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Downregulated osteopontin in placenta affects MMPs expression of trophoblastic cells

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Summary

Trophoblastic invasion plays a critical role in placental development. Shallow of invasion could cause the abnormal placentation which may probably resulted in preeclampsia (PE). Extracellular matrix (ECM) proteins are confirmed to mediate cell adhesion, migration, and invasion. Osteopontin (OPN) is a kind of ECM protein and has been found to be involved in invasion and metastasis of several types of cancers. The authors hypothesize that OPN could probably regulate trophoblastic cell invasion. Here, the results showed that the expression level of OPN significantly decreased in the placenta of PE. Furthermore the authors found that OPN-siRNA interference significantly decreased the expression of MMP-2, MMP-9 in villous explant, a crucial signaling molecule of trophoblast invasion. The findings suggest that OPN is a critical ECM protein for regulating trophoblastic cell invasion.

Key words: Osteopontin; Trophoblast; Invasion; Villous explant.

Introduction

Pre-eclampsia (PE) is a pregnancy-specific disease characterized by development of hypertension and proteinuria after 20 weeks of gestation. It affects about 2-8% of all pregnancies and it is a major contributor of maternal mortality worldwide [1]. This disease originates in the placenta, probably due to an inadequate cytotrophoblastic invasion and vascular remodeling. As a consequence, the high-resistance, non-dilated vessels cannot carry sufficient blood supply to meet the demand of the fetus in the second and especially third trimester of pregnancy [2]. This reduced placental perfusion ends with widespread maternal endothelial dysfunction associated with changed expression of numerous placenta-derived growth and angiogenic factors, such as soluble vascular endothelial growth factor receptor 1 (sFlt-1), which cause the maternal symptoms described above [3].

Within this concert of factors leading to PE, the authors could show that OPN proteins seemed to play an important role. The OPN protein can integrate and modulate the signals of integrins [4], matrix metalloproteinases (MMPs) [5], and vascular endothelial growth factor [6, 7]. OPN plays a basic role in vascular remodeling [8] and angiogenesis [9] in atherosclerotic cardiovascular disease. Recently it has been shown that OPN knockout mice inhibited neovascularization. A comprehensive study on breast cancer patients showed that high OPN expression was associated with a higher risk of metastasis [10], indicating again that OPN is associated with cell migration and invasion.

In recent years trophoblast cell research has underlined the striking similarities between the proliferative, migratory, and invasive properties of placental cells and cancer cells [11-13]. Previous studies have revealed that the OPN protein is expressed in the human placenta in the invasive interstitial extravillous trophoblast (EVT) giant cells [14]. As it has been shown for cancer cells, the present authors assume that impaired trophoblast invasion might be associated with deregulated OPN expression because the OPN molecules are able to react to oxidative stress, a characteristic for the placental environment in pre-eclampsia [15, 16].

Here the authors decided to investigate if the OPN protein fulfill different functions in migration and invasion properties using villous explant as a model for the invasive trophoblast.

Materials and Methods

Ten placentas were obtained from PE and normal pregnancy, respectively. Immediately after C-section, chorionic tissue from the central part of the placenta was collected and contamination with maternal decidua and amniotic membranes was excluded by morphological observation. Tissues were rinsed by sterile PBS (s-PBS) three times and snap frozen in liquid nitrogen immediately and stored at –80°C until extraction of RNA and protein samples. For immunohistochemical staining, tissue samples were fixed immediately after removal in 10% neutral buffered [in phosphate-buffered saline (PBS), pH 7.0] formalin for 24 hours at room

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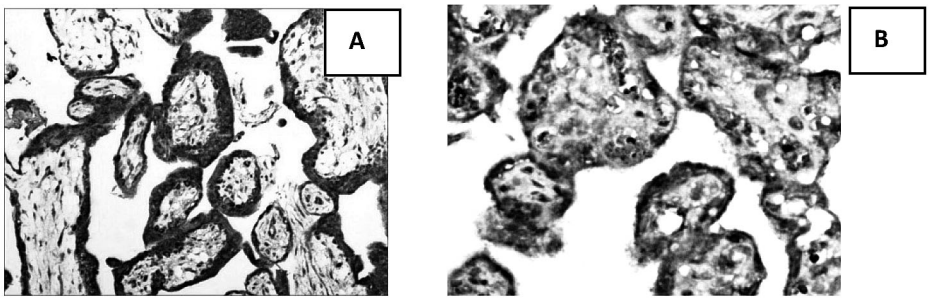

