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ORIGINAL PAPER

Placental Alpha Hemoglobin Stabilizing Protein (AHSP) and recurrent miscarriage

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Abstract AHSP inhibits cellular production of the reactive oxygen species. Reduced AHSP indicates reduced protection against oxidative stressors. Our objective was to investigate AHSP levels in recurrent miscarriage (RM). Trophoblast was collected from women of 10 weeks gestation: voluntary abortion controls (VA, $n=10$); spontaneous first miscarriage with subsequent normal pregnancy (SMSN, $n=15$) or with subsequent miscarriage (SMSM, $n=5$); RM previously investigated (RMPS, $n=5$) or not previously investigated (RM, $n=5$). AHSP mRNA and protein were determined using real-time quantitative polymerase chain reaction (PCR) and Western blot, respectively. One-way ANOVA was performed to assess statistical significance ($p<0.05$). ahsp mRNA levels were maximally reduced in RM and RMPS ($8.0\times10^{-6}\pm1.3$ and $8.1\times10^{-6}\pm0.7$, respectively) compared with SMSN and VA ($16.1\times10^{-6}\pm2.3$ and $26.1\times10^{-6}\pm2.7$, respectively). SMSM showed levels significantly reduced as well ($9.0\times10^{-6}\pm2.3$). In RM, a reduced defense from oxidative stressors is evident at first miscarriage, identifying women at high risk for subsequent eventful pregnancy. Reduced AHSP may identify women at risk of experiencing further miscarriages.

Keywords AHSP · Recurrent miscarriage · Trophoblastic tissue

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Introduction

Alpha hemoglobin stabilizing protein (AHSP) is a protein of recent discovery whose particular function is to stabilize temporarily other molecules in the cell's environment.

Miele et al. from the Roslin Institute of Midlothian in Scotland were the first to study this factor in Transmissible Spongiform Encephalopathies (TSE), demonstrating a close correlation between these conditions and AHSP expression levels. These studies have opened the way to the use of this molecular chaperone as a peripheral marker of TSE (Miele et al. 2001).

The main function of AHSP is in the red blood cells where it interacts with α globin and maintains free α globin in solution during the biosynthesis of the hemoglobin tetramer (Feng et al. 2004; Gell et al. 2002). Free α globin is highly toxic for the red blood cells because of its unstable monomeric structure. Its deleterious action creates reactive oxygen species (ROS) with subsequent oxidative damage and the formation of intracellular precipitates (Zhou et al. 2006; Halliwell and Gutteridge 1999).

AHSP closely resembles heat shock proteins (hsp) in their structure and function: these molecules are considered intracellular cyto-protective proteins (Dos Santos and Costa 2005).

Recurrent miscarriage (RM) is the main expression of a precocious lethal imbalance in the embryonic environment. Causes of recurrent miscarriage include genetic anomalies, pathological placental conditions, maternal thrombophylia, infections, among many other factors, and low socio-economic status is also associated. However, several studies

have shown that most RM are unexplained and are often related to unexplained intrauterine hypoxia (Hahn et al 2006). These studies stress that a high number of RM occur as a result of any imbalance in the early stages of embryonic development. In fact, this period requires a perfect compromise between the pro-oxidant and anti-oxidant forces which make up the environment for healthy fetal growth.

Any disruption in early gestation can cause conditions in which progressive early damage is reflected in the severity of the clinical outcome; that is, spontaneous first miscarriage with subsequent normal pregnancy (SMSN), spontaneous first miscarriage with subsequent miscarriage (SMSM), recurrent miscarriage not previously investigated (RM), and recurrent miscarriage previously studied (RMPS). Initial damage during gestation, can lead to several pathological obstetric outcomes such as intrauterine fetal death (IUFD), HELLP syndrome, intrauterine fetal restriction (IUGR), and pre-eclampsia.

The aim of this study was to investigate the expression levels of the ahsp gene in specimens of trophoblast from voluntary abortion (VA) and from patients who had experienced the kinds of miscarriage mentioned above. We looked for a possible correlation between the severity of the pathology and the expression levels of the ahsp gene both as an RNA messenger and as a protein.

We studied the "behavior" of AHSP during the particular gestational period known as the "hypoxic window" in comparison with other antioxidant molecules that are known to temporarily decrease at this stage.

