ORIGINAL ARTICLE

Cytokines, proteases, and ligands of receptor for advanced glycation endproducts (RAGE) released by primary trophoblasts from human term placenta under hypoxic stimulation

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Key words:

danger signal, placental hypoxia,
preeclampsia, protease, RAGE
ligand

Received: February 8, 2013

Revised: June 25, 2013

Accepted: August 24, 2013

Aim: In the placenta, hypoxia followed by reoxygenation (ischemia and reperfusion) is regarded as a trigger for the pathological onset of preeclampsia. In this study, we isolated primary trophoblasts from human term placenta, exposed them to hypoxic stress, and measured subsequent levels of cytokines, proteases, protease inhibitors, and ligands of receptor for advanced glycation endproducts (RAGE) to identify the trigger molecule released from hypoxic placenta into maternal circulation.

Methods: Ten placental samples were taken from healthy elective caesarean section patients. Trophoblasts were isolated using a Percoll-based method and cultured under 20% oxygen with or without 3 rounds of 0.1% hypoxic stimulation for 1 h.

Results: Cell viability did not differ between normal and hypoxic cultures (MTT assay), but cell injury was attenuated in hypoxic culture (LDH assay). In cell culture supernatants, concentrations of MMP-2, TIMP-2, IL-6, and IL-10 decreased in hypoxia compared to normal culture, while the RAGE ligand HMGB1 increased significantly in hypoxic culture. There were no significant differences in concentrations of MMP-9, TIMP-1, IL-8, or S100A12.

Conclusions: These results suggest that human term placenta under hypoxia and reperfusion releases the RAGE ligand HMGB1, a “danger signal”, that can trigger systemic inflammation and lead to preeclampsia or placenta-based complications in pregnancy.

Introduction

Hypoxia and possibly reoxygenation in placental tissue are regarded as triggers for preeclampsia.^{1,2)} Incomplete remodeling of the uterine spiral artery during the first trimester of pregnancy, a common pathological feature of preeclampsia,³⁾ leads to poor perfusion and hypoxia of the placenta after fetal growth later in pregnancy (the two-stage theory).²⁾ Also observed in preeclampsia are hypercoagulation⁴⁾ and/or vascular endothelial dysfunction,^{5,6)} which can cause systemic features and placental ischemia. Humoral factors released from hypoxic placenta have been studied elsewhere,^{7–10)} but

the exact trigger molecule released under hypoxic stress that induces preeclampsia has not been identified.

Ligands of receptor for advanced glycation endproducts (RAGE), also referred to as “danger signals”, induce activation of various intracellular pathways, especially those that mediate inflammatory responses.^{11,12)} After reviewing the literature, we hypothesized that the inflammatory response induced in normal pregnancy, preeclampsia, and preterm birth may be regulated by changes in RAGE ligands.^{13,14)} We subsequently reported increased serum levels of two soluble RAGE ligands in preeclampsia patients, high-mobility group box 1 (HMGB1) and S100/calgranulin family member

S100A12 (also known as extracellular newly identified RAGE-binding protein).¹⁵⁾ HMGB1 expression has also been reported in human term placenta.^{16,17)} In preeclamptic patients, HMGB1 expression tends to increase in the placenta¹⁷⁾ or decidua.¹⁶⁾ However, the effect of hypoxic stress, the most common change to occur in the placenta before preeclampsia, has not been studied as it relates to the production of RAGE ligands.

In this study, we isolated human primary trophoblast cells from placentas at term using an established method. We then cultured the cells under hypoxic stress to reproduce production of humoral factors in preeclampsia and investigated the release of cytokines, proteases, and RAGE ligands. Our purpose was to understand the pathophysiology of preeclampsia by gaining new insight into placental hypoxia and the production of danger signals.

Materials and methods

Sample collection

The study was approved by the Local Ethics Committee at Nara Medical University, and all participants provided written informed consent. At 38 weeks of gestation measured from the last menstrual period, with correction by detection of fetal heart beat or measurement of crown-rump length with ultrasound examination, 10 placental samples were obtained from women undergoing elective caesarean section because of breech presentation of the fetus or a previous caesarean section. After sterile collection of a healthy section without infarction or necrosis, the placental tissue was immediately suspended in cold sterile saline, transported to the laboratory, and washed several times in sterile phosphate buffered saline to remove excess blood.