Figure 1. — Immunoreactivity for OPN on human placental tissues. Immunoreactivity for OPN on normal term placentas (A; ×400), preeclampsia placentas (B; ×400), The expression of OPN was found in cytoplasms of placental cytotrophoblasts and syncytiotrophoblasts in normal pregnancy, preeclamptic group.

temperature, dehydrated in a series of ethanol and xylene, and then embedded in paraffin.

Eight placentas (5–12 weeks) obtained after legal termination of pregnancy were washed in sterile PBS (s-PBS), and areas rich in chorionic villi were selected and minced between scalpel blades. Study subjects were enrolled following informed consent and approval, as required by the Institutional Review Board of The First Affiliated Hospital of Sun Yat-sen University.

Sections were cut at 5 μm and mounted onto poly-L-lysine coated glass slides. Immunohistochemistry was performed using the avidin–biotin complex immunoperoxidase method. The sections were incubated in a moist chamber for one hour at room temperature with goat anti-human OPN (1500). Slides were washed in PBS for 3× three–minute cycles and then incubated further with biotinylated rabbit-anti-goat secondary antibody in PBS (1200) for 45 minutes at room temperature. Following another 3× three–minute washes in PBS, slides were incubated with an avidin-biotin-peroxidase complex using a specific kit. After a 45-minute incubation at room temperature, the sections were rinsed three times in PBS. For color visualization of the primary antigen–antibody complex, peroxidase substrate solution, (DAB stain kit) was applied to all sections followed by incubation in a dark, moist chamber for five minutes. After rinsing in tap water, a light hematoxylin counterstain was applied and the sections were dehydrated through graded concentrations of ethanol and cleared through three changes of xylene. Mounting media and coverslips were applied and the sections were examined by light microscopy.

To evaluate OPN staining, the authors used a case of lung cancer tissue as a positive control. This case had been confirmed to show high expression of OPN. Sections from each sample of human placenta tissue were used as negative controls with the primary antibody replaced with Tris-buffered saline. Semi-quantitative analysis of immunohistochemistry of OPN expression was performed according to a published method [17]. Briefly, ten fields were selected randomly and expression in 1,000 cells (100 cells/field) were evaluated using high-power (×200) microscopy. Specifically, no positive cell was scored as 0, 1–10% of positive cells as 1, 11–50% as 2, 51–80% as 3, and 81–100% as 4. Staining intensity was rated on a scale of 0–3: 0=negative, 1=weak, 2=moderate, and 3=strong. The percentage of positive cell score was multiplied by the intensity score to obtain a final number for further statistical analysis.

Individual clusters of 8–12 week gestation villi (20–30 mg wet wt) were dissected in sterile cold PBS containing calcium and magnesium, under a microscope. The proximal stem villi were inserted into the underside of sterile polystyrene cubes so as to float the villous trees in 750 μl of serum-free media (DMEM) with 100 units/ml penicillin, 100 units/ml streptomycin, 2 mM L-glutamine, 100 μg/ml gentamycin, and 2.5 μg/ml fungizone. These explants were maintained in physiologic 8% ambient oxygen 5% CO2 at 37°C [18]. For siRNA treatment, floating villous explants were incubated in the presence of 100 nM siRNA or 100 nM non-silencing control for up to two days

