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Systemic inflammatory stimulation by microparticles derived from hypoxic trophoblast as a model for inflammatory response in preeclampsia

Seung Mi Lee, MD, PhD^{1,2}, Roberto Romero, MD³, You Jeong Lee, MD, PhD⁴, In Sook Park, MT¹, Chan-Wook Park, MD, PhD¹, and Bo Hyun Yoon, MD, PhD¹

¹Department of Obstetrics and Gynecology, Seoul National University College of Medicine, Seoul, Korea

²Department of Obstetrics and Gynecology, Seoul Metropolitan Government Seoul National University Boramae Medical Center, Seoul, Korea

³NICHD, NIH, DHHS, Perinatology Research Branch, Detroit, MI

⁴Department of Pathology, Seoul National University College of Medicine, Seoul, Korea

Abstract

OBJECTIVE—To determine whether trophoblast-derived microparticles can induce different inflammatory responses of the peripheral blood mononuclear cells (PBMCs) depending upon the state of trophoblast when the microparticles are generated.

STUDY DESIGN—A trophoblast-derived cell line (ATCC No. CRL-1584) was cultured under normal or hypoxic conditions. Microparticles were isolated from the cell culture supernatants (MP_C: microparticles from normal trophoblast; MP_H: microparticles from hypoxic trophoblast). PBMCs were cultured alone or co-cultured with either MP_C or MP_H.

RESULTS—1) After 48 hours, the PBMCs co-cultured with MP_C released higher concentrations of IL-6 than PBMCs cultured alone; 2) The PBMCs co-cultured with MP_H showed higher concentration of IL-6 and TNF-α than PBMCs co-cultured with MP_C, after 24 hours and 48 hours.

CONCLUSION—More intense and rapid inflammatory response of PBMCs was observed with MP_H than with MP_C. This difference might explain the exaggerated systemic inflammatory response as a result of placental hypoxia in preeclampsia.

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Correspondence to: Seung Mi Lee, MD, PhD, Department of Obstetrics and Gynecology, Seoul Metropolitan Government Seoul National University Boramae Medical Center, Seoul, 156-707, Korea. Tel: 82-2-870-2346, Fax: 82-2-831-2826, lism@paran.com.

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Keywords

preeclampsia; microparticles; hypoxia; trophoblast; inflammation

Introduction

Microparticles are small membrane vesicles, which originate from blebbing membranes of either activated cells or cells undergoing apoptosis^{1, 2}. The syncytiotrophoblast forms the maternal-placental interface, and it has been suggested that microparticles from this cell (syncytiotrophoblast microparticles, STBMs) may be the factor which disturbs the maternal endothelium and mediates the inflammatory response^{3, 4}, which is the characteristic feature in preeclampsia^{5, 6}.

However, microparticles also showed immunosuppressive characteristics, and this immunoregulatory function of microparticles has been thought as the explanation of immune tolerance in normal pregnancy^{7–10}. The mechanism of this different action of microparticles (i.e. pro-inflammatory vs. immune-regulatory) in preeclampsia and normal pregnancy is not well determined.

Preeclampsia is initiated by reduced uteroplacental blood flow, resulting in placental hypoxia¹¹, and the experimental evidences and histological feature of placenta in preeclampsia supports this theory^{12, 13}. During normal placentation, the maternal spiral arteries are invaded by trophoblast, resulting in extensive remodeling of spiral arteries to ensure an adequate blood supply to the placenta and fetus. However, placentas of preeclamptic show shallow trophoblast invasion and defects in spiral artery remodeling, resulting in poor perfusion and ischemia^{14–19}. As a result of placental ischemia, it is believed that placenta release a number of soluble factors into maternal circulation, eliciting many of the symptoms of preeclampsia²⁰. In this context, our hypothesis was that the trophoblast in a different condition (i.e. normal vs. hypoxic; which represent normal pregnancy vs. preeclampsia) may shed microparticles which have different characteristics²¹, resulting in a different systemic inflammatory response to these microparticles.

To this end, we undertook this study to determine whether trophoblast-derived microparticles can induce different inflammatory response, according to the condition of the trophoblast.

Material and Methods

Cell culture

A trophoblast-derived cell line (ATCC No. CRL-1584), which has been used as the model for placenta in previous studies^{22–25}, was obtained from the Global Bioresource Center™ (<http://www.atcc.org>) and cultured in 10% MEM media (modified Eagle's medium, GIBCO, USA; with 10% fetal bovine serum and antibiotics). The cultures were maintained at 37°C in an atmosphere of 5% of CO₂.

