S100B protein expression in the amnion and amniotic fluid in pregnancies complicated by pre-eclampsia


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Our aim was to investigate the expression of S100B protein in the amnion and to assess the amniotic fluid concentration in pregnancies complicated by pre-eclampsia. Samples were obtained from women who developed pre-eclampsia (n = 7), pre-eclampsia with intrauterine growth retardation (IUGR) (n = 4), normotensive IUGR (n = 7) and gestational hypertension (n = 4) during pregnancy and healthy controls who delivered at term (n = 35). To determine the difference in the expression of S100B in the amnion, we performed immunohistochemistry, western blot analysis and RT–PCR. Using enzyme-linked immunosorbent assay (ELISA), we assessed the S100B concentration in amniotic fluid. The S100B mRNA expression in the amnion of pre-eclamptic patients and patients with pre-eclampsia with IUGR was significantly higher than that in the control. The amniotic fluid S100B protein concentration of the pre-eclampsia and normotensive IUGR cases was significantly higher than that of the control. This study shows that amnion could be a source responsible for the increased concentration of S100B in amniotic fluid. In pre-eclampsia, reactive oxygen species (ROS) are generated by oxidative stress. Some pathological conditions that develop during pregnancy and are related to hypoxic stress can affect the elevation of S100B concentration in the amnion.

Key words: amnion/amniotic fluid/IUGR/pre-eclampsia/S100B

Introduction
Pre-eclampsia is a pregnancy-specific syndrome that is diagnosed by the associated increase of blood pressure and proteinuria and affects 6–8% of all pregnancies. Pre-eclampsia is an idiopathic multisystem disorder specific to pregnancy and puerperium. It is associated with substantial risks. For fetuses, these include intrauterine growth restriction, death and prematurity with attendant complications, whereas the mother is at risk of seizures, renal failure, pulmonary oedema, stroke and even death. Delivery is the only effective treatment for pre-eclampsia. Endothelial cell dysfunction has been identified as the final common pathway in the pathogenesis of pre-eclampsia. However, little information about the association between neuroprotective factors and pre-eclampsia is available.

The S100 proteins are a family of low-molecular-weight (10–14 kDa) calcium-binding proteins consisting of two different subunits (α and β), which are present extracellularly, intracellularly and in the cytosol (Moore, 1988). They are multifunctional and trigger or activate several processes along the calcium signal transduction pathway via binding to a target protein. These target proteins include cytoskeleton-associated proteins with roles in signal transduction, cell motility and cytokinesis, cellular differentiation and cell cycle progression and inflammation. The S100B gene is located at 21q22.2–q22.3 (Allore et al., 1988; Morii et al., 1991). This protein may function in neurite extension (Winningham-Major et al., 1989), the regulation of energy metabolism and contraction (Zimmer et al., 1995), astrocytosis (Aberg and Kozlova, 2000), the stimulation of IL-6 secretion by neurons (Li et al., 2000) and the stimulation of nitric oxide (NO) secretion by astrocytes (Hu et al., 1996) and microglia (Petrova et al., 2000). S100B has been implicated in the modulation of learning and memory (Gromov et al., 1992) and in developmental plasticity, lesion-induced reactive synaptogenesis (McAdory et al., 1998). The S100B concentration in the blood of term neonates is higher than that in older individuals (Portela et al., 2002). Blood S100B concentrations decrease in the first two decades of life and remain relatively constant during adulthood (Wiesmann et al., 1998; Portela et al., 2002). It was reported that S100B concentrations are elevated in cerebrospinal fluid and/or serum in some pathological conditions and diseases, including cerebral palsy or delayed development in children (Park et al., 2004), Down’s syndrome (Allore et al., 1988) and Alzheimer’s disease (Pezzold et al., 2003). There have been several studies investigating the correlation between the S100B concentrations in the amniotic fluid, cord blood and maternal serum and some pathological conditions that develop during pregnancy and/or related to fetuses: intrauterine fetal death (Florio et al., 2004), pre-term delivery (Gazzolo et al., 2000), pregnancy-related hypertensive disorders (Schmidt et al., 2004), anencephaly (Sindic et al., 1984), open spina bifida (Anneren et al., 1988) and trisomy 21 fetuses (Gazzolo et al., 2003a). The aim of this study was to assess the association between S100B protein in the amnion and amniotic fluid and pre-eclampsia.

