Increased circulating heat shock protein 70 levels in pregnant asthmatics

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Abstract Asthma is one of the most common diseases complicating pregnancy and represents a risk factor for several maternal and perinatal complications. The natural history of asthma is known to change in pregnancy, but very few data are available in the terms of pathomechanism of this change during gestation. Circulating heat shock protein 70 (Hsp70) levels are decreased in healthy pregnancy, which might reflect physiological immunotolerance. The aim of our study was to determine the serum levels of Hsp70 in asthmatic women during gestation. Forty pregnant women with bronchial asthma and 40 healthy pregnant women matched for maternal and gestational age were involved in this case-control study. Serum Hsp70 levels were measured using the ELISA Kit of R&D Systems.

Spirometry and oxygen saturation measurements were performed in asthmatic patients. In asthmatic pregnant women, an increase of serum Hsp70 levels was observed compared to healthy pregnant women (median (25–75 percentile): 0.44 ng/ml (0.36–0.53) versus 0.21 ng/ml (0–0.27), $p < 0.001$). Fetal birth weight of asthmatic mothers was significantly smaller than of healthy controls, but in the normal range (3,230 g (2,690–3,550) versus 3,550 g (3,450–3,775), $p < 0.05$). A statistically significant negative correlation between maternal age and serum Hsp70 concentrations (Spearman $R = -0.48$, $p = 0.0018$) and a significant positive correlation between gestational age and serum Hsp70 levels (Spearman $R = 0.83$, $p < 0.001$) were detected in healthy pregnant women. In conclusion, this study proves an elevation of circulating Hsp70 levels during asthmatic pregnancy compared to healthy pregnant women. However, further studies are warranted to determine the role of circulating Hsp70 in the pathogenesis of maternal and perinatal complications of asthma in pregnancy.

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Introduction

Heat shock proteins (Hsps) are ubiquitous and phylogenetically conserved molecules, which indicate their functional importance. They are usually considered to be intracellular proteins with molecular chaperone and cytoprotective functions (Hightower 1991). However, Hsp60 and Hsp70 have been shown to be present in the serum and plasma of healthy nonpregnant women (Pockley et al. 1998, 1999). We have recently reported that serum Hsp70 levels are significantly lower in healthy pregnant women than in

healthy nonpregnant women (Molvarec et al. 2007a). The role of Hsp downregulation in normal pregnancy is not known, but we hypothesize that decreased circulating Hsp70 levels are due to mechanisms that maintain immune tolerance in pregnancy. In that study, circulating Hsp70 levels increased with advancing gestational age and decreased with increasing maternal age. The age-related decrease in Hsp70 levels observed in healthy pregnant women is in accordance with findings in nonpregnant subjects, and it might be explained by reduced ability of cells to respond to stress with increasing age (Rao et al. 1999; Rea et al. 2001; Jin et al. 2004). Increased circulating Hsp70 levels during pregnancy may be associated with maternal and fetal complications. Several previous studies reported elevated serum Hsp70 levels during pregnancy associated with preeclampsia, preterm delivery, and HELLP syndrome (Jirecek et al. 2002; Fukushima et al. 2005; Molvarec et al. 2006, 2007b, 2009; Madach et al. 2008). However, contradictory data also exist (Livingston et al. 2002).

