ORIGINAL ARTICLE

Expression of metalloproteinases 1, 2, 7, 9, and 12 in human cytotrophoblastic cells from normal and preeclamptic placentas

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Abstract

OBJECTIVES: Preeclampsia is a specific pregnancy disorder which could be due, at least in part, to impaired invasion of trophoblastic cells. Since matrix metalloproteinases (MMPs) are the predominant proteases involved in trophoblastic invasion, we investigated and compared expression of MMP-1, 2, 7, 9 and 12 of cytotrophoblastic cells (CTB) purified from preeclamptic (PE) placentas to control CTB.

MATERIAL AND METHODS: In order to evaluate invasive properties of cells, purified CTB were seeded on collagen-coated insert following boyden chamber principle and matrix metalloproteinases (MMPs) expression was evaluated by qPCR.

RESULTS: Our results showed that PE CTB are less invasive than control CTB *in vitro*. In parallel, expression of MMPs, except for MMP-2, tends to be decreased in PE CTB compared to control CTB.

CONCLUSION: At the exception of MMP-2, this study confirms the importance of MMPs in development of PE.

INTRODUCTION

Cytotrophoblastic cells (CTB) of the human placenta proliferate, migrate and invade the pregnant uterus to allow implantation and placentation (Red-Horse *et al.* 2004). The invasive property of CTB is due to their ability to secrete proteases such as matrix metalloproteinases (MMPs) which are able to degrade basement membranes and extracellular matrix (Bischof *et al.* 1995; de Jager *et al.* 2003; Fisher *et al.* 1985).

The metalloproteinases 2 and 9 (also known as gelatinase A and gelatinase B respectively) are the most studied MMPs in first trimester CTB (Isaka *et al.* 2003; Staun-Ram *et al.* 2004). *In vitro*, it was shown that human CTB secrete MMP-2 and MMP-9 (Bischof *et al.* 1991) which are essential for trophoblast invasion (Bischof *et al.* 1995). Accumulating evidences suggest that MMP-9 may play a more important role than MMP-2 in trophoblast invasion during pregnancy (Hurskainen *et al.* 1996; Librach *et al.* 1994; Librach *et al.* 1991; Polette *et al.* 1994; Shimonovitz *et al.* 1994; Xu *et al.* 2000). This was, however, challenged by Staun-Ram *et al.* who suggest that MMP-2 represents the main gelatinase in early trophoblast and that it could be the key enzyme in trophoblast invasion (Staun-Ram *et al.* 2004). If MMP-2 and 9 are the most studied in trophoblastic invasion, there are also many others MMPs found in placenta and involved in trophoblast invasion (Cohen *et al.* 2006; Vettraino *et al.* 1996). Indeed collagenase 1 or MMP-1 has the property to cleave fibrillar collagens within their triple helix at neutral pH and serves an essential

role in initiating efficient matrix turnover. Matrylisin, or MMP-7, has a broad capacity to degrade matrix components including elastin, fibronectin and proteoglycans (Cohen *et al.* 2006). MMP-12 was recently identified as another important mediator of uterine vascular remodeling during pregnancy by its capacity to degrade mural elastin during spiral artery remodeling (Harris *et al.* 2010). Together, these MMPs could cooperate to degrade main extracellular matrix components of the uterus and could be essential for human placentation.

Preeclampsia (PE) is a disorder that affects 5 to 8% of pregnancies. Preeclampsia and other pregnancy hypertensive disorders are a leading global cause of maternal and infant morbidity and mortality. The initiating event in PE has been postulated to be reduced uteroplacental perfusion as a result of abnormal CTB invasion of spiral arterioles(Gerretsen *et al.* 1981; Robertson *et al.* 1967). Placental ischemia/hypoxia is thought to lead to widespread activation/dysfunction of the maternal vascular endothelium. These endothelial abnormalities, in turn, cause hypertension by impairing renal function and increasing total peripheral resistance. Mechanisms that lead to shallow invasion or trophoblastic differentiation in PE are still unknown but, since MMPs seem to play an important role in trophoblast invasion, many authors speculate that MMPs expression is altered in PE (Campbell *et al.* 2004; Farina *et al.* 2009; Founds *et al.* 2009; Kolben *et al.* 1996; Montagnana *et al.* 2009; Myers *et al.* 2005; Shokry *et al.* 2009). However, studies examining the expression pattern of MMPs in human PE are limited. In the present paper, we have explored the potential changes in MMP-1, 2, 7, 9 and 12 expressions in PE CTB.

