Infectious Diseases in Obstetrics and Gynecology 7:180–185 (1999)

Heat Shock Proteins and Heat Shock Protein-Antibody Complexes in Placental Tissues

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ABSTRACT

Objective: The relationship between pregnancy outcome and expression of the heat shock proteins (hsps) or hsp-antibody complexes of 60kD (hsp60), 70kD (hsp70), and 90kD (hsp90) in placental tissue and circulating antibodies to hsps was evaluated.

Method: Expression of hsp60, hsp70, and hsp90 in placentae from 12 women with preterm birth, eight with intrauterine growth restriction (IUGR), and 10 with term birth, as well as the presence of the corresponding antibodies, was investigated by a new carbocyanine double fluorescence technique. Results were compared with microbiological findings and circulating antibodies to hsps in sera.

Results: In each placental specimen examined, hsp60, hsp70, and hsp90 were identified. However, hsp70-antibody complexes were detected in only four of the preterm labor cases. Similarly, hsp60-antibody complexes were detected in only five preterm labor patients and in one patient with IUGR. None of the placentae contained hsp90-antibody complexes. In the preterm birth group, all patients with hsp60-antibody complexes were also positive for circulating antibodies to hsp60. The presence of hsp70-antibody complexes also correlated with hsp70 antibody in sera.

Conclusions: Formation of hsp60- and hsp70-antibody complexes in the placenta may contribute to the induction of preterm birth. Women sensitized to these antibodies may be at increased risk for adverse pregnancy outcome. Infect. Dis. Obstet. Gynecol. 7:180–185, 1999. © 1999 Wiley-Liss, Inc.

KEY WORDS

preterm labor; placenta; carbocyanine double fluorescence; heat shock protein immunity

Heat shock proteins (hsps) protect cells from structural and functional damage following exposure to adverse environmental conditions. The functional involvement of hsps in folding and translocation of newly synthesized polypeptides, protecting hydrophobic domains from denaturation, and binding to receptor complexes are essential cell functions.[1,2] Heat shock proteins are also involved in immune responses; microbial hsps are potent immunogens in mammals. Conversely, initiation of mammalian hsp gene transcription has been associated with the inhibition of pro-

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Clinical Study

Received 2 November 1998
Accepted 28 January 1999

inflammatory cytokine production.[3–5] Further, autoimmunity to self-hsps has also been correlated with autoimmune diseases.[6,7]

Immunity to epitopes of the 60kD hsp (hsp60) molecule that are present in human hsp60 has also been associated with adverse pregnancy outcome. Women with cervical immunoglobulin (Ig) A antibodies to the *Chlamydia trachomatis* hsp60[8] or to a synthetic peptide corresponding to a conserved epitope present in both the chlamydial and human hsp60[9] have a decreased success rate with in vitro fertilization and embryo transfer. Similarly, cell-mediated immunity to the human hsp60, but not to the *Escherichia coli* hsp60, correlates with a history of spontaneous abortions.[10] Monoclonal antibody to mammalian hsp60 was also shown to inhibit the in vitro development of murine embryos.[11]

Divers et al. identified hsps in human placentae and found no differences in their expression between term and preterm pregnancies.[12] In this communication, we investigated the distribution of hsp60, as well as the 70kD (hsp70) and 90kD (hsp90) hsp and hsp-antibody complexes in placental tissue, and circulating antibodies to hsps in women with term and preterm birth and term birth with intrauterine growth restriction (IUGR).