Asthma is among the most common, potentially serious medical problems that complicate pregnancy. The most commonly reported maternal and fetal complications in asthmatic pregnant women are very similar to those with high Hsp70 levels: preeclampsia, preterm delivery, and infants with low birth weight or intrauterine growth restriction (Demissie et al. 1998; Kallen et al. 2000; Liu et al. 2001). A very recent study by Breton and coworkers of a database cohort of 13,100 pregnant asthmatics reported a 35% increased risk of perinatal mortality in the pregnancies of women with asthma, which may be partly explained by a higher rate of low birth-weight babies and preterm delivery (Breton et al. 2009). The confounding factors contributing to this increased perinatal mortality might be maternal obesity and smoking, as well as uncontrolled asthma (Schatz 2009). Another recent study of pregnant women with physician-diagnosed asthma ($n=719$) evaluated their asthma control repeatedly during pregnancy based on symptom frequency and interference with daily activities and sleep, as well as on reported hospitalizations and unscheduled clinic visits for asthma exacerbations. The incidence of preterm delivery, intrauterine growth restriction, and mean birth weight were evaluated relative to asthma symptom control and exacerbation measures. According to their results, the incidence of preterm delivery was significantly higher among patients with inadequate asthma symptom control during the first part of pregnancy compared with patients with adequate asthma control, and patients who were hospitalized for asthma during pregnancy had a higher incidence of preterm delivery compared with asthmatic women without a history of hospitalization, so there may be a risk for preterm delivery posed by poorly controlled maternal asthma (Bakhireva et al. 2008). Maternal asthma is also known as a risk factor for the

development of asthma in children (Celedon et al. 2002; Kurukulaaratchy et al. 2003; Latzin et al. 2007).

The natural history of asthma is known to change in pregnancy, but very few data are available in terms of the pathomechanism of this change during gestation (Schatz et al. 1988). In our previous study, we demonstrated culminating proliferation of circulating interferon- γ and interleukin-4 positive T-lymphocytes associated with uncontrolled asthmatic inflammation, which may potentially impair maternal airway symptoms as well as fetal development (Tamasi et al. 2005). It may be plausible that hypothesized altered Hsp70 levels in asthmatic pregnant women may play a role in decreased immunological tolerance that characterizes asthmatic pregnancies and result in perinatal and maternal complications.

The aim of our study was to evaluate: (1) the circulating levels of Hsp70 in asthmatic women during gestation and (2) the potential correlation between maternal Hsp70 levels and patient characteristics.

Methods

Subjects

Forty pregnant women with bronchial asthma and 40 healthy pregnant women matched for maternal and gestational age were involved in this case-control study. The study participants were enrolled in the Department of Pulmonology, in the 1st Department of Obstetrics and Gynecology and in the Department of Obstetrics and Gynecology of Kútvolgyi Clinical Center, at the Semmelweis University, Budapest, Hungary. All women were Caucasian and resided in the same geographic area in Hungary. Exclusion criteria were multifetal gestation, hypertensive disorders, diabetes mellitus, autoimmune disease, angiopathy, renal disorder, maternal or fetal infection, and fetal congenital anomaly. The women were fasting, none were in active labor, and none had rupture of membranes. All control subjects completed pregnancy without any complication.

Asthma was defined according to the current Global Initiative for Asthma (GINA) guideline, and all patients had persistent disease (www.ginasthma.com). All patients were prescribed inhaled corticosteroids. Long-acting beta-agonists were used by 21 patients. Fetal growth restriction was diagnosed if the fetal birth weight was below the 10th percentile for gestational age and gender, based on Hungarian birth-weight percentiles (Joubert 2000).

The study protocol was approved by the Regional and Institutional Committee of Science and Research Ethics of the Semmelweis University, and written informed consent was obtained from each patient. The study was conducted in accordance with the Declaration of Helsinki.

Biological samples

Maternal blood samples were obtained from an antecubital vein into native tubes and centrifuged at room temperature with a relative centrifugal force of $3,000\times g$ for 10 min. The aliquots of serum were stored at -80°C until the analyses were performed.