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E. Tskitishvili et al.

Materials and methods

Patients

For our study, we selected 57 patients with singleton pregnancies and without
outstanding hypertension, renal disease, diabetes mellitus, major fetal anomalies
or intrauterine fetal death. All the women were hospitalized in the Osaka
University Hospital for delivery between October 2003 and October 2005. Of
57 patients, 7 women had pregnancies complicated by pre-eclampsia, 4
patients had pre-eclampsia with intrauterine growth retardation (IUGR), 7
women had normotensive IUGR, 4 patients developed gestational hypertension
during pregnancy and 35 healthy women delivered at term. Pre-eclampsia
was defined by hypertension with systolic blood pressure ≥140 mmHg and/or
diastolic blood pressure ≥90 mmHg in association with proteinuria [24 h uri-
inary protein exceeding 300 mg per 24 h or persistent 30 mg/dl (1+ dipstick)
in random urine samples] with or without oedema. IUGR was defined by the
metrical type of IUGR was determined after delivery. The diagnosis of
IUGR, intrauterine growth retardation; NA, not available.

nullotensive IUGR (n = 4), normotensive IUGR (n = 7) and gestational hypertension (n = 4)
during pregnancy and 35 healthy controls who delivered. Twenty-four sam-
ple sets of amniotic membrane were obtained from women with pre-eclampsia
(n = 3), pre-eclampsia complicated by IUGR (n = 3), normotensive IUGR
(n = 3) and gestational hypertension (n = 3) and the controls (n = 12). For
immunohistochemistry, a portion of amnion was fixed in 10% formaldehyde
solution before the measurement of the S100B concentration. Two examiners
counted eight randomly selected fields from each section. The number of cells positively stained for S100B was quantified as the percentage of the sum of unstained and stained cells.

Western blot analysis of the amniotic membranes

The amniotic tissue pieces (each ~100 mg in weight, frozen at ~80°C) were
homogenized with 0.5 ml of lysis buffer consisting of distilled water, 0.5 mol/l
Tris–HCl (pH 6.8), 10% sodium dodecyl sulphate (SDS), glycerol, 6% α-
mercaptoethanol and 1% bromphenol blue. Homogenates were centri-
gufugated at 4°C for 20 min at 12000 g. An Immunomini NJ-2300 apparatus was
used to determine the protein concentration of the lysates. Fifty micrograms
of amniotic protein was subjected to electrophoresis on 15% SDS–polyacryl-
ide gels using a Bio-Rad Power-Pac 200 apparatus and transferred to a nitrocellu-
lose membrane for 1 h. The membrane was incubated with 5% dried milk powder (Amersham Biosciences, Arlington, IL, USA) followed by incubation with primary antibody polyclonal rabbit anti-S100 (DakoCytomation, Glostrup, Denmark) at a concentration of 9 µg/ml. After overnight incubation of the membrane at 4°C, we used the secondary antibody anti-
rabbit Ig, horse-radish peroxidase–linked whole antibody (from donkey) (Amersham Biosciences) at a concentration of 0.2 µg/ml. S100B immunoreac-
tivity was visualized with the enhanced chemiluminescence (ECL) western blott ing detection kit (Amersham Biosciences).

Table 1. Baseline, clinical characteristics and pregnancy outcome of study groups