MATERIAL AND METHODS

Reagents

Dulbecco's modified Eagle's medium (DMEM), Hanks balanced salted solution (HBSS) and antibiotics mixture (penicillin, streptomycin) were products of Invitrogen (Basel, Switzerland). Fetal bovine serum (FBS) was from Biochrom AG (Oxoid AG, Basel, Switzerland). Wst-1 cell proliferation assay was purchased from Roche (Diagnostics GmbH, USA). Collagen type I and collagenase were from Sigma (Sigma-Aldrich, Buchs, Switzerland).

Tab. 1. Demographic and clinical characteristics of the study groups.

	CTRL (n=9)	PE (n=6)	*p*-value
Maternal age (year)	33.2±1.8	34.6±1.9	0.66
Gestational age at delivery (week)	37.2±0.9	29.4±1	0.0003
BMI	26.8±1.8	27.4±3.1	0.86
Max systolic blood	122.8±3.2	166.8±7.6	0.0003
Max diastolic blood	80.1±2.2	106±4.8	0.0006
Parity	0.9±0.3	0	0.08
Gravidity	2.5±0.5	1	0.04
Baby weight (gram)	3021±299	1168±227	0.002
IUGR	n=0	n=2	

Study group

Severe PE was diagnosed using standard definitions from hypertension defined as a systolic blood pressure level ≥ 160 mmHg or a diastolic blood pressure level ≥ 110 mmHg on two occasions and proteinuria ≥ 3+ on a urine stick or ≥ 5g in a 24-hour urine specimen (ACOG practice, 2002).

9 control and 6 PE patients were recruited for this study. Their characteristics are given in Table 1.

Informed written consent was obtained from all patients before their inclusion in the study, for which approval was obtained from the local ethics committee.

Cell culture

CTB were isolated from placentas obtained after delivery as described (Bischof *et al.* 1995). In brief, fresh tissue specimen were isolated and washed several times in sterile HBSS. Tissue was then enzymatically digested 5 times for 20 minutes at 37 °C (0.25% trypsin, 0.25 mg/ml Dnase I). After incubation, the trypsin cocktail was neutralized with FBS, and the cells resuspended in DMEM. This cell suspension was filtered (100 μm mesh), laid onto a Percoll gradient (70% to 5% Percoll diluted with HBSS) and centrifuged for 25 min at 1200 × *g*. The 30–45% percoll layer containing CTB was collected, the cells washed and resuspended in DMEM. Cells were then immunopurified with immobilized anti-CD45 antibody according to a protocol published previously.

Proliferation assay

Wst-1 cell proliferation assay was used according to the manufacturer's protocol. Absorbance was recorded at 450nm using a 96-well plate reader.

Invasion assay

Cell invasion assay was performed in an invasion chamber based on the Boyden chamber principle. Each insert is fitted with an 8 μm pore size polycarbonate membrane (Costar, Vitaris, Baar, Switzerland) precoated with rat tail collagen I (5 μg/cm^2). Inserts were washed in DMEM and incubated for 30 minutes at room temperature. For each well, 2.5 × 10^5 CTB in 100 μl of mediawere added to the upper compartment of the transwell chambers. Cells were incubated for 24 h at 37 °C in a CO2 (5%) incubator. Cells that did not invade but were still attached to the collagen were swept away with a cotton swab. Viable cells that invaded collagen were stained with crystal violet cell stain (0.9% in ethanol). After washing the cells, the stain was extracted with a solution of 1% acetic acid:50% ethanol for 15 min at room temperature. 100μl of the dye mixture were transferred to a 96-well microtiter plate for colorimetric

measurement at 540 nm. Data were expressed as cells that invaded the collagen-coated membrane relative to the proliferation assay (reflecting the number of total cells). Collagen type I coated inserts incubated with medium without cells served as blank.

Zymography

After 24 of CTB culture, supernatants were collected and their proteolytic activity was assayed using gelatin-substrate gel electrophoresis as described previously (Martelli *et al.* 1993). Zymograms were scanned with an Epson Perfection 1 200 Photo scanner and the surface of the digestion bands measured by the Kodak 1D Image analysis software (Kodak, Rochester, NY).

