Altered subcellular distribution of cadherin-5 in endothelial cells caused by the serum of pre-eclamptic patients

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The main clinical features of pre-eclampsia are oedema and vascular leakage. Cadherin-5 mediates endothelial cell–cell contact in the vascular endothelium and may regulate permeability as a vascular function. Therefore, we addressed the question of whether pre-eclampsia alters cadherin-5 expression and intracellular distribution. Confluent human umbilical vein endothelial cells (HUVEC) were incubated with 20% serum from patients with pre-eclampsia (n = 18), haemolysis–elevated liver enzymes–low platelet syndrome (HELLP) (n = 12), pregnancy-induced hypertension (PIH) (n = 18) or normal pregnancy (n = 10). After incubation with sera from patients with pre-eclampsia, immunostaining analyses showed cadherin-5 accumulation in vesicular and tubular structures of the Golgi apparatus. Immunoblot analyses of HUVEC after pre-eclampsia serum incubation showed an increase of the stable form of cadherin-5 while degradation products decreased. Degradation of cadherin-5 takes place at the cell membrane, so this decrease may be due to a decrease of cadherin-5 in the cell membrane. The accumulation of cadherin-5 in the vesicular and tubular structures of the Golgi apparatus indicates that targeting of cadherin-5 to the plasma membrane could be disrupted. We suggest that intracellular retention of cadherin-5 caused by serum factors in patients with pre-eclampsia may decrease the number of adhesion complexes in the cell membrane, thereby contributing to endothelial dysfunction.

Key words: adhesion molecules/cadherin-5/endothelial dysfunction/pre-eclampsia/vascular endothelium

Introduction

In addition to hypertension, oedema and vascular leakage are the leading clinical characteristics of pre-eclampsia. Current pathogenetic concepts of pre-eclampsia therefore suggest that generalized dysfunction of the maternal vascular endothelium is a central event in this disease. The generally accepted pathogenetic model of pre-eclampsia holds that placentation is altered in these patients as a result of an unknown mechanism and that, as a consequence, the maternal vasculature is activated. The factors linking the dysregulated placentation and the development of the maternal disease remain to be fully elucidated (Roberts et al., 1989, 1991). Recently, it was postulated that syncytiotrophoblast membrane fragments shed from the placenta may enter the maternal circulation and activate the cellular immune system (Redman et al., 1999). This leads to secondary activation of the endothelium, which contributes to the endothelial dysfunction. In the present study, we did not aim to clarify either the underlying placental mechanisms or the linking serum factors but rather the underlying molecular mechanisms responsible for the obviously altered endothelial function.

The structural and functional integrity of the endothelium is an essential prerequisite for its function as a selectively permeable barrier between the bloodstream and the underlying tissues. Intercellular junctions formed by cell adhesion molecules are the major structural determinants of endothelial permeability (Lampugnani et al., 1993, 1997). The main structural protein of the intercellular junctions between endothelial cells is cadherin-5 (also termed VE-cadherin). Cadherins are transmembrane molecules whose extracellular domains, in the presence of calcium, perform homologous binding, thereby mediating cell–cell contact. Evidence for a link between cadherin-5 function and permeability in vitro has been reported (Lampugnani and Dejana, 1997). An increase in permeability of endothelial cell monolayers has been observed following thrombin incubation, which was correlated with cadherin-5 disappearance from the cell membrane (Lampugnani et al., 1991; Rabiet et al., 1996). In the present study, we addressed the question of whether serum from patients with pre-eclampsia and associated diseases, e.g. pregnancy-induced hypertension (PIH) and haemolysis–elevated liver enzymes–low platelet (HELLP) syndrome influences cadherin-5 expression and distribution.

Materials and methods

Patients

A total of 58 patients were recruited from the Department of Obstetrics and Gynecology of the University Hospital of Ulm, Germany. The study included patients presenting with pre-eclampsia (n = 18), with PIH (n = 18), with HELLP-syndrome (n = 12) and women with normal, uncomplicated pregnancies (n = 10). Pre-eclampsia was
Table I. Clinical and analytical data of patients with normal and pathological pregnancies. Values are expressed as median with the range shown in parentheses.