Total RNA was isolated using RNA extraction kit and first-strand cDNA synthesis kit. Gene expression was quantified using the qPCR master mix for SYBR green and a sequence detection system with primer as follows: OPN: forward 5'- ACCTGCCG-GTACTAGACAGC-3' reverse 5'- GAACTTGGGT-GAGTGGGCAC-3', MMP-2: forward 5'-ACCTGCCGGTACTAGACAGC-3' reverse 5'- TGCG-GATATTCAAGGATGCA-3', MMP-9 forward 5'-CCTGACCTT-TATAGAA CTCA-3' reverse 5'- GGGCCAAAATGCTCGTCATT-3'. For a quantitative measurement, GAPDH was used as an internal control. The PCR reactions were carried out in triplicate in a final volume of 25 μl cDNA, 1 × reaction buffer containing SYBR green and 10 pmol forward and reverse primers. PCR was performed 40 cycles of PCR under the following conditions: initial enzyme activation and template denaturation for 10 minutes at 95 °C followed by 10 seconds at 95 °C, 20 seconds annealing at 60°C, and extension phase for 35 seconds at 72°C. Specificity of the amplification products was confirmed by melting curve analysis. The PCR fragments were also visualized on 2% ethidium bromide-stained agarose gels (not shown). The quantity of cDNA in each sample was normalized to the GAPDH content.

Protein extracts were prepared from cells by homogenization with modified RIPA lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 1% NP-40, 0.25% Na-deoxycholate, 1 mM EDTA, 0.1% SDS) supplemented with EDTA-free complete protease inhibitors. Protein content was determined using a rotiquant protein assay. Protein samples were separated on a 10% polyacrylamide gel and electrophoretically transferred to nitrocellulose membrane. For the analysis of protein expression, membranes were blocked with 5% non-fat dried milk in Tris-buffered saline (TBS) with 0.03% Tween-20 and then incubated with polyclonal goat anti-human OPN (1: 500). Primary antibody binding was detected using the following secondary antibodies: anti-goat IgG antibody conjugated to horseradish peroxidase (1:10000). Detection was achieved with the ECL chemiluminescence kit according to the protocol using X-ray films. Blots were stripped in a buffer containing 62.5 mM Tris–HCl, pH 6.8, 2% SDS, 100 mM b-mercaptoethanol) at 55°C and reprobed with mouse anti-human GAPDH antibody (1:1000) for normalization of protein expression.

OPN,MMP-2,MMP-9 released into the culture media of villous explant was collected and detected by using an ELISA kit according to the manufacturer's instructions.

The data were analyzed for statistical significance by the student's *t*-test with the program SPSS 16.0. Differences with a *p*-value <0.05 were regarded as statistically significant.

Results

Gestational week of delivery and birth weight in the PE group were less than those of the normal group (all $p <$

Table 1. — *Demographic data of study subjects*

Group	Maternal age (Year)	Gestation week	BMI	Birth weight (kg)	Systolic BP	Diastolic BP	Proteinuria (mg/24h)
Normal	31.2±2.8	38.5±0.7	23.7±1.5	3.3±1.2	123.6±10.2	67.4±12.4	186.6±80.2
Pre-eclampsia	30.9±3.4	33.4±2.1	22.6±3.2	2.4±0.7	156.6±20.8	97.8±16.6	2509.4±708.9
P	*NS*	*P<0.01 NS P<0.01 p<0.01 P<0.01 P<0.01*					*Abbreviation: BMI, body mass index.*