Induction of hypoxic cell death

Two million trophoblast cells were cultured either in normal or hypoxic conditions. For normal condition, the cultures were maintained in an atmosphere of 5% of CO₂. For hypoxic condition, cells were cultured in the presence of 100μmol/L rotenone (Sigma-Aldrich, St Louis, MO), which induces chemical hypoxia by disruption of the mitochondrial electron transport chain²⁶. To confirm the process of apoptotic cell death after the addition of rotenone, floating cells were collected and adherent cells were detached with trypsin/EDTA, and the cells were pooled with centrifugation (300 × g, at room temperature, for 5 minutes), and were analyzed with flow cytometric analysis (FACS) using the FITC Annexin V Apoptosis Detection kit (BD Biosciences). For assessment, cells were resuspended in 100ul of Binding Buffer at a concentration of 1×10^6 cells/ml, and 5 uL of FITC Annexin V and 5 ul of PI was added. After incubation for 15 minutes at room temperature in the dark, 400ul of Binding Buffer was added and the analysis was performed using FACSCalibur (Becton Dickinson, CA, USA) and the data were evaluated with CellQuest software (Becton Dickinson). In the presence of rotenone, 15 to 40% of the trophoblast cells were stained with Annexin V after 24 hours of culture, and 50 to 80% of cells were stained with Annexin V after 48 hours of culture, although less than 15% of the trophoblast cells were Annexin V positive after 24 and 48 hours of culture in the normal condition (i.e. in the absence of rotenone).

Isolation and quantification of microparticles

After 24 hours of culture in normal or hypoxic condition, the supernatants were collected and microparticles were isolated by differential centrifugation. First, cell culture supernatants were separated from detached cells by two centrifugation steps (300 × g, 5 minutes and 800 × g, 5 minutes)²⁶. Then, the cell-free supernatants were ultra-centrifuged at 100,000 × g at 4°C for 90 minutes. After the removal of the supernatants, the pellet was washed twice with PBS and finally suspended in 10% MEM media.

The number of microparticles was counted by FACS, and adjusted to the concentration of trophoblast cells²⁷. Trophoblast cells were counted with a hemocytometer and 2×10^6 cells/ml of trophoblast cells were used for the experiment. In brief, the number of each microparticles and trophoblast cells were counted for 30 seconds at the “low-flow” modus and the concentration of microparticles was calculated by the following formula, then the concentration of microparticles was adjusted to standard concentration for the following experiment.

$$\text{The concentration of microparticles} = (\text{microparticles count/trophoblast cells count}) \times (2 \times 10^6) \text{ (counts/ml)}$$

Co-culture of peripheral blood mononuclear cells (PBMCs) with microparticles

Peripheral venous blood (40ml) was taken from healthy non-pregnant women (n=4). PBMCs were isolated by density gradient centrifugation using the Ficoll method (Sigma-Aldrich HISTOPAQUE®-1077). The final pellet was re-suspended in 10% MEM, and 2×10^6 cells/ml, 0.2ml aliquots were placed in a 96-well plate.

The PBMCs were cultured alone, or co-cultured with microparticles derived from the trophoblast in normal condition (MP_C, $5 \times 10^6/\text{ml}$), or co-cultured with microparticles derived from the trophoblast in hypoxic condition (MP_H, $5 \times 10^6/\text{ml}$), in the presence or absence of T cell mitogen, phytohemagglutinin (PHA, 5 $\mu\text{g}/\text{ml}$) up to 48 hours. Each experiment was done in duplicate. After either 24 or 48 hours of culture, cellular proliferation was measured with the CCK-8 (Cell Counting Kit-8), and the co-culture supernatants were obtained and assayed for interleukin-6 and TNF- α using the commercial ELISA kit (R&D Systems, Minneapolis).

The Institutional Review Board of the Seoul National University Hospital approved this study. The Seoul National University has a Federal Wide Assurance (FWA) with the Office for Human Research Protections (OHRP) of the Department of Health and Human Services (DHHS) of the United States.

Statistical analysis

Comparisons of continuous variables between groups were performed with the Mann-Whitney U test. A p-value <0.05 was considered as statistically significant.

Results

PBMCs co-cultured with microparticles from trophoblast in normal condition

After 24 hours of co-culture, the cytokine (both IL-6 and TNF- α) production and cellular proliferation was not different between PBMCs cultured alone and PBMCs cocultured with MP_C. This was the same in the addition of PHA (Figures 1 and 2). However, after 48 hours of co-culture, the supernatant of PBMCs co-cultured with MP_C had significantly higher concentrations of IL-6 than that of PBMCs cultured alone (Figure 1). Although the supernatant TNF- α concentration of PBMCs co-culture with MP_C was also higher than that of PBMCs cultured alone, this difference did not reach statistical significance. After 48 hours, the cellular proliferation of PBMCs was also increased when co-cultured with MP_C than when cultured alone (Figure 2). In the presence of PHA, the supernatant IL-6 concentration of PBMCs co-cultured with MP_C after 48 hours was also higher, although the cellular proliferation was not increased (Figures 1 and 2).

