

Placental expression of proteases and their inhibitors in patients with HELLP syndrome

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Abstract

In preeclampsia and hemolysis, elevated liver enzymes and low platelet (HELLP) syndrome, impaired trophoblast invasion and excessive fibrin deposition in the placental intervillous space is associated with fetal compromise. However, little information is available whether modulation of placental protease expression – potentially causing impaired trophoblast invasion – is associated with the HELLP syndrome. Total RNA and protein were extracted from placental tissue from 11 females with HELLP syndrome and 8 controls matched for gestational age. mRNA expression of matrix metalloprotease (MMP) -2 and -9, tissue inhibitors of metalloprotease (TIMP) -1, -2, and -3, and urokinase-type plasminogen activator receptor (uPAR) was determined by Northern blotting. Protein expression of MMP-2 and -9, and TIMP-1 and -2 was detected by Western blotting and that of uPA, uPAR, and plasminogen activator inhibitor (PAI) -1 by ELISA. In patients with HELLP syndrome, mRNA expression of MMP-2 and TIMP-2 was decreased, whereas TIMP-1 and -3 levels were unchanged. MMP-9 and uPAR mRNA was undetectable in both groups. Protein expression of all investigated proteolytic factors remained unchanged. Our findings at the mRNA level suggest a decrease in matrix remodeling in placentae from patients with HELLP syndrome compared with control pregnancies, although this is not supported at the protein level.

Keywords: matrix metalloprotease; placenta; preeclampsia; trophoblast invasion; urokinase-type plasminogen activator receptor.

Introduction

Hypertensive pregnancy disorders are a major cause of maternal and perinatal mortality and morbidity, affecting approximately 5–10% of all pregnancies. Patients with preeclampsia develop hypertension and proteinuria, and

severe cases are associated with lung edema, oliguria, and seizures. As a variant of severe gestational hypertensive disease, approximately 10% of all preeclamptic patients show a syndrome of hemolysis, elevated liver enzymes, and low platelet count, as described by Weinstein in 1982 as ‘HELLP syndrome’ (Weinstein, 1982). These patients are at high risk to develop disseminated intravascular coagulation, bleeding complications, and liver rupture. Not all of the preeclamptic patients develop HELLP syndrome, and some patients even show signs of HELLP syndrome without manifestation of hypertension and proteinuria, pointing towards differences in the pathophysiology of preeclampsia and HELLP syndrome.

The placenta is the key organ for preeclampsia and HELLP syndrome, as associated symptoms disappear only after removal of the placenta (Roberts and Cooper, 2001). In placentae from patients with preeclampsia, trophoblast cells fail to invade deeply into the maternal myometrium and to replace the endothelial layer in uterine spiral arteries. As a result, spiral artery remodeling is inadequate and placental perfusion impaired (Brosens et al., 1972). These changes could eventually lead to intrauterine growth retardation of the fetus and severe maternal symptoms.

Trophoblast invasion is a process closely resembling wound healing and tumor invasion, but strictly controlled both spatially and temporally (reviewed in Bischof and Campana, 2000). Matrix metalloproteases (MMPs), serine proteases and their specific inhibitors are involved in this process (Fisher et al., 1985; Librach et al., 1991; Estellés et al., 1994; Graham and McCrae, 1996; Hurskainen et al., 1996; Solberg et al., 2003). Librach et al. (1991) showed that the invasive behavior of trophoblast cells correlates with MMP-9 expression and that tissue inhibitors of metalloproteases (TIMPs) inhibit their invasive capacity. MMP-2 and -9 cooperate closely with the urokinase-type plasminogen activator (uPA) system, mutually activating their components (Nagase, 1997). Synergism of these proteolytic factors in placental development has been shown in animal experiments (Solberg et al., 2003). Plasminogen activator inhibitor (PAI) -1, expressed in villous and in invasive extravillous trophoblasts, as well as in maternal decidual tissue, is suggested to be closely involved in control of trophoblast invasion (Floridon et al., 2000).