RNA extraction

Control and PE CTB (5x10^6 cells) were cultured for 24 h before total RNA was extracted using RNeasy Mini kit (QIAGEN, Basel, Switzerland) following manufacturer's instructions.

Real-time quantitative reverse transcription-PCR

Reverse transcription was performed with 400 ng of total RNA in a final volume of 20 µL using QuantiTect Reverse Transcription kit (QIAGEN, Basel, Switzerland). The quantitative detection of the PCR product was performed using the qPCR Mastermix Plus for SYBR Green I (Eurogentec, Seraing, Belgium), supplemented with fluorescein (Bio-Rad, Reinach, Switzerland), with the iCycler iQ System (Bio-Rad). The relative expression was normalized to the housekeeping gene cyclophilin A. Oligonucleotide primers for qPCR were as follows: human cyclophilin A forward 5'-TACGGGTCCTGGCATCTTGT-3' and reverse 5'-CCATTTGTGTTGGGTCCAGC-3', human MMP-1 forward 5'-AAAGACAGATTCTACATGCG -3' and reverse 5'-TGCTTCACAGTTCTAGGGA-3', human MMP-2 forward 5'-ATAACCTGGATGCCGTCGT-3' and reverse 5'-AGGCACCCTTGAAGAAGTAGC-3', human MMP-7 forward 5'-CCAGATGTTGCAGAATACTC-3' and reverse 5'-CCACTGTAATATGCGGTAAG-3', human MMP-9 forward 5'-CTGAGAACCAATCTCACCGACA-3' and reverse 5'-AGATTTCGACTCTCCACGCA-3' and human MMP-12 forward 5'-CCAGCTCTCTGTGACCCCAA-3' and reverse 5'-TCCCACGGTAGTGACAGCATC-3'.

Proteomic analysis

In solution protein digestion

10 µg of total protein from each sample was suspended in 40 µl of distilled water. To this solution, 40 µl of urea 6 M were added and the mixture was incubated at 37 °C for 30 min. Then, 30 µl of DTT (38 mM in distilled water) were added and the reduction was carried out at 37 °C for 1 h. Alkylation was performed by adding 80 µl of iodoacetamide (108 mM in 50 mM ammonium bicarbonate) during 1 hour at room temperature in the dark.

Five µl of trypsin porcine (Sigma) solution (50 ng/µl in 50 mM ammonium bicarbonate) was added and the digestion was proceeded overnight at 37 °C. The sample was desalted with a C18 microspin column (Harvard Apparatus, Holliston, MA, USA), dried, and re-dissolved in H_20/CH_3CN/FA 94.9/5/0.1 before LC-ESI-MS/MS analysis.

Peptide fragmentation sequencing

LC-ESI-MS/MS was performed on a linear trap quadrupole (LTQ) Orbitrap Velos from Thermo Electron (San Jose, CA, USA) equipped with a NanoAcquity system from Waters. Peptides were trapped on a home-made 5 µm 200 Å Magic C18 AQ (Michrom) 0.1 × 20 mm pre-column and separated on a home-made 5 µm 100 Å Magic C18 AQ (Michrom) 0.75 × 150 mm column with a gravity-pulled emitter. The analytical separation was run for 65 min using a gradient of H2O/FA 99.9%/0.1% (solvent A) and CH3CN/FA 99.9%/0.1% (solvent B). The gradient was run as follows: 0–1 min 95% A and 5% B, then to 65% A and 35% B at 55 min, and 20% A and 80% B at 65 min at a flow rate of 220 nL/min. For MS survey scans, the orbitrap (OT) resolution was set to 60 000 and the ion population was set to 5×10^5 with an m/z window from 400 to 2 000. For protein identification, up to eight precursor ions were selected for collision-induced dissociation (CID) in the LTQ. The ion population was set to 1×10^4 (isolation width of 2 m/z) while for MS/MS detection in the OT, it was set to 1×10^5 with an isolation width of 2 m/z units. The normalized collision energies were set to 35% for CID.