MATERIAL AND METHODS

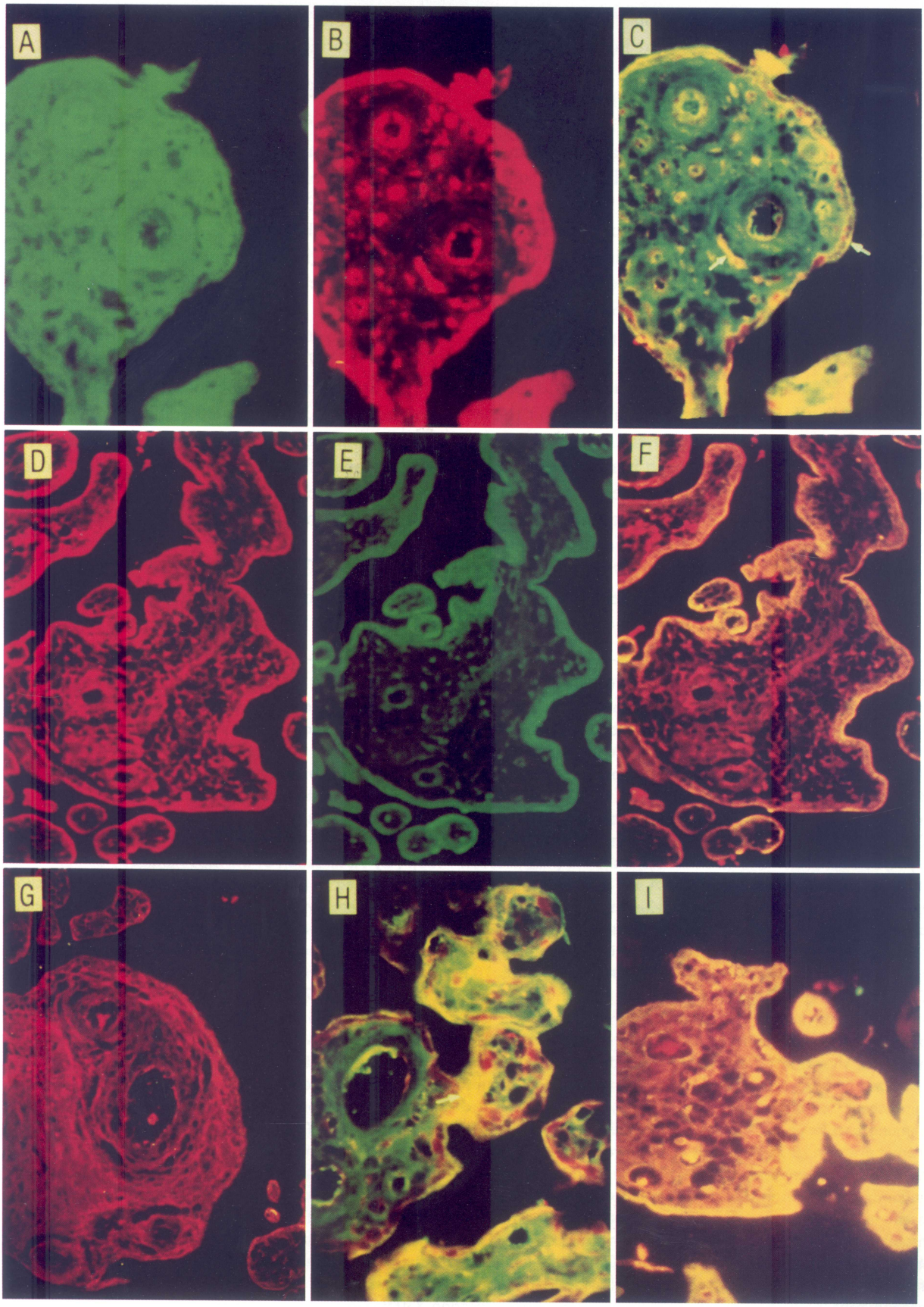
Material and Tissue Preparation

Placental tissue from 10 uncomplicated term births, 12 preterm births (28th–34th week of gestation), and eight term births with small-for-gestational-age babies (IUGR, below the tenth percentile in weight) were investigated by carbocyamine double fluorescence labeling.[13] The placental tissue samples were fixed with 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS) of pH 7.4. The tissue was subsequently equilibrated by suspension for one day each in PBS containing 10%, 20%, and 30% sucrose. Three frozen placental tissue sections (5mm) from different local regions of each placenta were mounted on fluorescence-free slides and washed three times for 5 min. in Tris-buffered saline (TBS), pH 7.4.