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>No. of previous pregnancies</th>
<th>Gestational age at sampling (weeks)</th>
<th>Gestational age at delivery (weeks)</th>
<th>Patients with normal pregnancies (n = 10)</th>
<th>Patients with HELLP syndrome (n = 12)</th>
<th>Patients with PIH (n = 18)</th>
<th>Patients with pre-eclampsia (n = 18)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 (22–37)</td>
<td>0 (0–2)</td>
<td>35 (26–39)</td>
<td>40 (36–41)</td>
<td>30 (21–38)</td>
<td>31 (22–42)</td>
<td>31.5 (20–41)</td>
<td>33 (22–41)</td>
</tr>
</tbody>
</table>

HELLP = haemolysis–elevated liver enzymes–low platelet syndrome; PIH = pregnancy-induced hypertension.

diagnosed in formerly normotensive and non-proteinuric patients if the diastolic blood pressure was >90 mmHg on two consecutive occasions at least 4 h apart and if proteinuria >300 mg in a 24 h urine specimen or if urine testing with a reagent test strip returned findings of at least 2+ proteinuria (scale: – to 3+). PIH was diagnosed in pregnant women with elevated arterial blood pressure but in whom proteinuria was either not detected or was less than that established as a criterion of pre-eclampsia. HELLP syndrome was diagnosed in cases with low platelet counts (<100 000/µl), elevated liver enzymes (aspartat aminotransferase >30 IU/l and alanin aminotransferase >30 IU/l), and positive haemolysis markers (lactic dehydrogenase elevation >250 IU/l and declining haptoglobin <0.3 g/l) regardless of pre-existing pre-eclampsia. Normal pregnancy includes patients who remained normotensive and non-proteinuric, delivered at term and displayed normal fetal growth. Women with pre-existing underlying diseases were excluded. All patients were of comparable chronological and gestational age (Table I). Serum was prepared from antecubital vein blood, centrifuged at 1800 g after clotting for at least 2 h at 4°C and stored in aliquots at −80°C. Samples were taken during the first admission to the department.

**Cell culture and treatment**

Confluent human umbilical vein endothelial cells (HUVEC; PromoCell, Heidelberg, Germany) were cultured in 20% fetal calf serum (FCS), 5% CO2, and 100% humidity at 37°C according to the manufacturer’s instructions and used for experiments until passage five. Cells which had grown to confluence in plain cell culture flasks (25 m²; Labor Schubert, Schwandorf, Germany) were washed three times with phosphate-buffered saline (PBS; 137 mmol/l NaCl, 2.7 mmol/l KCl, 1.4 mmol/l KH2PO4, 6.5 mmol/l Na2HPO4, pH 7.2) containing 2 mmol/l CaCl2 (PBS/Ca2+). Cells were grown to confluence on a glass slide with Mowiol. Confocal laser scanning microscopy was performed using a Leica TCS instrument (Leica, Heidelberg, Germany) based on a Leitz DMRBE interfaced with an argon–krypton laser adjusted to 488 and 568 nm. A total of 30–40 optical sections through cells were collected at a thickness of 100 nm using a 63 planapo lens (numerical aperture 1.40), pinhole 30. Sets of optical sections were subjected to the iterative Maximum Likelihood Estimation (MLE) deconvolution analysis (Bitplane software, Zürich, Switzerland) before running the co-localization software (Bitplane, Zürich, Switzerland).

**Protein extraction and immunoblot analysis**

For protein extraction, cell pellets were lysed in Tris/Triton buffer (10 mmol/l Tris–HCl, pH 7.4, 150 mmol/l NaCl, 1% Triton X-100, 2 mmol/l CaCl2, 1 mmol/l phenyl methyl sulphonyl fluoride and 20 IU/ml aprotinin). Soluble supernatant lysate was collected after centrifugation at 25 500 g for 10 min at 4°C. The supernatant was used for immunoblotting. Protein concentrations in the cell extracts were determined by Bradford protein quantification.