Protein identification

Peak lists were generated from raw orbitrap data using the embedded software from the instrument vendor (extract_MSN.exe). The monoisotopic masses of the selected precursor ions were corrected using an in-house written Perl script (Scherl *et al.* Proteomics 2008, 8, p. 2791). The peaklist files were searched against the SwissProt/trEMBL database (Release 15.10 of 03-Nov-2009) using Mascot (Matrix Sciences, London, UK). Human taxonomy (98529 sequences) was specified for database searching. The parent ion tolerance was set to 10 ppm. Variable amino acid modifications were oxidized methionine. Trypsin was selected as the enzyme, with one potential missed cleavage, and the normal cleavage mode was used. The mascot search was validated using Scaffold 3.00.08 (Proteome Software, Portland, OR). Only proteins matching with two different peptides with a minimum probability score of 95% were considered identified.

Statistical analysis

Data were expressed as means ± SEM for n different samples. Statistical differences between samples were assessed by the Student's t test and the p value < 0.05 was considered significant.

RESULTS

Patient characteristics

Table 1 reports the clinical information of women involved in this study. PE women had significantly elevated blood pressures and delivered earlier lower birthweight babies (Table 1) compared to normotensive patients (CTRL). Mean maternal age, parity and BMI were not significantly different between the 2 groups of patients.

Comparative invasive properties of CTB purified from CTRL and PE placenta

Despite that term CTB are not considered as invasive cells, we purified CTB from PE and CTRL placentas and compared their invasive properties on collagen. As shown in Figure 1, CTB obtained from severe PE placentas were significantly less invasive than CTRL CTB after 24 h of culture.

Expression of MMPs in CTB purified from CTRL and PE placenta

Table 2 reports the mean values of transcript expression of MMP-1, 7, 9 and 12 expressions in CTRL and PE CTB. MMP-1, 7, 9 and 12 mRNA expressions in PE CTB showed lower values than in CTRL CTB without reaching the level of significance. In contrast, MMP-2 mRNA expression was clearly not different between CTRL and severe PE CTB.

Tab. 2. Gene expressions of different MMPs in trophoblastic cells purified from CTRL and PE placenta.

	MMP-1	MMP-2	MMP-7	MMP-9	MMP-12
CTRL (n=9)	2.6±0.7	2.4±0.6	23.8±8.2	6.5±2	16.1±3.8
PE (n=6)	1±0.5	2.4±1.9	5.2±3.1	1.8±1.3	8.2±4.9
Fold decrease	2.6	1	4.5	3.6	1.9
p-value	0.12	0.99	0.1	0.16	0.22

Proteolytic activity of MMPs secreted by CTRL and PE CTB

Activities (Figure 2A) and levels (Figure 2B) of gelatinases were then evaluated in culture supernatant of PE and CTRL CTB. If MMP-2 activity was not affected in supernatant of PE CTB, MMP-9 activity is significantly decreased in supernatant of PE CTB compared to CTRL CTB (Figure 2A).

This result is reinforced by the observation of a tendency of decreased MMP-9 levels in supernatant of PE CTB compared to control CTB as detected by mass spectrometry (Figure 2B).

DISCUSSION

Trophoblast invasion depends on breakdown of the various components of the extracellular matrix. MMPs were identified as essential partners in this degradation (Cohen *et al.* 2006). Since preeclampsia is associated with impaired trophoblast invasion, we examined the expression of different MMPs in PE CTB. We have found that most studied MMPs mRNA expressions seemed to be decreased in PE CTB in comparison with normal CTB, explaining at least in part, the decreased invasive properties of PE CTB compared to control cells.

The present results indicate that MMP-9 mRNA expression, secretion and activity are decreased in PE CTB compared to CTRL CTB, confirming other studies (Campbell *et al.* 2004; Kolben *et al.* 1996). However, another study described the opposite (Wang *et al.* 2009). Recently, it was shown that MMP-9 gene expression is also decreased in first trimester villous samples in women who develop PE later in pregnancy compared to CTRL pregnant women (Farina *et al.* 2009). Together,