Immunofluorescence

For blocking nonspecific binding sites, the tissue was incubated in TBS plus 5% goat serum and

Fig. 1. Double labeling of hsp60 and hsp70 and the antibodies (IgG) in cryosections of placental tissues from a preterm labor case by combination of red (Cy3) and green (Cy2) immunofluorescence. Notice the yellow appearance of cells with both hsp60 and hsp70 and anti-hsp60 or anti-hsp70 antibodies. Pairs of photomicrographs (A and B as well as C and D) present the same structures mono-labeled for hsp (A, D), for antibodies (R, E) and double stained for the antibody attached to hsp60 or hsp70 (C, F). **(A)** Immunodetection of hsp60 from a case of preterm labor by green fluorescent Cy2-goat anti-rabbit IgG. Green fluorescent Cy2 immunostaining of human hsp60 in cells of the surface layer (syncytiotrophoblast) and stromal cells is shown (magnification ×150). **(B)** Immunodetection of antibodies against hsp60 from a case of preterm labor by red fluorescent Cy3-goat anti-human IgG. Red fluorescent Cy3 immunostaining of human hsp60-antibodies in cells of the surface layer (syncytiotrophoblast) and stromal cells is shown. (magnification ×150). **(C)** Visualization of the hsp60-antibody complexes in the same frame of cryosection as in A and B. Placental tissue was double labeled by using polyclonal rabbit anti-human hsp60 and, subsequently, red fluorescent Cy3-goat anti-human IgG and green fluorescent Cy2-tagged goat anti-rabbit IgG. The double exposure of both immunoreactivities leads to the yellow appearance of the hsp60-antibody complex (arrows). Green fluorescence shows hsp60 not attached by anti-hsp60 antibodies. Red fluorescence demonstrates other placental human IgG not specific for hsp60 (magnification ×150). **(D)** Staining of hsp70 from a case of preterm labor was revealed by red fluorescent Cy3-goat anti-rat IgG in cells of the surface layer and in stromal cells (magnification ×75). **(E)** Staining of hsp70 antibodies from a case of preterm labor was revealed by green fluorescent Cy2-goat anti-human IgG. Green fluorescent Cy2 immunostaining of human hsp70-antibodies in cells of the surface layer and in stromal cells is shown (magnification ×75). **(F)** Allocation of hsp70 (as shown in D) and hsp70-antibody complexes from a case of preterm labor. Placental tissue was simultaneously visualized by using monoclonal rat anti-human hsp70 and, subsequently, green fluorescent Cy2-goat anti-human IgG and red fluorescent Cy3-tagged goat anti-rat IgG (switch of the fluorochromes in comparison with C). The double exposure of both immunoreactivities leads to the yellow appearance of the hsp70-antibody complexes (magnification ×75). **(G)** Staining of hsp90 from a preterm labor patient by applying red fluorescent Cy3-goat anti-rabbit IgG in cells of the surface layer and in stromal cells. No hsp90-antibody complexes were seen in the placental tissue (magnification ×500). **(H)** Demonstration of hsp60-antibody complexes in the surface layer facing the cavum intervillosum and in stromal cells (yellow color, arrow). Note the predominant localization in cells of the surface layer (syncytiotrophoblast). Green fluorescence indicates the localization of hsp60. Red fluorescence shows other human placental IgG not specific for hsp60 and therefore not bound to hsp60 (magnification ×500). **(I)** Visualization of hsp70-antibody complexes in the surface layer facing the cavum intervillosum and in stromal cells. Red fluorescence reveals the localization of hsp70 not associated with anti-hsp70 antibodies. Notice the predominant localization in cells of the surface layer (magnification ×500).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0.3% Triton X-100 (TBS-T-NGS) in a moisture box at room temperature for 60 min. Immunostaining by double fluorescence utilized a primary polyclonal rabbit or monoclonal rat antibody against human hsp 60 (polyclonal rabbit anti-Hsp56/FKB59), hsp 70 (monoclonal rat anti-Hsp70), and hsp 90 (polyclonal rabbit anti-Hsp86) (StressGen, Victoria, B.C.) and fluorochrome-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA) directed against rabbit and rat immunoglobulins, as well as against human antibodies. Sections were incubated with primary antibodies at a concentration of 10µg/mL in TBS-T-NGS overnight at room temperature followed by rinsing three times for 5 min. in TBS. The concomitant detection of human anti-hsp60, anti-hsp70, and anti-hsp90 antibodies and different hsps in the tissue was performed by incubation with a cocktail of goat anti-human IgG conjugated to red fluorescent Cy3 (20µg/mL in TBS containing 2% bovine serum albumin [2% TBS-BSA]) and goat anti-rabbit IgG conjugated to green fluorescent Cy2 (10µg/mL in 2% TBS-BSA) for 1 hr. at room temperature. Thereafter, the sections were rinsed three times with TBS, followed by a short rinse with distilled water, air dried, and cover slipped with Entellan in toluene (Merck, Darmstad, Germany).