Laboratory methods

Serum Hsp70 levels were measured by using the ELISA Kit of R&D Systems (DYC1663E, Minneapolis, MN, USA). Ninety-six-well microtiter plates were coated with mouse antihuman Hsp70 capture antibodies ($100\text{ }\mu\text{l}$; $2\text{ }\mu\text{g/ml}$) in carbonate buffer (pH 9.5) overnight at 4°C . Plates were washed with PBS containing 0.1% Tween 20 three times and nonspecific binding sites blocked by incubation with $200\text{ }\mu\text{l}$ of PBS containing 0.5% gelatine and Tween 20 for 1 h at room temperature. After washing, $100\text{ }\mu\text{l}$ of the reference preparation (recombinant human Hsp70, 0–10 ng/ml) or samples (1:1) were added, and the plates were incubated for 2 h at room temperature. Plates were subsequently washed and Hsp70 binding was determined using biotinylated rabbit antihuman Hsp70 antibodies ($100\text{ }\mu\text{l}$; $0.5\text{ }\mu\text{g/ml}$) in PBS gelatine. After 1.5 h at room temperature, plates were washed and incubated with streptavidin-HRP (horseradish-peroxidase, 1:200) in PBS gelatine for 20 min at room temperature. Plates were washed and $100\text{ }\mu\text{l}$ of *o*-phenylene-diamine (Sigma, St. Louis, MO, USA) in citrate buffer was added. The optical density was measured at $\lambda=490\text{ nm}$ (reference at $\lambda=620\text{ nm}$). The detection range of the assay was 0.05–10 ng/ml, the intra/inter-assay variability $<10/16\%$, respectively.

Lung function

Spirometry (forced expiratory volume in 1 s; FEV_1) was measured by means of electronic spirometer (PDD-301/s, Piston, Budapest, Hungary) according to the American Thoracic Society guidelines. Three technically acceptable

maneuvers were performed, and the highest of them was evaluated. Asthma control was assessed by using the asthma control test (ACT) suggested in the GINA guidelines (www.ginasthma.com). Results are given in the mean $\pm$ SD of the total asthma control test score. Arterial partial oxygen pressure (pO_2) was also performed in asthmatic patients.

Statistical analysis

The normality of continuous variables was assessed using the Shapiro–Wilk's W test. As the continuous variables were not normally distributed, nonparametric statistical methods (Mann–Whitney U test, Spearman rank order correlation) were used. The Fisher exact test was applied to compare categorical variables between groups. The diagnostic accuracy of serum Hsp70 measurements was evaluated using the receiver operating characteristic (ROC) curve analysis. Multivariate logistic regression was carried out with adjustment for maternal age and gestational age at blood withdrawal.

Statistical analyses were performed applying the following software: STATISTICA (version 8.0; StatSoft, Inc., Tulsa, OK, USA), Statistical Package for the Social Sciences (version 15.0 for Windows; SPSS, Inc., Chicago, IL, USA), and MedCalc for Windows (version 10.0.1.0; MedCalc Software, Mariakerke, Belgium). For all statistical analyses, $p<0.05$ was considered statistically significant.

If not otherwise specified, data are reported as median (interquartile range) for continuous variables and as number (percent) for categorical variables.

Results

Patient characteristics and respiratory function parameters

Clinical characteristics of the study participants are shown in Table 1. There were no statistically significant differences between the two study groups in terms of maternal age and gestational age at blood withdrawal and delivery.

Table 1 Clinical characteristics and serum Hsp70 levels of healthy pregnant women and pregnant asthmatics

Variable	Controls ($n=40$)	Asthmatics ($n=40$)	Statistical significance (p value)
Age (years)	30 (28–32)	30 (29–33)	NS
Gestational age at blood draw (weeks)	23.5 (12.5–31)	24 (12–32.5)	NS
Gestational age at delivery (weeks)	39 (39–40)	38 (37–40)	NS
Fetal birth weight (grams)	3,550 (3,450–3,775)	3,230 (2,690–3,550)	<0.05
Fetal growth restriction	0 (0%)	2 (5.0%)	NS
Serum Hsp70 level (ng/ml)	0.21 (0–0.27)	0.44 (0.36–0.53)	<0.001

Data are presented as median (25–75 percentile) for continuous variables and as number (percent) for categorical variables

NS not significant; Hsp heat shock protein

The median fetal birth weight was significantly lower in pregnant asthmatics than in healthy pregnant women (3,230 versus 3,550 g, $p < 0.05$). However, no significant difference was found in the percentage of fetal growth restriction between the two groups. Respiratory function parameters of pregnant women with bronchial asthma are shown in Table 2. In asthmatic pregnant patients, the median of FEV₁ (% of predicted value) was 89.5%, and the median of PEF (% of predicted) was 74%. Median arterial blood partial oxygen tension was 92.2 mmHg, which is somewhat lower than the normal expected range in healthy pregnancy (De Swiet 1995). The level of asthma control in asthmatic pregnant women was satisfactory; the mean ACT total score was 20.66 ± 2.24 points.