Materials and methods

Tissue collection and population

Forty specimens of trophoblast were collected after uterine evacuation from women at 10 weeks gestation. Five were having recurrent miscarriage. Twenty women were experiencing a first spontaneous miscarriage. Those were followed up to their next pregnancy. Five of them experienced a repeated miscarriage. Ten women who had decided for voluntary abortion served as controls. At the end of the collection we had four groups: VA (voluntary abortion controls; n=10); SMSN (spontaneous first miscarriage with subsequent normal pregnancy; n=15); SMSM (spontaneous first miscarriage subsequent miscarriage; n=5); RM (recurrent miscarriage not previously investigated n=5); RMPS (recurrent miscarriage previously studied n=5).

All specimens were snap-frozen in liquid nitrogen and stored at −80°C until use. Assessment of purity was performed by immunocytochemistry using antibodies directed against cytokeratin isoform 7, according to Blaschitz et al (2000).

The study was approved by the Institutional Review Board and informed consent was obtained from all women.

Women who underwent voluntary abortion (VA) were not affected by any obstetric pathologies. VA was performed between the eighth and the twelfth week of pregnancy.

RNA extraction

A piece of the frozen tissue (20–40 mg) was homogenized in lysis buffer, and the total RNA was extracted with an RNA isolation kit (Promega, Madison, WI). RNA samples were tested by ultraviolet absorption at 260 nm in order to determine RNA concentration. The quality and concentration of the RNA samples were further confirmed by electrophoresis on denaturated 1% agarose gels. Two micrograms of RNA were reverse transcribed in a total volume of 25 μl for 60 min at 37°C with M-MLV Reverse Transcriptase (Promega, Madison, WI) using random nonamers in order to obtain complementary DNA (cDNA).

Real-Time quantitative PCR

cDNA was used for real-time quantitative PCR. To avoid false-positive results attributable to the amplification of contaminating genomic DNA in the cDNA preparation, the primers were selected to flank an intron and PCR efficiencies were tested and found to be close to 1. The following primers were used: 5′-CCAACCGCGAGAAGATGAC-3′ (forward), 5′-GAGGCGTACAGGGATAGCACA-3′ (reverse) for β-actin, and 5′-TGTCACCTGCTGCCTGTAAT-3′ (forward) and 5′-AAGGAGTTCAGCGTTCTGCT-3′ (reverse) for AHSP.

The genes were run in duplicate using SYBR Green chemistry. All samples were tested in triplicate using β-actin as the reference gene for data normalization to correct for variations in RNA quality and quantity. A serial dilution of the standard plasmid (harboring ahsp and β-actin cDNAs, respectively) was included in each run to obtain an estimation of absolute gene expression levels (under the assumption that reverse transcription efficiency is 100%). Direct detection of PCR products was monitored by measuring the fluorescence produced by SYBR Green I dye binding to double-stranded DNA after every cycle. The copy number of ahsp mRNA was then determined in each sample and divided by the amounts of β-actin mRNA to give copy number per unit β-actin mRNA.

Western blot

To confirm the results we made a Western blot. Tissue extracts were prepared with lysis buffer (PBS, containing 1% Nonidet P40, 0.1% SDS, 1 mM phenylmethylsulfonyl-

fluoride, and 2 μg/ml aprotinin). Samples containing 10-μg proteins were subjected to 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. As a positive control, cell extracts from *E. coli* BL21 expressing recombinant AHSP were also loaded in all Western blots. After regular blocking and washing, the membranes were incubated with rabbit polyclonal antibody (1:3000 dilution) against human AHSP (Dr. Mitchell Weiss, Children's Hospital of Philadelphia, Philadelphia, PA) for 1 h followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG (Pierce, Rockford, IL; 1:2000 dilution) for 1 h. AHSP protein was visualized using an enhanced chemiluminescent substrate (SuperSignal West Femto Maximum Sensitivity Substrate, Pierce). The intensities of AHSP bands were evaluated from pictures obtained using Quantity One 1-D Analysis Software.

Statistical analysis

All values were expressed as mean±Standard Deviation of the Mean (SD). All statistical analyses were performed by using the GraphPad Prism Software. One-way ANOVA was performed to assess statistical significance. Differences were considered significant at $p<0.05$.

