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Extracellular 70-kd heat shock protein in mid-trimester amniotic fluid and its effect on cytokine production by ex vivo–cultured amniotic fluid cells

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KEY WORDS
70-kd Heat shock protein
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Tumor necrosis factor-α
Toll-like receptor
Mycoplasma hominis

Objective: The 70-kd heat shock protein is released from cells in response to stress and functions as a regulator of innate immunity. We hypothesized that 70-kd heat shock protein in mid-trimester amniotic fluid might regulate local immune system activation.

Study design: Amniotic fluid that was obtained from 200 women who underwent amniocentesis at 15 to 19 weeks of gestation was tested by enzyme-linked immunosorbent assay for 70-kd heat shock protein, tumor necrosis factor-α, and interleukin-1β and -6. The amniotic fluid cellular fraction also was evaluated for *Mycoplasma hominis* by gene amplification. Whole amniotic fluids were incubated ex vivo in medium alone or medium that contained peptidoglycan, a TLR2 ligand, or lipopolysaccharide, a TLR4 ligand. After 24 hours, the supernatants were collected and assayed for 70-kd heat shock protein. The influence of exogenous 70-kd heat shock protein on tumor necrosis factor-α and interleukin-1β and -6 production by whole amniotic fluid was assessed similarly.

Results: The 70-kd heat shock protein was detected in all amniotic fluids with a median (range) of 11.5 ng/mL (1.2-76.7). The intra-amniotic 70-kd heat shock protein concentration was correlated positively only with amniotic fluid tumor necrosis factor-α levels ($P = .0002$). Detection of *M hominis* was associated with an increased 70-kd heat shock protein concentration (median, 17.2 ng/mL; $P = .01$). The addition of peptidoglycan resulted in a stimulation of 70-kd heat shock protein production, and exogenous 70-kd heat shock protein stimulated the release of tumor necrosis factor-α by amniotic fluid cells.

Conclusion: The 70-kd heat shock protein is released from cells in mid-trimester amniotic fluid as a consequence of TLR2 stimulation and potentiates tumor necrosis factor-α production.

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Heat shock proteins, so named because they were first discovered to be synthesized in response to elevated temperature, are a highly conserved family of proteins that are present in all prokaryotic and eukaryotic organisms. They function as intracellular chaperones and aid cell survival under adverse conditions. The production of the highly inducible 70-kd heat shock protein (hsp70) is up-regulated greatly in response to a wide variety of stressful stimuli, which including infection, inflammation, fever, malignancy, rapid cell differentiation, or tissue development.[1]

Recent evidence strongly suggests that extracellular heat shock proteins have a role as regulators of innate immunity. The synthesis and release of heat shock proteins into the extracellular milieu is stimulated by infection and by proinflammatory cytokines.[2-4] Released hsp70 can bind to Toll-like receptor (TLR)2 and TLR4 and initiate the activation of the primary proinflammatory cytokines, tumor necrosis factor-α (TNF-α), interleukin (IL)-1β and IL-6.[5,6] Conversely, extracellular heat shock proteins may also down-regulate the innate immune response. In the absence of infection, repetitive heat shock protein binding to TLRs may render these receptors unresponsive to more potent and potentially deleterious proinflammatory TLR activators.[4] Thus, the induction and release of heat shock proteins into the amniotic cavity may be a physiologic mechanism that is designed to modulate the extent of localized inflammation in response to variable circumstances.

In the present communication, we test the hypothesis that TLR stimulation results in the release of extracellular hsp70 into mid-trimester amniotic fluid and that the hsp70 stimulates TNF-α, IL-1β, and IL-6 production.

Material and methods

Study participants were 200 pregnant women with singleton pregnancies who underwent amniocentesis between 15 and 19 weeks of gestation for the typical clinical indications: advanced maternal age, maternal request, abnormal values on first- or second-trimester multiple serum marker testing, family history of chromosome abnormalities, or ultrasound identification of markers of aneuploidy. All subjects were free of detectable medical complications, negative for vaginal and cervical infections assessed by routine cultures during a previous prenatal examination, and had no clinical indications of myometrial contractions or infections at the time of amniocentesis. The study was approved by the Institutional Review Board at the Weill Medical College of Cornell University, and written informed consent was obtained. Samples were collected by transabdominal amniocentesis after disinfection of the skin. Excess unprocessed amniotic fluids that were not needed for clinical analysis were transported to the laboratory within 2 to 3 hours of collection. Aliquots were removed for ex vivo culture; the remainder of the sample was centrifuged, and supernatant and pellet fractions were stored separately at –80°C until they were analyzed.

Pregnancy outcome data were obtained from medical records only after the completion of all testing. The chart reviewer was blinded to all laboratory results. Spontaneous preterm birth was defined as delivery at <37 weeks of gestation that was preceded by preterm labor. Race/ethnicity was self-reported by the mother in consultation with her obstetrician.

For ex vivo culture, aliquots (0.18 mL) of fresh unfractionated amniotic fluid from white women were added to wells of a sterile microtiter plate. To quadruplicate wells were added 0.02 mL of either RPMI culture medium alone or medium that contained 100 ng/mL *Mycoplasma hominis* freeze-thaw cell lysate, 80 μg/mL *Staphylococcus aureus* peptidoglycan (a TLR2 inducer; InvivoGen, San Diego, CA), or 50 ng/mL *Escherichia coli* serotype 0111:B4 lipopolysaccharide (a TLR4 inducer; Sigma Chemical Co, St. Louis, MO). The optimal concentrations of peptidoglycan, lipopolysaccharide, and *M hominis* lysates were determined in pilot experiments. After 24 hours in a 5% carbon dioxide incubator at 37°C, the plate was centrifuged; like aliquots were pooled, and supernatants were frozen at –80°C. To analyze the influence of hsp70 on proinflammatory cytokine production by amniotic fluid cells, fresh amniotic fluids were incubated in 8 wells each that contained RPMI, lipopolysaccharide, or peptidoglycan. Recombinant low endotoxin hsp70 (StressGen, Victoria, British Colombia) was added to 4 of the wells with each unique component at a final concentration of 10 μg/mL. Incubation and processing was as described earlier. All reagents were filter sterilized before use, and a sterile technique was used throughout.

Amniotic fluids and culture supernatants were assayed in duplicate for concentrations of hsp70 (StressGen), TNF-α (Ultrasensitive ELISA, Biosource, Camarillo, CA), IL-1β (Biosource), and IL-6 (Biosource). Values were converted to nanograms per milliliter or picograms per milliliter by reference to a standard curve that was assayed in parallel to the test samples. For the culture supernatant analyses, values that were obtained from the amniotic fluids before the 24-hour incubation were subtracted from the experimental values. The lower limits of sensitivity were 0.5 ng/mL (hsp70), 0.09 pg/mL (TNF-α), 1.0 pg/mL (IL-1β), and 2.0 pg/mL (IL-6).

The presence of *M hominis* in the amniotic fluids was assessed by our polymerase chain reaction–enzyme-linked immunosorbent assay, as described previously.[7]

Possible associations between concentrations of hsp70, TNF-α, IL-1β, and IL-6 that are released by ex vivo amniotic fluid and the presence of exogenously added TLR agonists and gender and racial/ethnic