Serum Hsp70 levels

As presented in Table 1, serum Hsp70 levels were significantly higher in pregnant asthmatics than in healthy pregnant women. Using the ROC curve analysis, we determined a cutoff value of Hsp70 concentration (0.30 ng/ml), which can discriminate pregnant asthmatics from healthy pregnant women with 90.0% sensitivity and 85.0% specificity. High Hsp70 level (>0.30 ng/ml) was significantly associated with bronchial asthma in pregnancy (odds ratio, OR=51.0, 95% confidence interval, CI=13.2–196.6; $p < 0.001$), even after adjustment for maternal age and gestational age at blood draw in multiple logistic regression analysis (adjusted OR with 95% CI=93.9 (15.4–572.5); $p < 0.001$).

Investigating the relationship of patient characteristics to serum Hsp70 levels, we observed a statistically significant negative correlation between maternal age and serum Hsp70 concentrations (Spearman $R = -0.48$, $p = 0.0018$) and a significant positive correlation between gestational

Fig. 1 Relationship between ACT total scores and Hsp70 levels in asthmatic pregnant women ($r^2 = 0.9669$)

age and serum Hsp70 levels (Spearman $R = 0.83$, $p < 0.001$) in the group of healthy pregnant women. Moreover, ACT total scores showed a trend towards an increase in Hsp70 levels with the loss of asthma control (Fig. 1).

Discussion

In the present study, we found increased circulating Hsp70 levels in pregnant asthmatics as compared to maternal and gestational age-matched healthy pregnant women. In addition, a trend towards the loss of asthma control with increasing Hsp70 levels can be supposed (according to the Hsp70 and ACT data). In healthy pregnant women, serum Hsp70 levels showed a positive correlation with gestational age and an inverse correlation with maternal age. The latter two observations are in agreement with our previous findings and have already been discussed in detail (Molvarec et al. 2007a).

Yang and coworkers reported a significant increase in the rate of detected antibodies against Hsp70 in patients with asthma. In their study, the presence of autoantibodies against Hsp70 was associated with the severity of asthma. The presence of anti-Hsp70 was also correlated with higher levels of total IgE and IL-4 in asthmatic patients (Yang et al. 2005). Elevated serum levels of Hsp70 have recently been demonstrated in patients with chronic obstructive pulmonary disease (Hacker et al. 2009). However, according to our knowledge, no studies have been performed yet to evaluate the circulating Hsp70 levels in asthmatic pregnancy.

Hsp70 has been suggested to play a role in asthma and lung injury (Bonay et al. 1994; Wong and Wispe 1997; Bertorelli et al. 1998). Airway cells (epithelial cells and alveolar macrophages) as well as peripheral blood mononuclear cells showed increased expression of Hsp70 in

Table 2 Respiratory function and oxygen saturation of pregnant women with bronchial asthma

Variable	Median (25–75 percentile)
FVC (l)	3.61 (3.19–3.94)
FVC (%)	98.0 (90.0–106.0)
FEV ₁ (l)	2.83 (2.64–3.10)
FEV ₁ (%)	89.5 (84.0–99.0)
PEF (l)	5.32 (4.79–5.99)
PEF (%)	74.0 (68.0–85.0)
Raw	0.27 (0.21–0.32)
Raw (%)	124.0 (95.0–140.0)
pO ₂	92.2 (86.0–96.9)
pCO ₂	27.3 (25.6–28.2)
Saturation (%)	97.8 (97.3–98.3)