Results

Real-time PCR assay for ahsp gene expression

Trophoblastic ahsp mRNA levels were maximally reduced in RM and RMPS ($8.0\times10^{-6}\pm1.3$ and $8.1\times10^{-6}\pm0.7$, respectively) when compared to first miscarriage and controls ($16.1\times10^{-6}\pm2.3$ and $26.1\times10^{-6}\pm2.7$, respectively). Women experiencing first spontaneous miscarriage followed by subsequent repeated miscarriage showed levels significantly reduced, as well ($9.0\times10^{-6}\pm2.3$) (Fig. 1). All differences were highly significant ($p<0.001$).

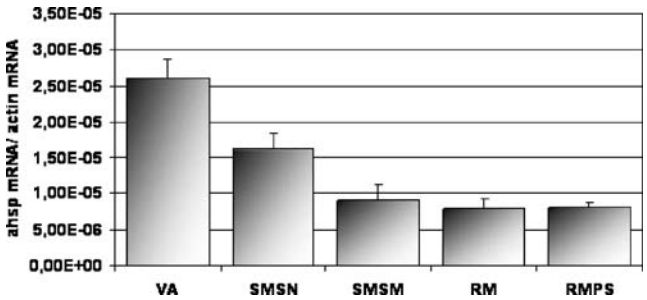

Fig. 1 Real-time quantitative PCR. ahsp mRNA levels in placenta of patients with normal or pathological pregnancies. Results are expressed as mean±SD

Western blot assay for AHSP protein

A representative Western blot is shown in Fig. 2. As reported above, the intensities of AHSP bands were evaluated from pictures obtained using Quantity One 1-D Analysis Software and expressed as arbitrary units. We found a significant decrease of AHSP protein in the RM group compared to the voluntary abortion group. Expression levels of AHSP protein are lower in all other groups compared to voluntary abortion group (Fig. 3).

Discussion

There is evidence that oxidative stress or an alteration in the correct oxidant and antioxidant balance in the uteroplacental tissues plays an essential role in causing changes in normal embryonic development (Jauniaux et al. 2006).

In the past, it was thought that the main function of the placenta was to provide the greatest quantity of oxygen and the widest exchange surface for the fetus (Jauniaux et al. 2003a). Further studies have shown, however, that during the first trimester of pregnancy the placenta limits the contribution of oxygen during the stages of organ genesis. In fact, the first phases of development take place at a low concentration of oxygen (Jauniaux et al. 2001, 2003a). This is a critical finding.

The fetal-placental exchanges are in a perfect balance in order to reduce the formation of free ROS at minimum levels. Embryonic cells are very susceptible to oxidative stress because of their active duplication and concomitant DNA exposure (Jauniaux et al. 2003b) to damaging agents. The placental syncitiotrophoblast is particularly vulnerable, both because it is exposed to high concentrations of maternal oxygen and because it contains surprisingly low concentrations of the main antioxidant molecules, particularly during the first stages of pregnancy (Watson et al. 1998).

Initially, placentation is not hemochorial: the extravillous trophoblast grows like a “shell” at the decidual level. The cells of this temporary structure of early pregnancy anchor the placenta to the maternal tissue but, at the same time, they create plugs in the uteral-placental arteries (Hustin and Schaaps 1987). At the end of the first trimester, these trophoblastic plugs gradually dissolve themselves allowing maternal blood to flow continually in a greater quantity in the intervillous environment (Jauniaux et al. 2003a).

As the pregnancy continues, decidual pressure of oxygen progressively increases reflecting a greater maternal blood flow in the uterine circulation.

Data in vivo show that partial pressure values of placental oxygen from the eighth to the twelfth gestational week are two or three times lower compared to partial pressure values after the twelfth gestational week (Jauniaux et al. 2000, 2001).

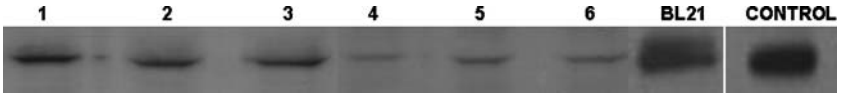

Fig. 2 Representative Western blot for AHSP. *1*, *2*, *3* voluntary abortion; *4*, *5*, *6* recurrent miscarriage not previously investigated. Protein lysate from trophoblast are shown on the first six lanes. *BL21 lane* shows the protein lysate from BL21 cells after induction of AHSP expression (positive control). The *control lane* shows the protein lysate which was present in all Western blots and used for normalization of expression

The gradual rise of oxygen pressure is followed by a simultaneous increase of the main antioxidant factors (both as RNA messenger and protein) in the villous tissue.