However, there is little information regarding differences in the pathophysiology of preeclampsia and HELLP syndrome. We have shown that the Rac1/JNK/c-Jun-dependent signaling pathway is downregulated in placentae of patients with preeclampsia and HELLP syndrome (Hannke-Lohmann et al., 2000). Morphologic investigations of the placentae proved that in HELLP syndrome chronic placental inflammation, the predominant feature of preeclampsia, is a less common event (Salafia et al., 1998). Although expression of components of

the uPA and MMP systems has been studied in patients with preeclampsia, there is only scarce information on the expression of these proteolytic factors in HELLP syndrome. Thus, the purpose of this study was to gain insight into the expression of these factors in placentae of patients with HELLP syndrome.

Results

We investigated RNA and protein extracted from placentae from 11 (respective 10) females with HELLP syndrome and 8 controls matched for gestational age. This is of importance because placental expression of proteases and other proteins might change during pregnancy. All HELLP patients exhibited severe clinical symptoms and clinical chemistry laboratory changes, thus meeting our inclusion criteria (Table 1). Mean birth weight of the newborns was significantly lower in the HELLP group than in the control group (1520.4 g vs. 2218.8 g, $p=0.020$). Birth weight was also more often below the 5th percentile (5 out of 11 vs. 1 out of 8), a feature commonly associated with HELLP syndrome as a symptom of clinically relevant placental insufficiency.

Trophoblast cells express a high amount of proteases and this accounts, in part, for their capacity to invade the maternal myometrium. To determine whether RNA levels of proteases and their respective inhibitors in placentae of patients with HELLP syndrome are different from control placentae, Northern blot analyses were performed. By Northern blotting, both MMP-9 and uPAR mRNA were undetectable in third trimester placentae of either group. This finding was confirmed by applying different hybridization and washing protocols. MMP-2, TIMP-1, -2, and -3 were clearly expressed. MMP-2 and TIMP-1 showed one band, whereas TIMP-2 and TIMP-3 displayed two bands each, reflecting mRNA splice variants. Quantitative analyses after normalization to 18S rRNA showed no significant difference between the respective splice variants of TIMP-2 and -3.

Compared with uncomplicated pregnancies, in patients with HELLP syndrome, expression of MMP-2 and TIMP-2 mRNA was decreased. Quantitative analysis after normalization to 18S rRNA showed a reduction of signal intensity for MMP-2 mRNA by 43% ($p=0.043$) and for TIMP-2 mRNA by 62% ($p=0.009$), respectively (Figure

1). TIMP-1 and -3 mRNA did not change significantly when comparing placentae from patients with HELLP syndrome to control pregnancies ($94.6\pm 13.4\%$ and $144.9\pm 30.9\%$ of control group, respectively). Thus, HELLP syndrome is associated with MMP-2 and TIMP-2 mRNA reduction, but does not affect TIMP-1 and -3 mRNA levels.

Expression of MMP-2, TIMP-1, and TIMP-2 protein showed no significant difference between HELLP patients and the control group (Figure 2). MMP-9 was

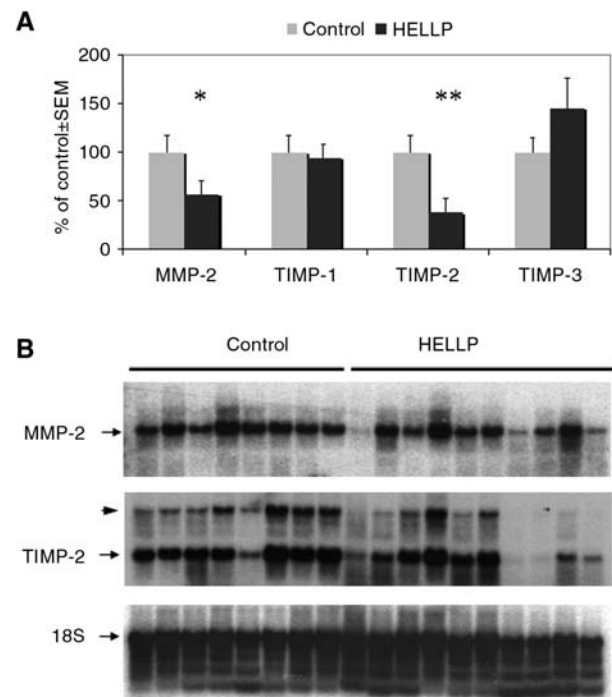


Figure 1 mRNA expression of MMP-2, TIMP-1, TIMP-2 and TIMP-3.