Control Experiments

In a first set of control experiments, primary antibodies were replaced by TBS-T-NGS and subsequently the cocktail of fluorochrome-conjugated immunoreagents (see above) was added. A further control for specificity of the hsp-antibodies for hsps was to switch the fluorochromes (see Fig. 1, hsp70) related to autoantibodies and primary antibodies directed against hsp. The binding sites of these exogenously applied primary antibodies and for endogenously occurring (human) antibodies were then concomitantly visualized by applying a cocktail of either green fluorescent Cy2-goat-anti-rabbit IgG or Cy2-goat-anti-rat IgG and red fluorescent Cy3-goat-anti-human IgG.

Fluorescence Microscopy

The slides were examined with a Zeiss Axioplan fluorescence microscope (CarlZeiss, Jena, Germany) equipped with appropriate filter combinations for the simultaneous visualization of green and red fluorescence. Cells containing hsp70 and hsp90 showed a red immunofluorescence using Cy3-labeling, while cells containing hsp60 showed green by using Cy2-labeling. The antibodies for hsp60 showed a red (Cy3) immunofluorescence and for hsp70, showed green (Cy2). Heat shock protein-antibody complexes appeared yellow after mixing of red and green immunofluorescence, demonstrating the double exposure of both immunoreactivities. Photomicrographs were made with Ektachrome 400 Elite slide film (Kodak, Rochester, NY).

Clinical, Microbiological, and Serological Factors

Clinical, microbiological, and serological evaluations were carried out to verify the immunohistochemical results. Clinical characteristics (e.g., smoking, single umbilical cord artery, preterm rupture of membranes, cervical dilation) in the pregnancy follow up were recorded. Vaginal swab samples were examined for bacteria and *Candida* species by standard clinical cell cultures. Detection of *C. trachomatis* from cervical samples and placental tissue was performed using polymerase chain reaction (PCR).[14] Antibodies to recombinant human hsp60 and hsp70 in maternal serum were determined by enzyme-linked immunosorbent assay (ELISA), as described elsewhere.[15]

Statistics

Fisher exact test was used to determine differences in discrete variables among the groups.

RESULTS

Immunofluorescence

In every placental specimen examined, we could demonstrate human hsp60 and hsp70, as evident by green or red immunofluorescence. Heat shock proteins were more evident in the apical surface of the syncytiotrophoblast layer than in stromal cells and muscle cells of the blood vessels (Fig. 1: A, D). Heat shock protein-70-antibody complexes were localized in four cases (4/12, $P = 0.018$) of preterm birth but in none of the two other groups (Table 1, Fig. 1: F, I). Heat shock protein-60-antibody complexes were seen in five cases of preterm birth (5/12, $P = 0.026$), in one case of IUGR (1/8), and in no term births (Table 1, Fig. 1: C, H). The occurrence of hsp70-antibody complexes (3/12; Table 1) coincided with placentae displaying hsp60-antibody complexes. In two cases, hsp70-antibody complexes were not detectable in the concomitant

TABLE 1. Relation between placental hsp60-anti-hsp60 or placental hsp70-anti-hsp70 complexes and pregnancy outcome

Group	No. of tissue samples	No. (%) with anti-hap60 complexes	No. (%) with anti-hsp70 complexes	No. (%) positive for both complexes
Term birth	10	0	0	0
IUGR	8	1 (12.5)	0	0
Preterm birth	12	5 (41.7)*	4 (33.3)**	3 (25)***

*P = 0.026 vs. term labor and IUGR
**P = 0.018 vs. term labor and IUGR
***P = 0.05 vs. term labor and IUGR

presence of hsp60-antibody complexes. Heat shock protein-90-antibody complexes were not found in any samples.

Clinical Findings

Term birth cases did not show any abnormal clinical findings, while in one case of IUGR, a single umbilical artery, and in four cases of IUGR maternal smoking resulting in placental calcification could be verified as a possible cause. Chromosomal disorders were excluded by chromosomal examinations of amniotic cells.