For immunoblotting, samples of 20 µg protein per lane were separated on 7.5% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE), and subsequently transferred to a nitrocellulose membrane. Filters were blocked in 10% low-fat milk rich, Switzerland) based on a Leitz DMRBE interfaced with an argon–krypton laser adjusted to 488 and 568 nm. A total of 30–40 optical sections through cells were collected at a thickness of 100 nm using a 63 planapo lens (numerical aperture 1.40), pinhole 30. Sets of optical sections were subjected to the iterative Maximum Likelihood Estimation (MLE) deconvolution analysis (Bitplane software, Zürich, Switzerland) before running the co-localization software (Bitplane, Zürich, Switzerland).

**Immunohistochemistry**

For indirect immunostaining HUVEC were grown to confluence on glass cover slips and stimulated as described above. After washing three times with PBS/Ca2+, cells were fixed and permeabilized by incubating with 3% paraformaldehyde (PFA) and 0.1% Triton X in PBS/Ca2+ for 10 min, then washed again three times with PBS/Ca2+ and incubated for 30 min with 1% BSA in PBS/Ca2+ to block non-specific binding. Incubation with the primary antibody, or a solution of two different primary antibodies was performed overnight at 4°C. Subsequently, cells were washed three times with PBS/Ca2+ and incubated with secondary antibodies conjugated to Cy2 or Cy3 (Dianova, Hamburg, Germany) for 2 h at room temperature. Finally, cells were washed with PBS/Ca2+ before the coverslips were mounted on a glass slide with Mowiol. Confocal laser scanning microscopy was performed using a Leica TCS instrument (Leica, Heidelberg, Germany) based on a Leitz DMRBE interfaced with an argon–krypton laser adjusted to 488 and 568 nm. A total of 30–40 optical sections through cells were collected at a thickness of 100 nm using a 63 planapo lens (numerical aperture 1.40), pinhole 30. Sets of optical sections were subjected to the iterative Maximum Likelihood Estimation (MLE) deconvolution analysis (Bitplane software, Zürich, Switzerland) before running the co-localization software (Bitplane, Zürich, Switzerland).

**Antibodies**

Cadherin-5 monoclonal antibody (TAE1/31) was purchased from Immunotech (Marseille, France), platelet endothelial cell adhesion molecule-1 (PECAM-1) monoclonal antibody (WM 59) was obtained from Sigma (Deisenhofen, Germany) and polyclonal serum against calreticulin was obtained from StressGen (Vancouver, Canada). Polyclonal antibodies against the Golgi protein, nucleobindin (Calnuc/ Nuc-1; Lin et al., 1998) were generated against a C-terminal peptide of the mouse protein (amino acids 401–420: QSAPPSKPDGQLQRPR-ADTDDD) and affinity purified by I.F. and L.H. in our laboratory. The rabbit polyclonal antibodies against Rab4 and Rab7 were raised against synthetic peptides derived from the C-terminus and prepared in our laboratory as outlined previously (Zerial et al., 1992). The rabbit polyclonal antibodies against Rab4 and Rab7 were raised against synthetic peptides derived from the C-terminus and prepared.
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Figure 1. Subcellular localization of cadherin-5, but not platelet endothelial cell adhesion molecule-1 (PECAM-1), is altered in confluent human umbilical vein endothelial cells (HUVEC) treated with serum from patients with pathological pregnancies. (A) Immunostaining for cadherin-5 in HUVEC incubated with serum of a patient with normal pregnancy shows slight intracellular staining of the perinuclear zone; (B) serum from a patient with haemolysis–elevated liver enzymes–low platelet (HELLP) syndrome and (C) serum from a patient with PIH leads to moderate intracellular staining of vesicular structures; (D) serum from a pre-eclamptic patient leads to a large amount of cadherin-5 staining of numerous intracellular vesicular structures. (E, F) HUVEC treated with serum from a patient with HELLP syndrome, and immunostained for (E) PECAM-1 or (F) cadherin-5 demonstrating that, in contrast to cadherin-5, no intracellular staining could be observed for PECAM-1. NB. HUVEC in (B) and (F) were treated with serum from two different patients with HELLP syndrome, one showing intense vesicle staining and the other showing only mild vesicle staining. Immunostaining was performed with monoclonal antibody TEA1/31 for cadherin-5 in (A–D, F) and for PECAM-1 with monoclonal antibody WM 59 in (E). Scale bar = 10 µm.