(A) Quantitative analysis of MMP-2, TIMP-1, TIMP-2 and TIMP-3 mRNA expression determined by Northern blotting in placental tissue of pregnancies complicated by HELLP syndrome and controls matched for gestational age at delivery. * $p<0.05$, ** $p<0.01$; SEM is standard error of the mean. (B) Northern blots for MMP-2 and TIMP-2. Lanes contain RNA extracts of 8 control placentae and of 10 HELLP placentae, beginning with higher weeks of pregnancy on the left side. The arrowhead shows a splice variant of TIMP-2. 18S rRNA signal shows degradation of the RNA.

Table 1 Patient characteristics.

	Control		HELLP		p
	Mean	SEM	Mean	SEM	
Number of patients (n)	8	–	11	–	
Cesarean delivery (n)	2	–	11	–	
Maternal age (years)	29.4	1.8	28.7	1.6	Not significant
Gestational age at delivery (weeks)	34.2	0.8	31.8	1.2	Not significant
Birth weight (g)	2218.8	181.5	1520.4	209.5	0.02
Growth retardation <5th percentile (n)	1	–	5	–	
Platelet count (G/l)	224.6	17.6	38.6	4.3	<0.001
ALT (U/l)	8.0	0.9	479.7	292.3	<0.001
LDH (U/l)			2979.4	1709.8	
Systolic blood pressure (mm Hg)	112.9	7.1	160.9	13.5	0.006
Diastolic blood pressure (mm Hg)	63.6	5.2	100.0	8.2	0.001

ALT, alanine aminotransferase; LDH, lactate dehydrogenase; SEM, standard error of the mean. LDH was not determined in control patients without hypertensive disease of pregnancy.

not detected by Western blotting. No significant differences in protein concentrations of uPA (HELLP vs. control: 1.22 ± 0.13 vs. 1.28 ± 0.17 ng/ml protein), PAI-1 (HELLP vs. control: 26.62 ± 4.43 vs. 26.70 ± 5.73 ng/ml protein), or uPAR (HELLP vs. control: 0.43 ± 0.08 vs. 0.52 ± 0.09 ng/ml protein) were observed (Figure 3).

Discussion

Proteases, such as MMPs, and factors of the plasminogen activation system, e.g., the uPA, its inhibitor PAI-1 and the receptor uPAR have been implicated in tissue remodeling in various physiological and pathological processes, including wound healing and tumor invasion and metastasis. Yet, the role of proteolytic factors in the development of the human placenta during uncomplicated or impaired pregnancy is still not fully understood (Nagase, 1997; Bischof and Campana, 2000).

MMP-2 and -9 are members of a large family of zinc-dependent MMPs. MMP-2 is broadly, and often constitutively, expressed, whereas MMP-9 expression is more restricted, and, typically, low or absent in normal, quiescent tissues (Stamenkovic, 2000).

In the present study, MMP-9 mRNA and protein were not detected in placental tissue. This is in line with previous studies by Polette et al. (1994) who did not detect MMP-9 mRNA in term placentae, and Isaka et al. (2003) who also did not find MMP-9 mRNA or protein in normal

term placentae, except MMP-2. In contrast, Campbell et al. (2004) noted MMP-2 as well as MMP-9 protein secretion in term trophoblasts co-cultured with decidual cells, reflecting the different study conditions in culture, more similar to first trimester placental development.

Importantly, our findings regarding detection of MMP-2, TIMP-1, -2, and -3 mRNA and protein, respectively, are also in agreement with those of other research groups (Polette et al., 1994; Hurskainen et al., 1996; Hupertz et al., 1998; Isaka et al., 2003).