Trophoblast isolation

Trophoblast cells were isolated from placental tissue using our previously published method^{18–20)} with some modifications. In brief, placental chorionic villi were washed in Hank's balanced salt solution (HBSS; Sigma-Aldrich Co., St. Louis, MO), and villous material was dissected, minced, and digested for 35 min at 37°C in 0.25% trypsin (Invitrogen Co., Carlsbad, CA) and 0.5 mg DNase I (Sigma-Aldrich Co.) twice without stirring and a third time with agitation. Supernatants were collected at the end of the third digestion. After centrifugation, cell pellets were resuspended in HBSS, spread onto a 5% step-layer Percoll gradient (Sigma-Aldrich Co.), and centrifuged (1,200×g, 30 min). The layer containing trophoblast cells (35%–45% Percoll) was collected, supplemented with complete culture medium (DMEM-F12 containing 10% fetal bovine serum, 1,000 U/ml penicillin, 1 mg/ml streptomycin, and 1.5 mg/ml

amphotericin B, from Sigma-Aldrich Co.), centrifuged, and resuspended in complete medium.

Cell culture

Trophoblasts ($3.0–5.0 \times 10^6$ cells/500 μ l of medium/well) were plated in a 24-well plate coated with fibronectin (Sigma-Aldrich Co.). Cells were first cultured for 12 h in a 5% CO₂/20% O₂ incubator at 37°C for attachment of trophoblasts to the plate. Cells were then cultured for 24 h at 20% O₂ (normal conditions) or given 3 rounds of 0.1% hypoxic stimulation for 1 h using AnaeroPack (Mitsubishi Gas Chemical Co., Japan) with 1 h of 20% O₂ reoxygenation between stimulations. Cell culture supernatants were collected, centrifuged, and stored immediately at –80°C until analysis. Cell viability was evaluated by the MTT assay ($n=3$; Chemicon International, Inc., Billerica, MA; $1.0–3.0 \times 10^4$ cells/100 μ l of medium/well on a 96-well plate), and cell injury was assessed by the lactose dehydrogenase (LDH) cytotoxicity assay ($n=5$; Cayman Chemical Co., Ann Arbor, MI). The phenotype and purity of cell cultures were assessed by immunocytochemistry for cytokeratin 7 at the end of the culture period, as previously described.¹⁸⁾

Concentrations of cytokines, proteases, protease inhibitors, and RAGE ligands in cell culture supernatants

Concentrations of matrix metalloproteinase (MMP)-2, MMP-9, tissue inhibitor of MMP (TIMP)-1, TIMP-2, interleukin (IL)-6, IL-10, and IL-8 were measured in duplicate using the Searchlight multiplex ELISA assay (currently Ciraplex™ assay, $n=5$; Aushon BioSystems, Billerica, MA). Concentrations of HMGB1 ($n=10$; Shino-Test Co., Kanagawa, Japan) and S100A12 ($n=10$; CircuLex™ ELISA kit, CycLexCo., Ltd., Nagano, Japan) were measured in duplicate using a quantitative sandwich ELISA. Results were within the coefficient of variations provided by the suppliers.

Statistical analysis

Data are presented as the ratio of hypoxia values to normal values, as well as concentration (mean $\pm$ standard error). Statistical calculations were performed using SPSS 15.0 J (SPSS Japan Inc., Japan). Pairwise comparisons were made between normal and hypoxic placental trophoblast cultures, and analyses were performed using the Wilcoxon signed-rank test, with $P<0.05$ indicating a statistically significant difference.

Results

Immunocytochemistry

Trophoblasts in culture and immunocytochemistry for

Figure 1. Representative photomicrographs of trophoblasts isolated from the same sample (at 38 weeks of gestation) after 36 hours of culture under normal oxygen conditions.

A), B) Phase contrast photomicrograph of trophoblasts in culture. C), D) Trophoblasts immunostained for cytokeratin 7. Original magnification: $\times 100$ for A) and C), and $\times 400$ for B) and D).

cytokeratin 7 are shown in Figure 1. Isolated trophoblasts attached to the surface of culture plates coated with fibronectin (Figure 1A, 1B) were immunopositive for cytokeratin 7 (Figure 1C, 1D). Purity was consistently $> 95\%$ at the end of each culture period, with and without hypoxic stimulation.

Cell viability and injury

As assessed by the MTT assay, cell viability in hypoxic culture tended to be higher than in normal culture, but the difference was not statistically significant (1.35 ± 0.14 fold increase, Figure 2). On the other hand, the LDH cytotoxicity assay showed that cell injury under hypoxia was significantly lower than under normal conditions (0.34 ± 0.37 fold decrease, $P = 0.039$, Figure 2).

