of the chlamydial hsp60 is presented in Table 1.

Table 1 Properties of the *Chlamydia trachomatis* hsp60

1.	The second most abundant protein in *C. trachomatis* lysates
2.	Loosely associated with the cell surface (easily removed by gentle washing)
3.	Present in both reticulate bodies and elementary bodies
4.	The only protein produced following induction of a non-culturable persistent state
5.	Released into the extracellular milieu during persistence
6.	48% amino acid sequence homology to the human hsp60
7.	Potent inducer of mononuclear delayed-type hypersensitivity response in animals and humans previously exposed to *C. trachomatis*
8.	Three genes coding for hsp60 are present—Ct110, Ct604, and Ct755
9.	The three hsp60 genes are expressed independently and differently during active and persistent infections.
10.	Ct110 is the chlamydial homolog of *E. coli* GroEL.

Clinical consequences

The immunopathogenic consequences resulting from a persistent *C. trachomatis* infection lead to the conclusion that prompt diagnosis and treatment of a newly acquired chlamydial infection might prevent development of these sequelae. The difficulty with applying this seemingly obvious solution is that *C. trachomatis* infections are most often asymptomatic and thus, difficult to detect. A solution to this problem is that all sexually active women should undergo screening for this microorganism, preferably by a protocol involving gene amplification technology. Ideally, screening should be repeated following every new sexual contact and each time the woman's partner has a new sexual contact. Unfortunately, most young women, as well as their clinicians, are currently not aware of this reality and a program of public health education regarding the value of testing for chlamydial infections is sorely needed.

However, as has been pointed out recently, implementation of public health measures to diagnose newly acquired *C. trachomatis* infections is not without adverse consequences (Brunham and Rekart, 2009). Because an effective anti-chlamydial immune response takes many months to develop (Molano et al. 2005), prompt diagnosis and treatment of this infection leaves the individual susceptible to reinfection. In fact, the incidence of *C. trachomatis* reinfection has been increasing in the United States since the mid-1990s. However, the concomitant decrease in the rate of upper genital tract infection suggests that it is more important to prevent a persistent chlamydial infection than to develop natural immunity to prevent a reinfection. This trade-off, therefore, is apparently worthwhile.

As described above, the persistence in vivo of genital tract chlamydial serovars is related, in many cases, to the

lack of available tryptophan. A co-infection with another microorganism that is capable of producing tryptophan may, therefore, lead to the transient reestablishment of RB replication and the return of a productive chlamydial infection (Caldwell et al. 2003). Repeated long-term cycles of productive infection and persistence with intermittent release of the chlamydial hsp60 may further facilitate the breaking of tolerance to self hsp60 and development of high titer anti-hsp60 autoantibodies.

It has been suggested that exposure to the *C. trachomatis* hsp60 may also be a risk factor for development of cancer (Di Felice et al. 2005). The proposed mechanism involves the hsp60-mediated inhibition of apoptosis. The enhanced susceptibility of chlamydial hsp60-positive cells that express oncogenes or have mutated DNA to survive and continue to proliferate could foster evolution of the malignant phenotype. Conversely, it has also been proposed that development of anti-chlamydial hsp60 immunity may be protective against development of malignant tumors (Cappello et al. 2009). Since some tumors have hsp60 presented on their surface (Shin et al. 2003), the presence of antibodies directed to shared conserved hsp60 epitopes might promote development of an anti-tumor immune response.

An immune response to self hsp60 expressed on the surface of endothelial cells or into secretory granules of islet beta cells has been associated with development of atherosclerosis (Perschinka et al. 2003) and diabetes (Brudzynski 1993), respectively. While other sources of anti-hsp60 are possible, a prior or concomitant persistent chlamydial infection may very well contribute significantly to development of cross-reacting anti-human hsp60 autoantibodies and initiation or facilitation of disease processes.

Conclusions

A *C. trachomatis* infection of the female genital tract can result in induction of unique hsp60-mediated pathology. The ability of this microorganism to migrate from the cervix to the upper genital tract and survive in epithelial cells in a non-replicative, intracellular persistent state results in a long-term release of chlamydial hsp60. In addition to stimulating a local pro-inflammatory immune response that results in tissue scarring and occlusion, the continuous exposure of the immune system to this microbial hsp60 increases the likelihood of eventual immune sensitivity to conserved regions of hsp60 that are also expressed in the homologous human hsp60. The generation of autoantibodies and T cell responses to one's own hsp60 can have negative consequences for future pregnancy outcome as well as elevate susceptibility to development of autoimmune disorders, atherosclerosis, and possibly also progression of malignancy. The prevalence of a *C. trachomatis* genital tract infection among 14–19-year-old female adolescents in the United States has very recently been estimated to be 3.9% (Forhan et al. 2009). Given this high prevalence, concentrated efforts to diagnose and effectively treat sexually transmitted *C. trachomatis* infections prior to their ascension to the upper genital tract deserve special attention. Since the only way to determine whether a sexually active woman is infected with this asymptomatic pathogen is to have a screening test for *C. trachomatis*, efforts to increase public awareness of the need for such testing are badly needed. In addition, identification of the subset of women who are at high risk for developing a persistent upper genital tract infection after being exposed to *C. trachomatis* should be a research priority. Studies have demonstrated that women with functional polymorphisms in the gene coding for HLA DQ (Kinnunen et al. 2002), mannose-binding lectin, an innate immune system antimicrobial component (Sziller et al. 2007) as well as other genes with immune functions (summarized in Brunham and Rekart 2009), are at increased likelihood to develop fallopian tube damage after being infected with *C. trachomatis*. Delineation of mechanisms to prevent development of autoimmunity to hsp60 or, when present, to limit its negative impact on pregnancy outcome and autoimmunity are other understudied areas of investigation.

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