<table>
<thead>
<tr>
<th>Variable</th>
<th>Pre-eclampsia group (n = 7)</th>
<th>Pre-eclampsia and IUGR group (n = 4)</th>
<th>Normotensive IUGR group (n = 7)</th>
<th>Gestational hypertension group (n = 4)</th>
<th>Control group (n = 35)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years, mean ± SD)</td>
<td>31.6 ± 2.8</td>
<td>30.25 ± 3.9</td>
<td>31.1 ± 3.4</td>
<td>36.5 ± 3.0</td>
<td>30.4 ± 4.8</td>
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<tr>
<td>Parity</td>
<td></td>
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<tr>
<td>Nullipara (n, %)</td>
<td>6 (85.7)</td>
<td>3 (75)</td>
<td>3 (42.9)</td>
<td>3 (75)</td>
<td>10 (28.6)</td>
</tr>
<tr>
<td>Oedema (n, %)</td>
<td>3 (42.9)</td>
<td>2 (50)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Proteinuria (n, %)</td>
<td>7 (100)</td>
<td>4 (100)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mean blood pressure</td>
<td>109 ± 9.4</td>
<td>115 ± 8.9</td>
<td>92.7 ± 4.5</td>
<td>106.0 ± 14.4</td>
<td>76.4 ± 11.8</td>
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<tr>
<td>Gestational age at delivery (weeks)</td>
<td>36.4 ± 2.8</td>
<td>32.5 ± 4.5</td>
<td>37.1 ± 3.2</td>
<td>37.7 ± 2.1</td>
<td>39.2 ± 1.1</td>
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<tr>
<td>Mode of delivery</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Vaginal birth (n, %)</td>
<td>5 (71.4)</td>
<td>1 (25)</td>
<td>6 (85.7)</td>
<td>3 (75)</td>
<td>30 (85.1)</td>
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<td>Birthweight (g)</td>
<td>2590.6 ± 627.5</td>
<td>1204.5 ± 498.1</td>
<td>2084 ± 438.3</td>
<td>2608.5 ± 456.4</td>
<td>3113.0 ± 315.7</td>
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<tr>
<td>Umbilical cord blood</td>
<td></td>
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<tr>
<td>pH</td>
<td>7.25 ± 0.12</td>
<td>7.28 ± 0.03</td>
<td>7.27 ± 0.08</td>
<td>7.29 ± 0.06</td>
<td>7.29 ± 0.05</td>
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<td>PO2 (mmHg)</td>
<td>17.7 ± 5.8</td>
<td>11.37 ± 4.05</td>
<td>15.7 ± 7.5</td>
<td>NA</td>
<td>16.8 ± 6.68</td>
</tr>
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<td>PCO2 (mmHg)</td>
<td>50.22 ± 13.68</td>
<td>52.32 ± 3.4</td>
<td>47.54 ± 12.3</td>
<td>48.6 ± 0.72</td>
<td>47.68 ± 7.4</td>
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<td>Base excess (mmol/l)</td>
<td>−6.35 ± 4.49</td>
<td>−2.87 ± 2.2</td>
<td>−5.77 ± 2.9</td>
<td>−4.17 ± 3.4</td>
<td>−3.62 ± 2.0</td>
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<td>Apgar score</td>
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<td></td>
<td></td>
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<tr>
<td>At 1st min</td>
<td>6.57 ± 2.57</td>
<td>7.25 ± 1.5</td>
<td>7.29 ± 2.36</td>
<td>7.25 ± 2.12</td>
<td>8.12 ± 0.48</td>
</tr>
<tr>
<td>At 5th min</td>
<td>7.57 ± 2.94</td>
<td>8.5 ± 1.0</td>
<td>8.14 ± 1.86</td>
<td>9.25 ± 0.5</td>
<td>8.97 ± 0.52</td>
</tr>
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</table>

IUGR, intrauterine growth retardation; NA, not available.

Values are given as mean ± SD. Mean arterial pressure was calculated as [systolic pressure + (2 × diastolic pressure)]/3.
used oligo (dT) 12-18, 10 mM dNTPs, 5× first-strand buffer, 0.1 M dithiothreitol, RNase inhibitor (Toyobo, Osaka, Japan) and Superscript II RT (Invitrogen, Japan). We performed PCR amplification using the RT mixture after the incubation as described earlier (20 μl), with sequence-specific primers for S100B 5′-CATTTCCTAGGAAATC-3′ (sense) and 5′-ATGTCCAAAGACTCGTG-3′ (antisense) (Applied Biosystems, Japan) or G3PDH 5′-ACCACAGTCCATGCCATAAC-3′/5′-TCCACCACCTGTTGCTGTA-3′. PCR was carried out for 35 cycles using a thermal cycler (Perkin-Elmer/Cetus, Norwalk, CT, USA). Each cycle consisted of denaturation at 94°C (40 s), annealing at 52°C (40 s) and extension at 72°C (40 s). For amplification of G3PDH, 25 cycles of 94°C for 40 s, 52°C for 40 s and 72°C for 40 s were performed. The PCR-amplification product size for S100B was 147 bp. The amplification products (1 μl of cDNA from each sample) were electrophoresed on a 1.7% agarose gel stained with ethidium bromide. Amplified products were visualized by UV illumination. Molecular sizes were estimated using a 100-bp DNA ladder.