Taking these factors into consideration, we evaluated the expression of AHSP (both as transcript of the gene and protein) in trophoblasts of the classes of miscarriage described above, and compared its level to that detected in the voluntary abortion (control) group.

The aim was to explore a possible correlation between AHSP levels and the gravity of the obstetric pathology and to verify the existence of a possible fluctuation of AHSP expression levels during the first period of the embryonic development. In fact, we studied the behavior of this chaperone during the physiologic hypoxic window. This temporary hypoxia protects the developing embryo by limiting the ROS formation owing to the lower availability of free oxygen.

Furthermore, this hypoxic window selects the more vital embryonic cells which can survive in this particular condition.

Our results showed that all patients had mRNA levels significantly lower than that detected in the respective control group (VA). Particularly, there was a trend toward decreasing values of ahsp mRNA in, progressively, VA, SMSN, SMSM, and, eventually, in RM and RMPS. These data underline that there is a wide range of situations in which the status of the initial reduced defense from oxidative cell stressors leads to a specific clinical outcome.

These data support the results previously obtained studying AHSP expression in several obstetric pathologies. The levels of the primary transcript in pregnancies at term are higher than that of pregnancies complicated by HELLP syndrome, IUGR, and IUFD (Emanuelli et al. 2008).

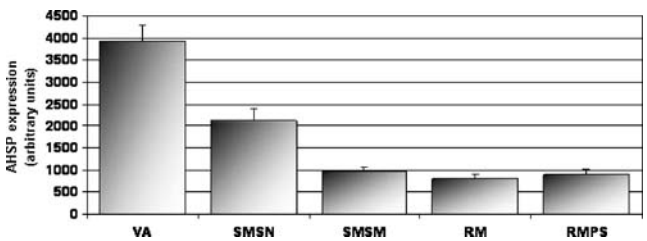

Fig. 3 Western blot data analysis. Expression of AHSP protein in trophoblasts of patients with voluntary or spontaneous abortions. Results are expressed as mean±SD

In trophoblasts from voluntary abortion, the expression of mRNA of the ahsp gene is much lower than in pregnancies at term. This fact could be due to the hypoxic window (eighth to twelfth gestational week) during which, voluntary abortions are performed. During the hypoxic window, there is a lower need for of antioxidant molecules; this explains the low levels of ahsp mRNA.

Western blot data agree with real-time PCR results: AHSP protein expression reflects the decreasing detection of mRNA in, progressively, VA, SMSN, SMSM, and, at the end, RM and RMPS. These results emphasize that the lower the level of AHSP expressed, the worse the outcome is. Our results can not indicate at this stage, however, exact values that are predictive of recurrent miscarriage, although all recurrent miscarriages showed values below 10.0×10^{-6}.

AHSP expression in voluntary abortion is lower than that of full-term pregnancy and the observed AHSP decrease seems to be in agreement with the behavior shown by other antioxidant molecules during the physiological hypoxic window.

Our results stress that decreased levels of AHSP may indicate a failure of the cell to withstand oxidative stress.

AHSP is a molecular chaperone and, due to this function, it can be considered a marker of cell vitality. Its production is an index of the intrinsic capacity of the cell to defend itself; therefore, AHSP can be considered a marker of the cell survival potential against an oxidative injury. The cell can produce AHSP in a variable quantity and this can "decide" its survival. This fact explains that a high production of AHSP can indirectly express cell vitality as a reaction to an increase of free radicals of oxygen.

In the above classes of miscarriage, the levels of AHSP that the cell produces are lower than the hypothetical threshold needed for survival. Death is the natural consequence. This aspect shows that a variable production of AHSP correlates with a corresponding variable evolution of pregnancy, which can result in early failure, miscarriage, or in the obstetric pathologies recently studied such as IUFD, HELLP syndrome, and the IUGR. The status of reduced defense from oxidative cell stressors may be evidenced already at a first miscarriage, thus, identifying women who are at high risk for subsequent eventful pregnancy.

The future challenge is to find a correlation between AHSP expression levels and fetal health to perform early therapeutic procedures. This could be made easier by the

availability of new and routine analytical methodologies which will allow a non-invasive investigation of this protein at different sites.

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