Categorization of the qualitative results

In order to correlate frequency and intensity of the results of the immunostaining experiments, we categorized the observed intracellular staining of cadherin-5 as follows: 1 = poor intracellular staining (Figure 1A,B); 2 = moderate number and size of cadherin-5 positive intracellular foci (0.5–2.5 µm in diameter, Figure 1C); and 3 = high number and larger sized cadherin-5 positive foci (2–4 µm in diameter.

Figure 2. Cadherin-5 is co-localized with calreticulin (endoplasmic reticulum) and nucleobindin (Golgi apparatus) in intracellular vesicles of confluent human umbilical vein endothelial cells (HUVEC) treated with serum from patients with pre-eclampsia. (A) Immunostaining of HUVEC treated with pre-eclampsia serum for cadherin-5 and (B) for calreticulin (marker-protein of the endoplasmatic reticulum); (C) co-localization (yellow signal) of cadherin-5 and calreticulin by simultaneous two channel scan; (D) calculated colocalization of cadherin-5 and calreticulin using Maximum Likelihood Estimation (MLE) deconvolution and co-localization software. Immunostaining of HUVEC treated with the pre-eclampsia serum for (E) cadherin-5 and (F) nucleobindin (marker-protein of the Golgi apparatus); (G) co-localization (yellow signal) of cadherin-5 and nucleobindin by simultaneous two channel scan; (H) calculated colocalization of cadherin-5 and nucleobindin using MLE deconvolution and co-localization software. NB. HUVEC in (A–D) and (E–H) were treated with serum from two different PE patients, one showing moderate vesicle staining and the other showing extreme vesicle staining. Scale bar = 10 µm.
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Figure 1D. Categorization of the immunostaining experiments was carried out by three different observers at three different points in time. Observers were blinded to the sources of the sera being tested.

The cadherin-5-specific antibody TEA1/31 recognizes two protein bands in HUVEC extracts, a single band of 130 kDa and a double band of ~100 kDa which is reported to be a proteolytic degradation product (Lampugnani et al., 1992; Esser et al., 1998). In order to relate the different immunoblot results to the four clinical groups, we categorized the different immunoblot pattern as follows: 1 = mainly expressing the 100 kDa double band; 2 = 100 and 130 kDa bands of equal signal intensity; 3 = mainly expressing the 130 kDa single band.

**Statistical analysis**

Statistical analyses of the results were carried out using the χ² test for independence followed by adjustment for multiple tests using the Holm procedure.

**Results**

**Cadherin-5 accumulates in tubular-vesicular structures of the endoplasmic reticulum and Golgi complex after incubation of HUVEC with pre-eclampsia serum**

Immunofluorescence staining using the TEA1/31 antibody specific for human cadherin-5 of HUVEC grown in 20% FCS or in 20% serum from women of the control group showed staining of the plasma membrane and of the perinuclear zone (Figure 1A). When HUVEC monolayers were incubated with sera of patients belonging to the HELLP, PIH or pre-eclampsia groups, cadherin-5 was also found in intracellular foci of varying number and size (Figure 1B–D). Staining of intracellular foci was strongest in cells treated with pre-eclampsia serum (Figure 1D). Moderate occurrence of cadherin-5-positive vesicles was observed upon treatment with serum from patients with PIH (Figure 1C) and HELLP (Figure 1B).

In order to determine whether the observed vesicle formation was cadherin-5-specific or simply a non-specific phenomenon occurring for transmembrane adhesion molecules, we conducted staining of HUVEC for PECAM-1. PECAM-1 belongs to the immunoglobulin superfamily of adhesion molecules and, like cadherin-5, is a transmembrane adhesion molecule expressed at the endothelial cell surface. In contrast to cadherin-5, however, PECAM-1 was not found to accumulate in intracellular vesicles of HUVEC incubated with vesicle forming serum from pre-eclampsia, HELLP or PIH patients (Figure 1E, F). Thus, accumulation in intracellular vesicular structures in HUVEC treated with sera from patients with pre-eclampsia or pre-eclampsia-associated diseases did not appear to be a common adhesion-molecule related phenomenon and could be specific for cadherin-5.