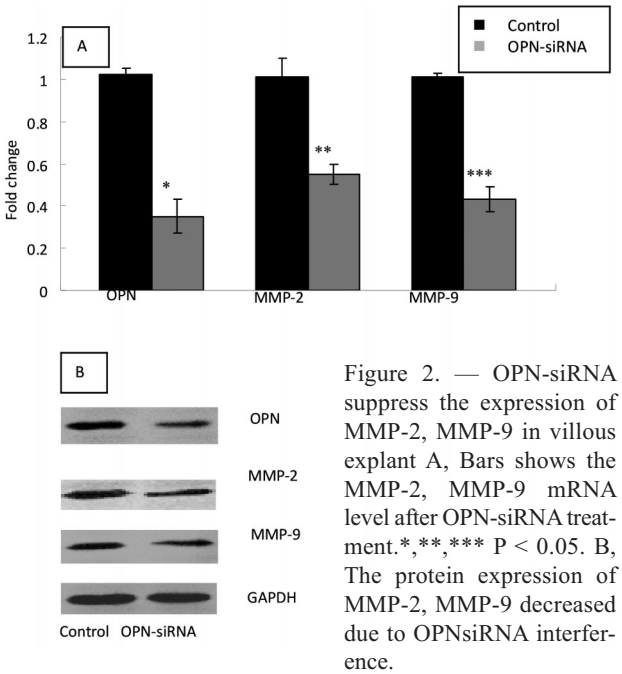

Figure 2. — OPN-siRNA suppress the expression of MMP-2, MMP-9 in villous explant A, Bars shows the MMP-2, MMP-9 mRNA level after OPN-siRNA treatment.*,**,*** P < 0.05. B, The protein expression of MMP-2, MMP-9 decreased due to OPNsiRNA interference.

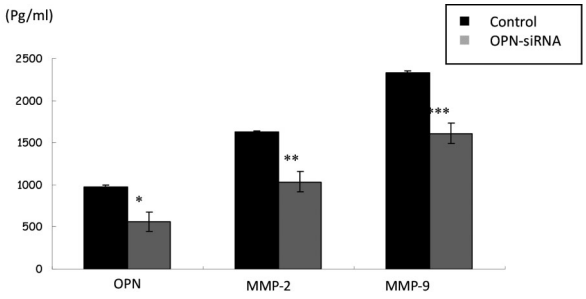

Figure 3. — OPN-siRNA decrease the generation of OPN, MMP-2, MMP-9 in culture media of villous explant.*,**,*** P < 0.05. The generation level of OPN, MMP-2, MMP-9 decreased due to OPN siRNA interference.

0.01). Blood pressure, proteinuria in the PE group were more than those of the normal group (all $p < 0.01$). There were no significant difference in maternal age and pre-pregnancy body mass index among two groups (all $p > 0.05$, Table 1).

Expressions of OPN protein in PE group and normal control group are shown in Figure 1 by Western blotting and in Figure 2 by immunohistochemistry. Strong expression of OPN protein was observed in normal group, while the expression of OPN protein remained low in PE group. There was a significant difference between the PE group and the normal control group ($p < 0.05$)

Expressions of OPN mRNA in PE group and normal control group are shown in Figure 3 by RT-PCR. The expression level of OPN mRNA was higher in normal group. There was a significant difference between the PE group and the normal control group ($p < 0.01$)

In order to explore the exact effect of OPN on invasion of villous explant, the authors examined MMP-2, MMP-9 expression level in the absence and in the presence of OPN-siRNA (Figure 2). The results showed that the mRNA and protein expression level of MMP-2, MMP-9 significantly decreased in the OPN-siRNA group compared with control.

The authors also examined OPN, MMP-2, MMP-9 generation level in the absence and in the presence of OPN-siRNA (Figure 3). The results showed that the generation level of OPN, MMP-2, MMP-9 significantly decreased in the OPN-siRNA group compared with control.

Discussion

The placental development and specific properties are crucial for further survival of the embryo [19]. In recent years trophoblastic cell research has underlined the striking similarities between the proliferative, migratory, and invasive properties of placental cells and cancer cells. There are several groups who have found that OPN could be a diagnostic marker for malignant tumors like ovarian cancer or breast cancer and have recently demonstrated the production of OPN mRNA in ovarian cancer cells [20, 21]. The human placenta is unique in its ability to proliferate and invade another tissue in a controlled fashion and is, thus, a very interesting model for the study of molecular mechanisms involved in these processes and for differentiating them from those implicated in tumor progression.

Several studies have defined OPN as an important glycoprotein with multiple functions and to play a role in basic cellular processes, such as neovascularization and tissue remodeling, which are essential to placental morphogenesis and embryo implantation [22-24]. From the present results, implications for the functional role of OPN at the maternal-fetal interface can be discussed. OPN could play a role in mediating cell adhesion at the maternal-fetal interface. Furthermore, several lines of evidence have implicated OPN in angiogenesis, and vascular endothelial growth fac-