Real-time PCR

For real-time PCR, we used 1 μl of cDNA from each sample, Taq Man Gene expression assays (Hs99999905_m1 G3PDH, 250 μl, 20× mix and Hs00389217_m1, 250 μl, 20× mix) (Applied Biosystems) and Taq Man® Universal PCR Master Mix (Roche, Branchburg, NJ, USA). The final volume for the reaction was 20 μl. The absolute-quantification real-time PCR method was performed using computer program ABI PRISM (Applied Biosystems). 5(6) Carboxy Fluorescein Amidite (FAM) was used as detector manager. Each reaction was performed in triplicate, and the results were normalized to the expression of G3PDH in each sample of cDNA but in separate tubes. The thermal profile was as follows: 50°C (2 min), 95°C (10 min), 95°C (15 s) and 60°C (1 h). The comparative Ct method was carried out on the data.

Enzyme-linked immunosorbent assay to determine S100B concentrations

To determine the S100B concentration in amniotic fluid, maternal serum and cord blood serum, we used a commercial enzyme-linked immunosorbent assay (ELISA) kit serum (Sangtec®100ELISA, DiaSorin, USA). All the procedures were performed according to the manufacturer’s protocol. The intra- and inter-assay variabilities were 2.9–9.4 and 3.5–7.2%, respectively.

Statistical analysis

The data were subjected to one-way analysis of variance by using the Statview statistics package (Abacus Concepts, Berkeley, CA, USA). P < 0.05 was considered significant.

Results

To examine the presence of S100B protein in amniotic membranes, we performed immunohistochemical staining of amniotic samples. As shown in Figure 1, the amniotic cells were positively stained. The intensity of immunostaining by anti-S100B protein of the epithelial cells of patients with pre-eclampsia (Figure 1A), pre-eclampsia with IUGR (Figure 1B), normotensive IUGR (Figure 1C), and gestational hypertension (Figure 1G) was stronger than that of the women with gestational hypertension and control women. Scale bar: 100 μm.