Results are given as median (25–75th percentile) for all variables

asthma (Vignola et al. 1995; Tong and Luo 2000). Since Hsp70 is recognized to have a role in chaperoning antigenic peptides and in facilitating class II peptide assembly, Hsp70 overexpression in professional and nonprofessional antigen presenting cells (APCs) implies a potential role for this protein in antigen processing and/or presentation resulting in an increased activity of APCs, which is essential for the initiation and modulation of the asthmatic immune response in chronic asthma (Bertorelli et al. 1998). Furthermore, Hsp70, in T_H2 environment, can upregulate CD23 expression of THP-1 cells and alveolar macrophages and, thus, might play an important role in maintaining the chronic bronchial inflammation in asthma (Harkins et al. 2003). However, the source of circulating Hsps in healthy individuals, as well as in patients with pathological conditions, has not been completely determined yet. It is also not known whether circulating Hsp70 concentration reflects intracellular expression. Furthermore, not only can Hsps be released from viable cells exposed to stressful stimuli by active mechanisms but might also be discharged from necrotic cells in a passive manner.

Asthmatic women are at an increased risk for several complications during pregnancy. Schatz and coworkers followed 366 pregnancies of asthmatic women, and in 35% of them, asthma worsened during pregnancy, but in most of the patients, symptoms reverted within 3 months after delivery (Schatz et al. 1988). The epidemiological observations indicate that pregnancy may exacerbate asthma. A pregnant woman with asthma is at risk for experiencing preeclampsia, gestational diabetes, placenta praevia, chorioamnionitis, cesarean delivery, and increased maternal hospital length of stay. Adverse perinatal outcomes associated with asthma include preterm delivery, low birth weight, intrauterine growth restriction, perinatal death, and increased infant hospital length of stay (Demissie et al. 1998; Kallen et al. 2000; Liu et al. 2001).

In our previously published study, reduced maternal peak expiratory flow and fetal birth weight were associated with increased maternal circulating INF- γ and IL-4 positive T cell counts in pregnant asthmatics (Tamasi et al. 2005). According to these data, a connection between asthmatic inflammation and reduced fetal birth weight can be suspected in asthmatic pregnancies.

Several studies have associated elevated Hsp70 levels with higher risk for complications during pregnancy. Among patients who were at risk for preterm delivery, the mean serum concentration of Hsp70 was higher in patients who delivered preterm than in those who delivered at term (Fukushima et al. 2005). High serum concentrations of Hsp70 were also reported in pregnant patients with preeclampsia (Jirecek et al. 2002; Fukushima et al. 2005; Molvarec et al. 2006). Increased serum Hsp70 levels have recently been found to be associated with systemic

inflammation, oxidative stress, and hepatocellular injury in preeclampsia (Molvarec et al. 2009).

These data suggest that circulating Hsp70 might play a connecting role in the pathomechanism of asthmatic inflammation and obstetrical/perinatal complications in asthmatic pregnant patients. The fetus is a semi-allograft, since its antigen structure is partly of paternal origin. Several mechanisms have been implicated in the maintenance of immunological tolerance to the embryo/fetus in pregnancy. Pregnancy is characterized by a shift towards a T helper type 2 (T_H2) immune response, which maintains pregnancy (Wegmann et al. 1993). Abnormal extracellular Hsp70 levels during pregnancy associated with asthma could initiate proinflammatory immune responses (e.g., proinflammatory cytokine (tumor necrosis factor- α (TNF- α), interleukin (IL)-1 β and IL-6) and chemokine production, conversion of dendritic cells from tolerogenic to immunogenic and stimulation of the cytolytic activity of NK cells and γ/δ T cells), which might lead to pregnancy complications (Pockley 2003; Asea et al. 2000; Asea 2005).

In summary, according to our results, higher circulating Hsp70 levels can be detected in pregnant asthmatic women than in healthy pregnant women. Abnormal, asthma-dependent Hsp70 levels may play a role in the altered immunological tolerance in pregnancies of asthmatics, which might result in obstetrical/perinatal complications. Nevertheless, further studies are needed to determine the role of circulating Hsp70 in the pathogenesis of maternal and perinatal complications of asthma in pregnancy.

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