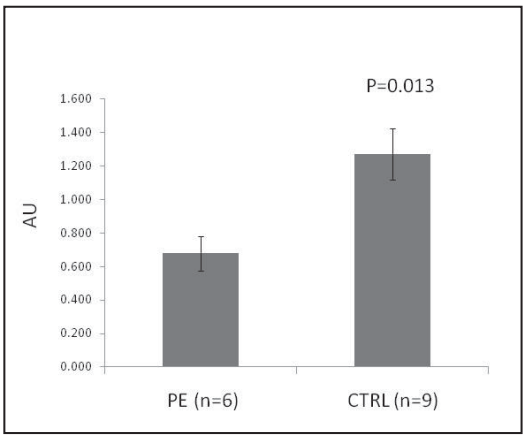

Fig. 1. Invasive property of CTB. AU: absorbance unit

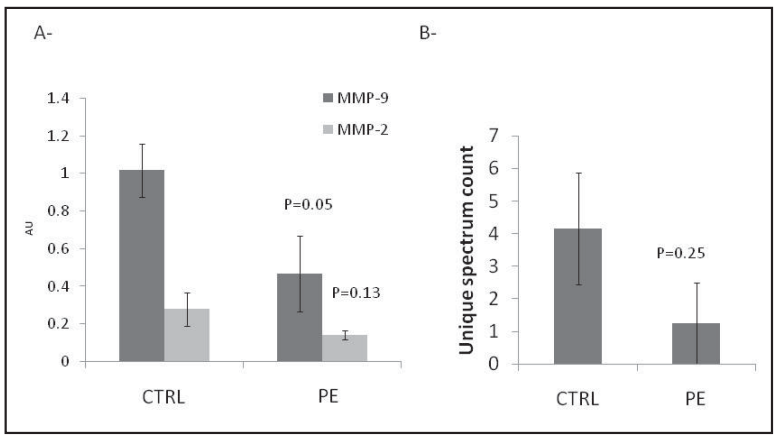

Fig. 2. Secretion of MMP-9 from CTB purified from control and PE placenta. **A** - zymography of culture supernatant of control (CTRL) and preeclamptic (PE) CTB; AU: absorbance unit; **B** - LC-MS/MS analysis of pooled supernatants of CTRL and PE CTB.

these studies tend to suggest that alteration of MMP-9 expression could be associated with the pathogenicity of PE.

In contrast, MMP-2 mRNA expression and activity are not altered in PE CTB. This result is in agreement with MMP-2 and -9 immunostaining studies showing no difference of MMP-2 protein expression and a decreased expression of MMP-9 protein in PE compared to CTRL placenta (Shokry *et al.* 2009). Although it is difficult to speculate on the first trimester events with results obtained from term CTB, the present data are in agreement with the hypothesis that MMP-9 could play a more important role in trophoblast invasion than MMP-2 (Hurskainen *et al.* 1996; Librach *et al.* 1994; Librach *et al.* 1991; Polette *et al.* 1994; Shimonovitz *et al.* 1994; Xu *et al.* 2000). Moreover they suggest that MMP-2 does not contribute to the pathogenesis of PE. However, several studies showed an increase of MMP-2 level in serum of PE patients compared to healthy pregnant women (Montagnana *et al.* 2009; Myers *et al.* 2005). These authors suggest that MMP-2 activity could play a major role in the endothelial dysfunction associated to the pathophysiology of PE. Nevertheless, another study failed to confirm this observation (Palei *et al.* 2008). The different methods used to measure MMP-2 protein in these papers could explain this discrepancy. Indeed, two studies of them have used zymography which is a qualitative and not a quantitative method for MMP-2 activity determination (Myers *et al.* 2005; Palei *et al.* 2008) whereas Montagnana *et al.* quantified circulating MMP-2 protein by ELISA (Montagnana *et al.* 2009). Since placental MMP-2 level seems to be identical in PE and CTRL patients, we suggest that the origin of the increase of circulating MMP-2 level in PE women is not placental.

Decreased mRNA expression of MMP-1, 7 and 12 in PE CTB corroborates the study of Lian et al even if the fold changes between expression of these different MMPs in PE and CTRL placentas differ (Lian *et al.* 2010). Decrease of MMP-12 mRNA expression was also confirmed by microarray analysis in first trimester placentas of women destinated to develop PE compared to CTRL trophoblast (Founds *et al.* 2009). Due to its capacity to degrade elastin, collagen IV, laminin, fibronectin, heparan sulfate, and vitronectin, it was recently shown that MMP-12 could be necessary for the vascular remodeling during pregnancy (Harris *et al.* 2010). Together, these studies tend to confirm the importance of MMP-12 in placentation and implicating MMP-12 as a potential therapeutic target.

At the exception of MMP-2, this study tends to confirm the importance of MMPs in development of PE. Nevertheless, this study was conducted on third trimester CTB, at the moment when cells are supposed to have lost their invasive capacity *in vivo*. Even if some of these results seem to corroborate with those obtained from first trimester CTB, they would have to be confirmed on first trimester CTB.

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