MMP-2 mRNA and protein expression is localized to extravillous trophoblast cells (Polette et al., 1994; Hupertz et al., 1998; Isaka et al., 2003) and almost unchanged during placental development. However, compared with normal placental development, we observed lower levels of MMP-2 and TIMP-2 mRNA in placentae from patients with HELLP syndrome. Interestingly, although mRNA expression was reduced, no significant reduction of MMP-2 and TIMP-2 protein, compared with control placental tissues, was observed.

TIMP-2 binds to both the hemopexin C domain of pro-MMP-2 and the activation domain of the membrane-bound MT1-MMP. This trimeric complex of MT1-MMP and TIMP-2 presents a cell surface-bound MMP-2 zymogen:proteolytic cleavage of MMP-2, which results in MMP-2 activation, and is then mediated by a second, TIMP-2-free MT1-MMP molecule (Imai et al., 1996; Kinoshita et al., 1998; Zucker et al., 1998). As a consequence, reduced expression of TIMP-2 could lead to diminished activation of the MMP-2 proenzyme and these changes of both MMP-2 and TIMP-2 might reduce remodeling potential of placentae in patients with HELLP syndrome.

In the aforementioned co-culture experiments (Campbell et al., 2004), both MMP-2 and -9 secretion by term cytotrophoblasts were reduced in those obtained from preeclamptic pregnancies compared with controls, whereas no difference in amounts of latent or active forms of the enzymes could be noted.

High expression of the MMP inhibitor TIMP-1 is particularly found in the placental bed throughout pregnancy (Polette et al., 1994). In our study, tissue samples contained no decidual materials. Therefore, unchanged TIMP-1 expression in HELLP placentae was not surprising. This is also true for the MMP inhibitor TIMP-3, consistent with the absence of MMP-9 expression, as TIMP-3 is also highly expressed in decidual cells probably involved in the control of MMP-9 mediated cell invasion during early pregnancy (Hurskainen et al., 1996).

Recently, the key role of the placental expression of angiogenic factors, such as vascular endothelial growth factor (VEGF) and placenta growth factor and their receptor soluble fms-like tyrosine kinase 1 (s-Flt-1), in the pathogenesis of preeclampsia has been elucidated (summarized in Levine et al., 2006). The simultaneous overexpression of sFlt-1 and soluble endoglin, a receptor for transforming growth factor β 1 (TGF- β 1), was found to result in a combination of preeclampsia and HELLP syndrome in pregnant rats (Venkatesha et al., 2006). This finding provided a first clue with regard to the special pathogenesis of HELLP syndrome.

TIMP-2 is known to be upregulated by TGF- β 1 in cultured trophoblast cells (Karmakar and Das, 2002). Inter-

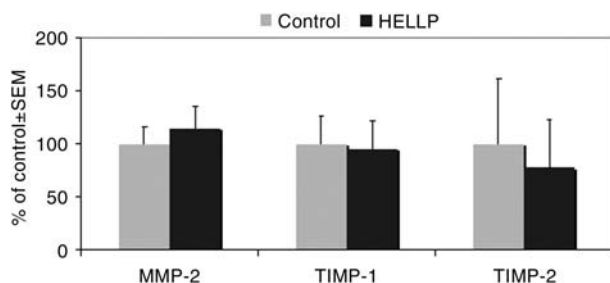


Figure 2 Quantitative analysis of MMP-2, TIMP-1, and TIMP-2 protein expression in placental tissue of pregnancies complicated by HELLP syndrome and controls matched for gestational age at delivery measured by Western blotting. Statistical analysis revealed no significant differences. SEM is standard error of the mean.