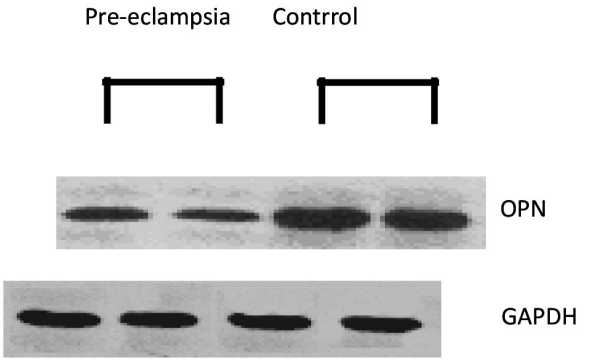

Figure 4. — Western Blotting analysis result of OPN protein expressions in different groups, There was significant decreased exression of OPN in preeclamptic placenta.

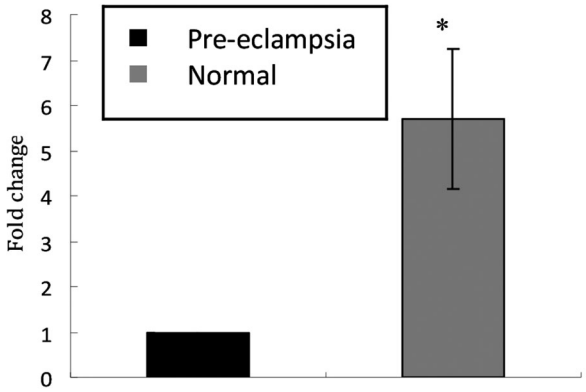

Figure 5. — Real-time quantitative PCR analysis of OPN mRNA expression in the placenta of pre-eclampsia compared with control. There were approximately 6- fold decreases (*$p < 0.01$).

tor may induce expression of OPN in endothelial cells [25-27]. Apparao *et al*. [28] demonstrated the coexpression of OPN and αvβ3 integrin in the human endometrium during menstrual cycle. Using the Ishikawa cell line, they demonstrated a different regulation for both. OPN was regulated through progesterone, whereas αvβ3 integrin was modulated through growth factor epidermal growth factor. Omigbodun *et al*. [29] found that the secretion of progesterone by trophoblast regulates the expression of OPN. Specific expression of the receptor (CEACAM1) of OPN implicates a possible role in regulating the normal processes taking place at the maternal-fetal interface during implantation and placentation [30, 31].

In the present study, the authors investigated for the expression pattern of the OPN in the human placenta of eclampsia. As shown by immunohistochemistry, all placentas presented expression of OPN in the trophoblast cells. Strong expression was observed in trophoblast (Figure 1). The authors also demonstrated the expression of OPN significantly decreased in the placenta of PE. OPN expression in placentas was confirmed by Western-blot analysis and RT-PCR (Figures 4 and 5).

Previous studies have investigated the expression pattern of OPN in gestational trophoblastic diseases (GTDs) [32]. GTDs (including hydatidiform moles, choriocarcinomas, placental site trophoblastic tumor, and placental site nodule) are the result of pathological placental development and are associated with abnormal proliferation and/or invasion of trophoblast [33]. It was found that in hydatidiform moles, OPN is highly expressed in the villous trophoblast proliferations on the villous surface [32]. Also the implantation site showed a positive nuclear staining as did the invasion site in three cases of an invasive mole.

To investigate a potential functional link between OPN and trophoblastic invasion, villous explant was transfected with OPN-siRNA and shown decreased expression of MMP-2 and MMP9 (Figures 4 and 5), indicating that OPN potentially enhance invasiveness of trophoblast cells. In PE placental tissues, MMP-2 and/or MMP-9 proteolytic activity of trophoblasts were found to be defective [34, 35], which is hypothesized to induce shallow invasion of the trophoblast cells. A role for both MMPs in trophoblast invasion has been proved in rats where inhibition of MMP activity could indeed impair trophoblast invasion with subsequent changes in vascular remodeling and placental perfusion [36]. Using an *in vitro* model of BeWo cells transfected with OPN-siRNA, the present authors have now demonstrated decreased cellular invasiveness and the expression level of MMP-2, MMP-9 after OPN-siRNA interference, indicating a probable function of OPN in regulating trophoblastic invasion via modulating MMP-2 and MMP-9 expression.

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