Figure 1. Immunohistochemical staining of S100B protein-producing cells in the amnion. The epithelial amniotic cells (e) of the amnion were stained as described in the text. Three amniotic samples from each pathological group were stained immunohistochemically and compared with 12 samples from the control group. (A) Pre-eclampsia case; (B) pre-eclampsia with intrauterine growth retardation (IUGR) case; (C) normotensive IUGR case; (G) gestational hypertension case; and (D–H) control cases. The intensity of immunostaining by anti-S100B protein of the epithelial amniotic cells of patients with pre-eclampsia, pre-eclampsia with IUGR and normotensive IUGR was stronger than that of the women with gestational hypertension and control women. Scale bar: 100 μm.
complicated by IUGR (Figure 1B) and normotensive IUGR (Figure 1C) was stronger than that of the women with gestational hypertension (Figure 1G) and healthy controls (Figure 1D–H). At the same time, there was no significant difference in the intensity of immunostaining between women with gestational hypertension and the healthy controls. Figure 2 shows that the percentage of positively stained amniotic epithelial cells was higher in pre-eclampsia ($P < 0.0001$), pre-eclampsia with IUGR ($P < 0.05$) and normotensive IUGR cases ($p < 0.05$) than that in the healthy controls. Similarly, we found that the percentage of positively stained amniotic epithelial cells was significantly higher in cases of pre-eclampsia ($P < 0.05$) and pre-eclampsia complicated by IUGR ($P < 0.05$) than in the gestational hypertension patients. However, there was no significant difference in the percentage of positive cells between cases of gestational hypertension and the control cases. To compare the expression of S100B protein in the amniotic membranes with or without pre-eclampsia, we performed western blot analysis. Figure 3A demonstrates that S100B expression in the amniotic membranes from women with pre-eclampsia, pre-eclampsia with IUGR and normotensive IUGR was higher than that of the healthy controls. As shown in Figure 3B, the S100B expression in the amnion of pre-eclamptic patients was higher than that of healthy controls. Figure 3C shows densitometric analysis, which revealed that S100B expression in the amniotic membranes of women with pre-eclampsia was significantly higher than that in the control ($P < 0.05$). RT–PCR using specific primers for S100B was performed to detect the expression of S100B transcripts in amniotic membrane. Figure 4A shows the expression of S100B transcripts in the amniotic membranes. As shown in Figure 4B, the G3PDH level was constant in all tracks. To compare the expression levels of the transcripts, we performed the real-time PCR. Figure 5 shows the expression levels of S100B transcripts in pre-eclamptic patients in comparison with the levels in pre-eclampsia complicated by IUGR, normotensive IUGR, gestational hypertension and the control cases. S100B expression was significantly higher in pre-eclampsia ($P < 0.05$) and in pre-eclampsia with IUGR ($P < 0.0001$) samples compared with the control. Moreover, S100B transcript expression levels were significantly higher in pre-eclamptic patients complicated by IUGR than those in normotensive IUGR cases ($P < 0.05$). A significant difference was also detected between pre-eclampsia and gestational hypertension cases ($P < 0.05$) and pre-eclampsia complicated by IUGR and gestational hypertension patients ($P < 0.05$). We did not find any significant difference in the S100B transcript expression between the normotensive IUGR and the control or between gestational hypertension and the control. To compare the concentration of S100B protein in amniotic fluid, maternal serum and umbilical cord serum, we performed ELISA for S100B protein. As shown in Figure 6, the concentration of S100B protein in the amniotic fluid of patients with pre-eclampsia ($0.47 \pm 0.39 \mu g/l$) and normotensive IUGR ($0.60 \pm 0.80 \mu g/l$) was significantly higher than that in healthy controls ($0.18 \pm 0.11 \mu g/l$) ($P < 0.05$). The concentration of S100B in the maternal sera of patients with pre-eclampsia ($0.12 \pm 0.10 \mu g/l$), pre-eclampsia complicated by IUGR ($0.09 \pm 0.04 \mu g/l$), normotensive IUGR ($0.09 \pm 0.04 \mu g/l$) and gestational hypertension ($0.13 \pm 0.16 \mu g/l$) was not significantly different from that of the control ($0.09 \pm 0.08 \mu g/l$). There was no significant difference between the S100B titres of the cord blood in the neonates born from mothers with pre-eclampsia ($0.34 \pm 0.17 \mu g/l$), pre-eclampsia complicated by IUGR ($0.34 \pm 0.15 \mu g/l$), normotensive IUGR ($0.28 \pm 0.21 \mu g/l$), gestational hypertension ($0.25 \pm 0.11 \mu g/l$) and healthy controls ($0.37 \pm 0.33 \mu g/l$). We analysed the relationship between the concentration of S100B in
The relationship between S100B protein in the amnion and pre-eclampsia

Costa et al. demonstrated that pre-eclampsia, pre-eclampsia complicated by IUGR and also normotensive IUGR (but not gestational hypertension) affected the amount of S100B protein in the amniotic membranes using immunohistochemical analysis and western blot analysis. We also demonstrated that the transcripts of S100B protein were present in amniotic membranes of pregnant women in the third trimester. Real-time PCR revealed that the expression of transcripts in the amnion of women with pre-eclampsia and pre-eclampsia complicated by IUGR (but not the normotensive IUGR cases) was significantly higher than that in the gestational hypertension and the controls. S100B expression was significantly higher in pre-eclamptic patients with IUGR compared with normotensive IUGR cases. Interestingly, we did not find any significant difference in S100B expression between gestational hypertension and the controls. Moreover, significantly higher expression of S100B protein transcripts in pre-eclampsia and pre-eclampsia complicated by IUGR cases than that in gestational hypertension was revealed. Taken together, the production of S100B protein in the amnion might be transcriptionally regulated in cases of pre-eclampsia and pre-eclampsia complicated by IUGR. However, further investigations would be necessary to determine the mechanism of S100B production by the amniotic membranes of pregnant women complicated by pre-eclampsia.