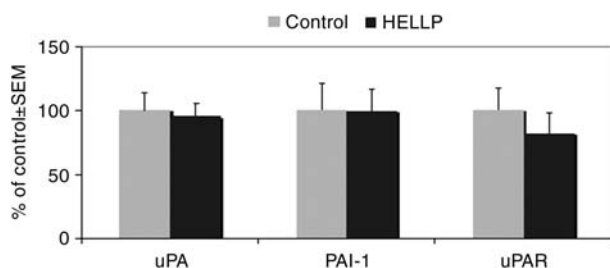


Figure 3 Quantitative analysis by ELISA of uPA, PAI-1, and uPAR protein expression in placental tissues of pregnancies afflicted with HELLP syndrome and of control pregnancies, matched for gestational age at time of delivery. Statistical analysis revealed no significant differences. SEM is standard error of the mean.

estingly, an increase in circulating soluble endoglin, found to be associated with clinical symptoms of HELLP syndrome, could lead to reduced TGF- β 1 levels, similar to circulating sFlt-1 and lower VEGF levels present in preeclampsia (Levine et al., 2006). Thus, reduced TGF- β 1 levels could induce the placental changes of TIMP-2 described in our study with placental tissue of HELLP syndrome patients, linking our results to recent findings concerning angiogenetic changes in preeclampsia and HELLP syndrome.

uPAR (CD 87) is the major cellular binding site for the serine protease uPA, allowing focused cell surface-oriented plasminogen activation. Beside its major role in fibrin degradation, plasmin is known to activate certain MMPs, e.g., transforming pro-MMPs to their proteolytically active forms (Nagase, 1997). In our study, uPAR mRNA was not detected in third trimester placental tissue. This finding is in line with studies by Zini et al. (1992) who showed that differentiation of trophoblasts is paralleled by a decrease in uPAR mRNA and protein expression. Also, the protein concentrations of uPA, PAI-1, and uPAR detected by ELISA were similar to those published previously (Kolben et al., 1996; Uszynski et al., 2001).

Kolben et al. (1996) showed significant higher PAI-1 levels in maternal plasma in preeclampsia and HELLP syndrome. Another, later study on the concentrations of uPA and PAI-1 in maternal plasma showed no differences between preeclamptic patients and controls if matched for gestational age, whereas umbilical cord arterial and venous plasma showed elevated PAI-1 levels if mothers suffered from severe preeclampsia (Roes et al., 2002). Obviously, in our study the tissue sampled near the decidual plate did not contain much fetal or maternal plasma, otherwise resulting in elevated PAI-1 protein levels.

Graham and McCrae (1996) showed that trophoblast cells isolated from preeclamptic placentae displayed reduced capacity of plasminogen activity. Reduced uPA or PAI-1 levels were not detected, thus they hypothesized that reduced cell surface uPA activity could result from diminished expression or function of cell surface uPAR. They argued that this reduced cell-bound plasminogen activation capacity might be responsible for high fibrin deposition observed in the intervillous space in placentae of women with preeclampsia. This seems probable because high uPAR expression was observed in trophoblast cells of first and third trimester placentae associated with deposits of fibrin-type fibrinoid as shown by immunostaining (Pierleoni et al., 1998). However, we could not confirm changes in expression of uPAR protein.

We studied expression of proteolytic factors of the MMP family and the plasminogen activation system, performed in placental tissues obtained from non-hypertensive pregnancies and matched patients suffering from HELLP syndrome after third trimester delivery, which is approximately 20 weeks after trophoblast invasion was completed. But still, our findings suggest a decrease in matrix remodeling capacity in placentae from patients with HELLP syndrome, being part of a change in protease expression pattern. These changes could be related to inappropriate adaptation of the placenta to increasing requirements for oxygen and nutrients of the fetus.

Materials and methods

Patients

Pregnant volunteers and HELLP patients who received care at our department gave written informed consent for research use of their third trimester placentae. A total of 11 patients showed severe symptoms of HELLP syndrome defined by the presence of all of the following criteria: platelet count $<100\,000/\mu\text{l}$, serum alanine aminotransferase $>70\text{ U/l}$, serum lactic dehydrogenase $>600\text{ U/l}$, and right upper quadrant-abdominal pain. All patients presented with hypertension $>160/110\text{ mm Hg}$ and showed proteinuria of 3+ in a dipstick random sample or $>5\text{ g}$ in 24 h. Exclusion criteria were multiple pregnancies, multiparity, and other medical conditions, such as chronic hypertension, renal disease, autoimmune disorders, or diabetes. All patients with HELLP syndrome were delivered by cesarean following institutional guidelines. Eight females with similar gestational age at time of delivery were chosen as controls. None of the control subjects showed evidence of hypertensive or cardiovascular disease, chorioamnionitis, or any other placental abnormality that would have been detected histologically. Placental tissue samples were obtained after removing decidual material within 1 h after delivery and stored at -80°C .