Searchlight multiplex ELISA assay

Supernatant concentrations of MMP-2 (normal, 3.53 ± 0.85 ng/ml; hypoxia, 2.04 ± 0.54 ng/ml), TIMP-2 (normal, 6.93 ± 2.04 ng/ml; hypoxia, 3.42 ± 0.76 ng/ml), IL-6 (normal, 47.00 ± 10.84 pg/ml; hypoxia, 29.04 ± 6.66

Figure 2. Cell viability measured by the MTT assay (MTT, $n = 3$) and cell injury measured by the lactose dehydrogenase cytotoxicity assay (LDH, $n = 5$) for trophoblast cultures.

Data are expressed relative to the values of normal culture (mean $\pm$ SEM). * $P < 0.05$ by paired analysis.

Figure 3. Concentrations of proteases, inhibitors, and cytokines in cell culture supernatant.

Values are expressed relative to the concentrations of normal culture (mean ± SEM). * $P < 0.05$ by paired analysis.

Figure 4. Concentrations of ligands of receptors for advanced glycation endproducts in cell culture supernatants.

Values are expressed relative to the concentrations of normal culture (mean ± SEM). * $P < 0.05$ by paired analysis.

pg/ml), and IL-10 (normal, 2.60 ± 0.59 pg/ml; hypoxia, 1.52 ± 0.23 pg/ml) were significantly lower in hypoxia than in normal conditions (fold decreases: MMP-2, 0.57 ± 0.04 , $P = 0.044$; TIMP-2, 0.62 ± 0.13 , $P = 0.044$; IL-6, 0.62 ± 0.02 , $P = 0.044$; IL-10, 0.66 ± 0.09 , $P = 0.047$; Figure 3). There were no significant differences in concentrations of MMP-9 (normal, 50.28 ± 7.33 ng/ml; hypoxia, 44.02 ± 18.36 ng/ml), TIMP-1 (normal, 3.75 ± 0.53 ng/ml; hypoxia, 4.80 ± 1.12 ng/ml), and IL-8 (normal, 18.16 ± 5.72 ng/ml; hypoxia, 15.70 ± 2.23 ng/ml) between normal and hypoxic cultures (Figure 3).

RAGE ligands

The concentration of S100A12 (normal, 24.80 ± 5.10 ng/ml; hypoxia, 23.96 ± 4.45 ng/ml) did not differ significantly between normal and hypoxic cultures (Figure 4). However, the concentration of HMGB1 was significantly higher in hypoxia (3.93 ± 0.88 ng/ml) than in normal conditions (2.57 ± 0.47 ng/ml; 1.46 ± 0.19 fold increase in hypoxia, $P = 0.0069$; Figure 4).

Discussion

This study identified three significant alterations in the concentrations of humoral factors released by trophoblasts from human term placenta under hypoxic stimulation. First, levels of the protease MMP-2 and its inhibitor TIMP-2, which localized more strongly in the placental bed than the systemic MMP-9,^{21,22} decreased in hypoxia. Second, concentrations of the major inflammation-related cytokines IL-6 and IL-10²³ also decreased in hypoxic culture supernatant. Finally, HMGB1, a molecule recently described as a transducer of the innate immune system via its function as a danger signal,²⁴ increased under hypoxic stress.

MMP-2 and MMP-9 are considered key enzymes in the degradation of basement membranes,²⁵ and their activities in term trophoblast cells have been previously demonstrated.²⁶ TIMP-2 is a dominant inhibitor of MMP-2 but can also activate pro-MMP-2 by binding to membrane type-1-MMP.²⁷ In hypoxic conditions, MMP-2 activity has been found to decrease in invasive trophoblast cells from early human pregnancy,²⁰ even though MMP-2 and other proteases are regarded as target molecules of hypoxia-induced factor (HIF) in placental development.²⁸ Although gelatinase activity was not measured in the current study, reduced activity of the MMP-2/TIMP-2 pathway under hypoxic stress may

occur also in term placental trophoblasts. Additionally, an increase in activity of the MMP system in term placenta was reported in maternal type-2 diabetes mellitus.²⁹⁾ Decreased MMP-2 and TIMP-2 levels in hypoxic conditions may relate to a loss of placental maintenance function, but its contribution to the pathology of pregnancy complications remains unclear.