PBMCs co-cultured with MP_C or MP_H

The inflammatory response of PBMCs was compared according to the type of microparticles. The supernatant IL-6 and TNF- α concentration of PBMCs co-cultured with MP_H was significantly higher than that of PBMCs co-culture with MP_C, after both 24 and 48 hours of culture (Figure 3). In the presence of PHA, PBMCs co-cultured with MP_H also showed more intense proinflammatory response. The cellular proliferation was not different between PBMCs cocultured with MP_C and MP_H (Figure 4).

When cultured alone for 24 or 48 hours, neither MP_C nor MP_H in the absence of PBMCs produced any measurable amounts of IL-6 or TNF- α , both in the absence or presence of PHA (data not shown).

Comment

The principal findings of this study

1) The PBMCs co-cultured with MP_C released higher concentrations of IL-6 and showed higher cellular proliferation than PBMCs cultured, alone after 48 hours of culture; 2) The PBMCs co-cultured with MP_H showed more intense inflammatory response (higher concentration of IL-6 and TNF- α) than PBMCs co-cultured with PM_C, and this higher inflammatory response appeared both after 24 hours and 48 hours of co-culture; 3) The cellular proliferation was not different between PBMCs co-cultured with MP_C and MP_H.

Preeclampsia and maternal inflammatory response

The proposed pathogenesis in preeclampsia is the maternal systemic inflammatory response and endothelial activation. It is well known that poor placentation plays an important role in the initiation of this inflammatory process, and it has been postulated that some placental factors are differently released from preeclamptic placenta into maternal circulation²⁰. In this context, several bioactive factors from the syncytiotrophoblast have been shown to be altered in preeclampsia²⁸. These pro-inflammatory placental factors include sFlt-1²⁹, endoglin³⁰, placental growth factor³¹, corticotropin releasing hormone³², Activin-A³³, Inhibin A³³, Leptin³⁴, and trophoblast microparticles⁷.

Microparticles in preeclampsia

The finding of the current study indicates that different inflammatory response between microparticles from normal trophoblast and microparticles from hypoxic trophoblast may be the mechanism for the difference between normal pregnancy and preeclampsia. Microparticles from normal trophoblast are pro-inflammatory, but microparticles from hypoxic trophoblast provoke more intense inflammatory response.

This is consistent with the previous suggestions, that normal pregnancy is characterized by a mild systemic inflammatory state, and preeclampsia is associated with more exaggerated systemic inflammatory change^{35–37}. In addition, microparticles from syncytiotrophoblast (STBMs) forming the maternal-placental interface have been thought as candidate for this pro-inflammatory placental factor. Germain et al suggested that STBMs are potential contributors to altered systemic inflammatory responsiveness in pregnancy and preeclampsia, because the inflammatory priming of PBMCs during pregnancy and more enhanced inflammatory response in preeclampsia were confirmed, and STBMs prepared by perfusion of normal placental lobules stimulated production of inflammatory cytokines in PBMCs³⁷. In the study of von Dadelszen et al., endothelial cells co-cultured with STBM (prepared from placenta of normal pregnant women) caused significant activation of peripheral blood leukocytes, in terms of increase in intracellular free ionized calcium, falls in intracellular pH, and release of reactive oxygen species, also suggesting that STBM could activate leukocytes in preeclampsia via endothelial cell activation³. In the study of other investigators³⁸, placental perfusion-derived STBM also significantly induced T cell proliferation and increase in IFN γ release.

But all the above studies did not compare the different stimulatory effect of STBMs between from normal pregnancy and from preeclampsia^{4, 21}. Aly et al examined this difference⁴, and showed that STBMs isolated from preeclamptic women induced significant greater superoxide production by neutrophils, than STBMs from normal pregnant women. The finding of the current study further indicates that this difference of STBMs from preeclamptic women and normal pregnant women may be attributed to the different characteristics of the trophoblast (normal vs. hypoxic).

In contrast to the current study, other mechanisms for the difference of STBMs between preeclampsia and normal pregnancy have been suggested by other investigators. In the study of Gupta et al., STBMs prepared by different methods (mechanical dissection, villous explants culture, and perfusion of placental cotyledons) showed different effect on both endothelial cells³⁹ and peripheral T lymphocytes³⁸. Placental perfusion-derived STBMs induced enhanced proliferation and IFN γ release of T cell, whereas mechanically- and villous explants-derived STBMs inhibited activation, proliferation, and cytokine release of T lymphocytes³⁸. However, the difference in the mode of preparation of STBMs may not represent the difference between normal pregnancy and preeclampsia.