Serological Results

In maternal serum samples of the preterm birth group, IgG antibody to the human hsp60 were more prevalent than in the IUGR and term labor groups (Table 2). This antibody was present in seven cases in the preterm birth group, in two in the IUGR group, and in two in the term birth group ($P < 0.05$). Anti-hsp70 IgG was present in four cases of preterm birth and in none among the IUGR or term labor groups ($P = 0.018$) (Table 2).

Microbiological Findings

The microbiological findings of all subjects are shown in Table 3. There were no differences between groups in the prevalence of any infection. Antibiotic treatment in those patients with positive bacterial culture from the vagina did not influence preterm labor. There was no relation between microbiological findings and hsp antibodies or hsp-antibody complexes.

DISCUSSION

All placental tissues examined expressed hsp60, hsp70, and hsp90. Comparing the term, preterm,

TABLE 2. IgG antibody to human hsp60 and hsp70 in sera from pregnant women

Group	No. of women	hsp60-antibody no. (%) positive	hsp70 no. (%) positive
Term labor	10	2 (20.0)	0
IUGR	8	2 (25.0)	0
Preterm labor	12	7 (58.3)*	4 (33.3)**

*P = 0.05 vs. term labor and IUGR, P < .05 vs. term labor
**P = 0.018 vs. term labor and IUGR

TABLE 3. Microbiological evaluation of pregnant subjects

Vaginal/cervical findings	No. of positive/no. tested		
	Term	IUGR	Preterm
Normal	6/10	6/8	5/12
Bacterial vaginosis	1/10	0/8	2/12
Ureaplasma/mycoplasma	1/10	2/8	2/12
E. coli	1/10	1/8	1/12
Anaerobic bacteria	1/10	0/8	1/12
C. trachomatis	0/10	0/8	1/12[a]
Candida	1/10	0/8	2/12

[a]Positive only in placental tissue by PCR.

and IUGR groups, we did not find any differences in the intensity of hsp expression, confirming that the presence or absence of hsp seems to have no influence on preterm labor.[12] However, the concomitant presence of hsp immunity may be relevant to the pathogenesis of preterm labor. Using the carbocyanines Cy2 and Cy3, which were previously shown to be suitable for sensitive double immunofluorescence labeling of neuropathology,[13] we demonstrated the simultaneous detection of hsp60- and hsp70-antigen-antibody complexes only in placental tissues from some women who delivered preterm. The detection of circulating antibodies to hsp60 and hsp70 correlated with the placental findings of antigen-antibody complexes.

Thus, immune sensitization to hsp60 and hsp70 can result in the appearance of hsp60- and hsp70-immune complexes in the placenta. The subsequent activation of the pro-inflammatory cytokine cascade by these complexes might adversely influence pregnancy outcome by the induction of preterm labor.[16,17] There is more than one cause of preterm labor, so it is not surprising that only a subgroup of our preterm labor patients had placental hsp-antibody complexes. However, although the sample size was limited, the results suggest

that the appearance of these complexes correlate with preterm birth in some women.

Comparing the term labor and IUGR group, we cannot find any differences in hsp expression or immunity in sera or placenta. The assumption of an impaired placental metabolic exchange caused by hsp-antigen-antibody complexes in IUGR cannot be confirmed by the present experiments.

Induction of an immune response to human hsp60 and hsp70, either prior to or subsequent to pregnancy, might be due to a genital tract infection with bacteria sharing cross-reactive epitopes with the human hsp60 or hsp70.[7,9,18] Once antibody production is induced, the stress response is continued due to host hsp expression, which might further sustain the immune reaction. In such cases antibiotic treatment may not be able to stop the induced immune process. Additional prospective studies are required to clarify the influence of hsp60 and hsp70 antibodies in the immune network, especially in pregnant women, for more efficient diagnostic and therapeutic strategies.

In conclusion, systemic immunity to human hsp60 or hsp70 and antibody binding to these hsps in placental tissue might be a morphological and pathophysiological indicator of preterm labor. Further studies are necessary to determine possible synergistic actions of hsp60 and hsp70 antibodies and their immune complexes with cytokine activation to better understand the immune mechanism of preterm labor. Determination of circulating antibody to hsp60 or hsp70 in women, or antibody to specific hsp epitopes, might have diagnostic or prognostic relevance for predicting risk of preterm delivery.

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