To further characterize these vesicular structures, we performed immunostaining experiments with antibodies specific for different intracellular compartments. Calreticulin was used as a marker for the endoplasmic reticulum, nucleobindin for the Golgi apparatus, and Rab4 and Rab7 for early and late endosomes respectively. In double immunostaining experiments using antibodies against these compartment markers, in combination with the TEA1/31 antibody, we did not observe a co-localization of cadherin-5 with Rab4 or Rab7 (data not shown). Instead, co-localization of TEA1/31 staining was found with staining for calreticulin (Figure 2A–D) and nucleobindin, a calcium-binding protein which has been recently identified as a Golgi-resident protein (Figure 2E–H). The perinuclear co-localization of cadherin-5 with calreticulin was found in all cells independently of incubation conditions, confirming cadherin-5 detection in the endoplasmatic reticulum. Cadherin-5 co-localization with nucleobindin was only observed in HUVEC with cadherin-5 staining in large and numerous intracellular vesicular structures, i.e. those treated with pre-eclampsia serum. Thus, pre-eclampsia serum leads to accumulation of cadherin-5 in vesicular and tubular structures of the Golgi apparatus.

Incubation with serum from patients with pre-eclampsia-induced accumulation of cadherin-5 in numerous large, vesicle-like structures, in 13 out of 18 cases. When HUVEC were incubated with serum from normal pregnant patients, we did not observe staining of intracellular vesicles in nine of 10 cases. In one case, some small cadherin-5 positive vesicles were found. In the PIH and HELLP group, however, all three categories of cadherin-5 positive vesicles occurred with comparable frequency (Figure 3B). The difference between the pre-eclampsia and control groups was statistically significant (P = 0.0048).

**Cadherin-5 proteolytic degradation decrease in HUVEC treated with pre-eclampsia serum**

To support our immunostaining data, endothelial cells treated with different sera were subjected to immunoblot analysis. Protein extracts from control-treated HUVEC revealed as a main signal the double band at 100 kDa (Figure 3C, control). Immunoblots of pre-eclampsia serum-treated cells showed a strong signal representing the 130 kDa band while the double band of smaller size was faint (Figure 3C, pre-eclampsia). When HUVEC were treated with serum from patients with a diagnosis of PIH or HELLP, the signal intensities of the two bands varied among the three categories (Figure 3C, PIH and HELLP). When we related the frequency of the different patterns to the four clinical categories (control, HELLP, PIH and pre-eclampsia) a switch in signal intensity from the lower band of smaller size was observed in the control group (Figure 3A). The pattern (1) of the control group was not observed after pre-eclampsia serum treatment. Instead, in one-third of these experiments both bands were of equal intensity, while two-thirds showed the 130 kDa form as the dominant signal (Figure 3A). The difference between the pre-eclampsia and control groups was statistically significant (P = 0.0055).

**Discussion**

In this study, we present the first report of molecular changes in HUVEC, possibly demonstrating altered protein metabolism in endothelial cells due to pre-eclampsia. Our analysis indicates that the serum of pre-eclamptic patients affects membrane targeting of cadherin-5 in HUVEC. This is suggested by the accumulation of cadherin-5 in the vesicular and tubular structures of the Golgi complex. Sera from patients with pre-
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Figure 3. Frequency of altered cadherin-5 patterns in immunostaining and immunoblotting analyses correlated to the clinical groups. (A) Immunoblot pattern of cadherin-5 in confluent human umbilical vein endothelial cells (HUVEC) was categorized as follows: 1 = main signal found in 100 kDa double band; 2 = similar intensity of 130 and 100 kDa bands; 3 = main signal found in 130 kDa band. (B) Subcellular distribution of cadherin-5 was categorized as follows: 1 = no intracellular vesicle staining; 2 = moderate numbers of cadherin-5 positive vesicles; 3 = increased numbers of cadherin-5 positive vesicles of >2 µm in diameter. Control = patients with normal pregnancies; hellp = haemolysis–elevated liver enzymes–low platelet (HELLP) syndrome; pih = pregnancy-induced hypertension; pe = pre-eclampsia. (C) Immunoblot patterns of cadherin-5 in HUVEC treated with serum from patients with normal pregnancy (control), HELLP (hellp), PIH (pih) and pre-eclampsia (pe). Three blots representative for each respective clinical group are shown.