S100B was studied in the placenta, umbilical cord tissue, fetal membranes (Marinoni et al., 2002), amniotic fluid (Gazzolo et al., 2001), cord blood (Gazzolo et al., 2000), maternal serum (Schmidt et al., 2003) in uncomplicated and complicated pregnancies. Pathological conditions that develop during pregnancy affect the amnion, leading to the elevation of S100B protein concentration in the amnion and amniotic fluid. Moreover, a controlled period of fetal hypoxiaemia with associated acidemia leads to persistent elevations in plasma S100B concentrations and is correlated with haemodynamic changes, which occur during fetal blood flow redistribution (Giussani et al., 2005). We can speculate that one of the reasons for elevated S100B protein concentrations in our study might be hypoxic stress as well, especially in pre-eclamptic patients complicated by IUGR. In our study, we were not able to match completely the gestational age of the study population and the control cases. At this stage, it is not possible to know how this might limit the interpretation of our results. Further investigations will be necessary to clarify this.

S100B protein has been reported to be associated with the poor outcome of pregnancy. Interestingly, S100B concentrations have been shown to be higher in IUGR fetuses with the redistribution of fetal-placental blood flow, the so-called brain sparing effect and correlated

maternal blood and amniotic fluid and that between the concentration of S100B in cord blood and amniotic fluid. There was no significant relationship between the S100B titres in maternal blood and amniotic fluid nor between the S100B titres in cord blood and amniotic fluid.

Discussion

In this study, we confirmed the presence of S100B protein in amniotic membranes. In previous studies, S100B was demonstrated to be located in the trophoblasts and amnion, and there was no difference in the intensity of positive staining of amniotic cells during pregnancy (Marinoni et al., 2002) and no difference in the localization or intensity of S100B staining in placental tissues or cord between uncomplicated and IUGR pregnancies (Gazzolo et al., 2002). In this study, we
with the degree of fetal haemodynamic impairment, whereas IUGR fetuses without the ‘brain sparing effect’ showed S100B concentrations similar to those of non-IUGR fetuses. Thus, the impairment of fetal growth per se does not affect S100B concentrations in the fetal circulation, as also demonstrated by the lack of correlation between protein levels and birthweight (Gazzolo et al., 2002). Our findings are consistent with previous studies. High amounts of the amniotic fluid S100B protein in mid-gestation have been associated with intrauterine fetal death (Florio et al., 2004). S100B protein in biological fluids increases at an early stage when standard monitoring procedures are still silent, and later on, brain damage develops (Micheiti and Gazzollo, 2002). The sources of S100B protein in amniotic fluid are fetal urine, fetal pleural fluids, amniotic membranes and the mother. The concentration of S100B in the maternal and cord sera of patients from study cases was not significantly different from that in the controls. There was no significant relationship between the S100B titres in the maternal blood and amniotic fluid nor between the S100B titres in the cord blood and amniotic fluid. The amnion of patients with pre-eclampsia and pre-eclampsia complicated by IUGR contained a significantly higher amount of S100B protein compared with the control amnions. Moreover, we showed that the S100B protein concentration in amniotic fluid was significantly higher in pre-eclamptic and normotensive IUGR patients than that in the healthy controls. These results suggested that one of the sources of S100B protein in amniotic fluid was the amniotic membrane, especially in case of pre-eclampsia, where real-time PCR revealed a significant expression of S100B transcripts in pre-eclamptic patients compared with controls but not in normotensive IUGR cases.

Pre-eclampsia has been linked to oxidative stress. In pre-eclampsia, reactive oxygen species (ROS) are generated by hypoxic stress (Banerjee et al., 2006). Such conditions might induce S100B protein elevation in the amnion. The function of increased S100B protein in amniotic fluid is still unknown. S100B protein has various functions, such as neuroprotection, the regulation of energy metabolism and contraction, the stimulation of IL-6 secretion and the stimulation of NO and reactive oxygen species (ROS) are generated by hypoxic stress (Wainwright et al., 2004). Moreover, a small amount of S100B protein might contribute to the protection of the fetus from the condition of insufficient oxygen when the mothers suffer pre-eclampsia. Further investigations would be necessary to determine the function of S100B protein in the amniotic fluid of patients with pre-eclampsia.

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The relationship between S100B protein in the amnion and pre-eclampsia


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