Northern blotting

Total RNA was extracted from frozen placental tissue using a modified Chomczynski method (Chomczynski and Sacchi, 1987), as described elsewhere (Krüger et al., 1997). The quality of the resulting RNA was checked by use of the 260/280 nm quotient. Then, 15 μg RNA was electrophoresed for 3 h at 60 V, 50 mA, on a 1% agarose-formaldehyde gel containing 0.036 M 3[n-morpholino]propanesulfonic acid sodium salt. Transfer of RNA to a positively charged nylon membrane (Hybridization Transfer Membrane, Genescreen; Perkin Elmer, Boston, MA, USA) by capillary action using a solution of 1.5 M NaCl and 0.15 M sodium citrate (pH 7.0) was performed overnight. The membrane was fixed at 80°C for 3 h and the RNA crosslinked. The Northern blot was probed at 65°C with random primed, [^{32}P]-labeled cDNAs specific for matrix metalloproteases MMP-2 and -9, their tissue inhibitors TIMP-1, -2, and -3, and uPAR mRNA and subsequently washed at 62°C using 0.5 M Na_2HPO_4 (pH 7.2) containing 1% (w/v) sodium dodecyl sulfate. Because there was not enough tissue of one HELLP placenta for performing all of the experiments, only 10 of the 11 HELLP placental extracts were available for the blots which were probed with MMP-2, -9, and TIMP-2 cDNAs. Loading efficiencies were checked by reprobing of the membrane with 18S rRNA. This revealed remarkable amounts of degradation products, characteristic for tissue samples obtained under suboptimal conditions. Quantitative analysis of specific RNA expression was performed using the PhosphorImager™ 445 SI scanner and the ImageQuaNT4.1 software (Molecular Dynamics/Amersham, Uppsala, Sweden). Intensities of the specific signal were normalized to 18S rRNA intensity.

Western blotting

Placental tissue was pulverized in frozen state with a Microdisembrator II (ball mill; Sarstedt, Melsungen, Germany) and resuspended in a buffer 0.02 M Tris-HCl (pH 8.5), 125 mM NaCl, and 1% Triton X-100 under gentle rotation at 4°C for 15 s. The lysate was ultracentrifuged (100 000 g, 45 min, 4°C) and an aliquot of the supernatant assayed for protein concentration.

Western blotting was performed as described elsewhere (Ried et al., 1999). Loading efficiencies were checked by reprobing the blot with an antibody against β -actin. Immunostaining was visu-

alized by ECL according to the manufacturer's recommendation (Amersham Pharmacia Biotech, Buckinghamshire, UK). Primary antibodies employed were as follows: anti-MMP-2 (Ab-3, mouse, 1:1000), anti-MMP-9 (Ab-1, mouse, 1:1000), anti-TIMP-2 (Ab-1, mouse, 1:1000), all from Calbiochem, San Diego, CA, USA, and anti-TIMP-1 (RP1T1 at 1:1000, rabbit; Triple Point Biologics, Forest Grove, OR, USA). Quantitative analyses were performed using densitometric scanning and Scan Pack 3.0 software (Biometra, Göttingen, Germany).

Enzyme-linked immunosorbent assay (ELISA)

uPA, PAI-1, and uPAR protein concentrations were determined by ELISA according to the manufacturer's instructions (uPA: Imubind # 894, PAI-1: Imubind # 821, uPAR: Imubind # 893; American Diagnostica Inc., Stamford, CT, USA). Concentrations of analytes were expressed as ng/mg protein. Protein was determined using the BCA method (Jänicke et al., 1994).

Statistical analysis

Statistical analysis was performed using the Mann-Whitney *U*-test employing SPSS 11.0 for Windows (SPSS, Inc., Chicago, IL, USA). A *p*-value <0.05 was considered statistically significant.

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Received February 6, 2009; accepted June 9, 2009