eclampsia yielded the most pronounced results, while those from patients with PIH or HELLP were associated with more moderate alterations. Furthermore, the retention of cadherin-5 in intracellular vesicles was correlated with a decreased protein degradation. Our immunoblot results show that less cadherin-5 is found as a degradation product upon incubation with pre-eclamptic serum. This result is striking because cadherin-5 is very sensitive to degradation as has been observed under conditions of prolonged cell extraction (Lampugnani et al., 1995; Esser et al., 1998) and thrombin treatment (Lampugnani and Dejana, 1997). Coagulation tendency is increased in pre-eclamptic patients (Perry and Martin, 1991; Weiner, 1991). Therefore, an increased proteolytic activity caused by enzymes of the blood coagulation system would be expected in these sera. Instead, we observed enhanced cadherin-5 stability as indicated by the accumulation of the 130 kDa band (Figure 3C). We propose that intracellular retention of cadherin-5 makes it inaccessible for degradation by serum factors. Consequently, our immunoblot results indirectly show that less cadherin-5 is integrated in the plasma membrane.

Concomitant intracellular accumulation of cadherin-5 in the vesicular and tubular structures of the Golgi complex and a decrease in membrane integrated cadherin-5 indicate disruption in membrane targeting of the protein resulting from pre-eclampsia serum treatment. The molecular mechanism for this intracellular retention remains unclear. However, it may specifically affect cadherin-5 membrane targeting as there was no increase in intracellular PECAM-1.

The strength of the defect in cadherin-5 targeting was correlated with the severity of pre-eclampsia and PIH. Patients with pre-eclampsia differ from those with PIH by the extent of the two major clinical symptoms, hypertension and proteinuria. Of these, proteinuria could be caused by altered endothelial permeability. Indeed, it has been shown that pre-eclampsia serum increased the permeability of a HUVEC monolayer (Haller et al., 1997, 1998). Intracellular retention of cadherin-5 and a consecutive decrease in the number of adhesion complexes in the cell membrane might be responsible for alterations in permeability.

Failures in membrane targeting could be caused by an incomplete processing of the protein, e.g. accumulation of an uncleaved premature form or incomplete glycosylation. However, our immunoblot data revealed neither an enrichment of premature cadherin-5 nor an accumulation of an unglycosylated form following incubation with pre-eclampsia serum. Defects either in signal peptide cleavage or glycosylation should also affect PECAM-1 processing and targeting, which was not observed (Figure 1E,F). Furthermore, studies on E-cadherin have demonstrated that neither process is important for membrane targeting. Signal peptides containing either
E-cadherin and unglycosylated E-cadherin are able to integrate into the membrane (Ozawa et al., 1989; Shore and Nelson, 1991). However, there is evidence that membrane targeting is coupled to the assembly of the E-cadherin/β-catenin complex shortly after E-cadherin synthesis (Chen et al., 1999). Whether this sorting mechanism also holds true for cadherin-5 and is affected by serum factors from pre-eclamptic patients is a challenging question for further studies. Considering the concept of Redman et al. (Redman et al., 1999) characterizing pre-eclampsia as a decompensated immune response, future studies should address questions of altered intracellular signal transduction involving immune-related signals. In this context, the alteration of cadherin-5 distribution in HUVEC after treatment with pre-eclampsia serum shown in the present study could work as a model for the endothelial dysfunction in pre-eclampsia as a decompensated immune response, future studies should address questions of altered intracellular signal transduction involving immune-related signals. In this context, the alteration of cadherin-5 distribution in HUVEC after treatment with pre-eclampsia serum shown in the present study could work as a model for the endothelial dysfunction in pre-eclampsia. This could form the basis for studies aimed at identifying the causative serum factors.

In conclusion, cadherin-5 seems to play a role in the endothelial pathogenesis of pre-eclampsia. Elucidation of the mechanisms involved will require further studies including investigation of cadherin-5 metabolism and its function in HUVEC after incubation with syncytiotrophoblast membrane fragments which may be involved in the pathogenesis of pre-eclampsia.

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References