IL-6 has been classified as a pro-inflammatory cytokine, but recent studies also demonstrated its ability to exert anti-inflammatory effects by increasing IL-10 and soluble TNF-receptors.^{23,30)} This functional relationship was recently demonstrated in the central nervous system.³¹⁾ In term placenta, IL-10 suppressed toll-like receptor (TLR) and the inflammatory action of its ligands.³²⁾ In the same study, IL-6 was induced by TLR ligands as a pro-inflammatory cytokine. In another study, IL-6 and IL-10 production was higher in preeclamptic placenta than in healthy placenta, and a hypoxic culture of the preeclamptic placenta showed an increase in IL-6 and a decrease in IL-10.³³⁾ Perhaps because of the purity of trophoblasts isolated using 5%-step layer Percoll and a different study design, our results differed in some respects from these reports. For example, IL-10 concentrations measured in this study were lower but within measurable limits. Additionally, human placenta physically exists in 5 to 10% O₂ conditions. Primary trophoblasts may thus be stressed oxidatively in 20% O₂ conditions, which suggests that low oxygen culture does not increase pro-inflammatory cytokines. Further research that includes HIF will be needed to establish the functions of IL-6 and IL-10 under hypoxic conditions.

RAGE was first identified as the receptor for advanced glycation endproducts in the inflammatory pathology of hyperglycemic patients.¹¹⁾ A wide range of other endogenous ligands, called RAGE ligands, has been identified with similar inflammatory actions¹²⁾ and include members of the S100 protein family³⁴⁾ and HMGB1.²⁴⁾ HMGB1 in particular is known as the initiator of acute inflammation, chronic cellular dysfunction, and tissue destruction.¹¹⁾ In preeclampsia, the expression of RAGE increased in placental cells, a change that may reflect oxidative stress.³⁵⁾ Little research on RAGE ligands in normal or complicated placenta has been reported, but some studies have shown a tendency towards higher HMGB1 expression in the preeclamptic decidua.^{16,17)} A significantly high expression of S100A12 was found in the amniotic fluid of women with chorioamnionitis, with S100A12 levels correlated to the degree of inflammation.³⁶⁾ However, expression of S100A12 in villous trophoblasts was not found.³⁶⁾ In the current study, we found a notably high concentration of S100A12 in the trophoblast culture supernatant, but its localization should be defined by further research. Furthermore, a pathway on trophoblasts to increase S100A12 under hypoxic

conditions remains unclear.

There is no established evidence that HMGB1 contributes to the pathology of preeclampsia, although some publications, including our own,^{15,16)} suggest a relationship between HMGB1 and preeclampsia.¹³⁾ As expected from previously reported serum concentrations,¹⁵⁾ our current study strongly suggests that hypoxia and reoxygenation in the preeclamptic placenta contributes to systemic release of the danger signal HMGB1. Cellular debris (microparticles) from syncytiotrophoblasts is regarded as a possible mediator of the systemic inflammation that connects placental failure in the first trimester to later onset of preeclampsia; HMGB1 and S100 family members are considered candidate functional surface antigen molecules on the microparticles.³⁷⁾ The timing of debris release might be different in mechanisms underlying early placental failure and late placental hypoxic shock, but the link between the two can be inferred from the difference in HMGB1 levels, since a recent report showed the release of microparticles from normal trophoblasts under hypoxic conditions.³⁸⁾

The appropriate oxygen tension to mimic healthy or preeclamptic placenta remains controversial. We regard 5% oxygen as the “normal” condition for trophoblasts in the first trimester,²⁰⁾ but used 20% oxygen in this study because the oxygen concentration might be higher in term placenta, and it may change dramatically with complications in pregnancy and during the course of labor. We cannot exclude the possibility that our culture conditions subjected trophoblasts to hyperoxidative stress, since cell injury shown by the LDH assay decreased with hypoxic stimulation. Moreover, a recent study using near-infrared spectroscopy suggested that oxygen tension is higher in the preeclamptic placenta because of poor oxygen exchange.³⁹⁾ Reoxygenation rather than hypoxia may thus have an effect on the onset of disease locally or systemically.

In conclusion, our study shows that the release of MMP-2, TIMP-2, IL-6, IL-10, and HMGB1 by trophoblasts purified from term placenta is altered under hypoxic conditions. These findings could contribute to understanding the pathology of preeclampsia and lead to further improvements in the diagnosis and treatment of this disease.

Acknowledgements

Part of this research was presented at the Award Lecture of the Japan Society for the Study of Hypertension in Pregnancy, Fukushima, 2008. The authors dedicate this study to the memory of Professor Akira Sato.

This research was supported by KAKENHI (Japan Society for the Promotion of Science Grants-in-Aid Nos.

21791571 and 24659735) and the Mitsui Life Social Welfare Foundation.

Disclosure

The authors declare that they have no conflict of interests.

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