Elevated concentrations of circulating STBMs in preeclampsia have been observed in several reports, and it has been suggested that this higher concentration may be associated with endothelial dysfunction and maternal inflammatory response in preeclampsia^{4, 37, 40}. However, by flow-cytometric analysis, Lok et al could not find the association between the numbers of microparticles and the severity of preeclampsia, suggesting that the number of microparticles alone does not explain the reported vascular effect of microparticles⁴¹. Similarly, in the current study, microparticles from the hypoxic trophoblast induced more intense inflammatory response than microparticles from the normal trophoblast, even in the same concentration of both types of microparticles.

Unanswered questions and further studies

The exact mechanism of the different inflammatory response to microparticles from hypoxic and normal trophoblast was not addressed in the current study. In the study of Pap et al⁴², among the cells in PBMCs from healthy non-pregnant women, T lymphocytes, but not B lymphocytes or NK cells, were targets of trophoblast-derived microparticles, confirmed by binding of microparticles to T lymphocytes in confocal microscopy. And they also demonstrated that microparticles-lymphocyte interaction induced STAT3 phosphorylation in T cells. The result of the current study also suggests that the targets of hypoxic microparticles might be T lymphocytes, because the pro-inflammatory responses provoked by hypoxic microparticles were more intense in the presence of PHA, which is T cell mitogen. The targets and different mechanism of action of hypoxic microparticles in the pathogenesis of preeclampsia needs further studies.

The endothelial activation is also a key factor in the pathophysiology of preeclampsia^{3, 43}. As previously described, von Dadelzen et al³ showed that human umbilical vein endothelial cells cultured with STBMs activated leukocytes, and suggested that the activation of leukocytes in preeclampsia may be the result of endothelial activation caused by circulating

STBMs shed in excess amount from the placenta. The role of hypoxic microparticles in endothelial cell activation also needs to be determined.

We used rotenone to induce chemical hypoxia to represent placental hypoxia in preeclampsia. Huppertz et al²¹ reported that severe placental hypoxia showed both apoptotic and necrotic characteristics, suggesting aponecrosis. Aponecrosis is a form of cell death with morphologic features of both apoptosis and necrosis in response to hypoxia. And Orozco et al suggested that cells treated with rotenone can mimic this phenomenon²⁶. The type of cell death and characteristics of microparticles also needs to be determined in further studies.

It may be argued that the IL-6 and TNF- α may be also from microparticles besides from PMNC. However, when we cultured microparticles themselves without PMNC in the presence of absence of PHA for 24 and 48 hours, the concentration of supernatant IL-6 and TNF- α from microparticles alone was at negligible level (Data not shown).

In conclusion, microparticles from hypoxic trophoblast induced more intense inflammatory response than those from normal trophoblast, and this difference may be a key mechanism linking placental hypoxia and maternal systemic inflammatory response in preeclampsia.

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A

After 24 hours

B

After 24 hours

c

After 48 hours

D

After 48 hours

Figure 1.

The cytokine production of peripheral blood mononuclear cells (PBMCs), after 24-hour and 48-hour culture, in the presence or absence of MP_C, with or without PHA; (A) supernatant concentration of IL-6 after 24-hour culture, (B) supernatant concentration of TNF-α after 24-hour culture, (C) Supernatant concentration of IL-6 after 48-hour culture, and (D) Supernatant concentration of TNF-α after 48-hour culture.

A

After 24 hours

B

Figure 2.

The mean value of cellular proliferation (absorbance of CCK-8) of peripheral blood mononuclear cells (PBMCs) after 24-hour and 48-hour culture, in the presence or absence of MP_C, with or without PHA; (A) The cellular proliferation after 24-hour culture, and (B) The cellular proliferation after 48-hour culture. Each error bar represents mean $\pm$ standard deviation.

A

After 24 hours

B

After 24 hours

c

After 48 hours

D

After 48 hours

Figure 3.

The cytokine production of peripheral blood mononuclear cells (PBMCs) after 24-hour and 48-hour culture with MP_C or MP_H, with or without PHA; (A) Supernatant concentration of IL-6 after 24-hour culture, (B) Supernatant concentration of TNF-α after 24-hour culture, (C) Supernatant concentration of IL-6 after 48-hour culture, and (D) Supernatant concentration of TNF-α after 48-hour culture.

A

After 24 hours

B

After 48 hours

Figure 4.

The mean value of cellular proliferation (absorbance of CCK-8) of peripheral blood mononuclear cells (PBMCs) after 24-hour and 48-hour culture, with MP_C or MP_H, with or without PHA; (A) The cellular proliferation after 24-hour culture, and (B) The cellular proliferation after 48-hour culture. Each error bar represents mean $\pm$ standard deviation.