Published in final edited form as:

J Hypertens. 2016 July ; 34(7): 1380–1388. doi:10.1097/HJH.0000000000000944.

Upregulation of P53 promoted G1arrest and apoptosis in human umbilical cord vein endothelial cells from preeclampsia

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Abstract

Objective—Preeclampsia is a leading cause of maternal and perinatal morbidity and mortality. Current research has focused on endothelial dysfunction regarding pathogenesis of preeclampsia. However, very limited or no studies so far have been performed to assess possible damaged endothelial cell growth/development in the placenta–umbilical cord circulation system in human preeclampsia.

Methods—We isolated and cultured human umbilical cord vein endothelial cells (HUVECs) from normal and preeclampsia pregnancies *in vitro*. We used 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay to measure cell growth and flow cytometric analysis to determine cell-cycle distribution. Annexin V-fluorescein isothiocyanate/propidium iodide double staining was employed for cell apoptosis experiments.

Results—The study showed that the cell growth was significantly suppressed, accompanied by the increased G1 arrest and apoptosis in cultured HUVECs from preeclampsia pregnancies comparing with normotensive controls. Protein P53 was upregulated in the cultured HUVECs from preeclampsia pregnancies, which induced G1 arrest, followed by upregulating P21 expression, and downregulating cyclin E expression and CDK2–cyclin E complexes. On the other hand, upregulation of P53 also activated *Bax* gene and repressed *Bcl-2* and *BIRC5* genes, resulting in an increase of the Bax/Bcl-2 ratio and subsequently activating caspase cascade, ultimately led to an initiation of the apoptotic machinery.

Conclusion—These results indicated that in preeclampsia, vascular endothelial cells could be damaged and cellular proliferation was depressed in human placenta–umbilical cord circulation, adding new information on endothelial cell injury for better understanding the pathogenesis of preeclampsia.

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Authors' contributions: Q.G., X.Z., and Z.X. took part in the conception and design of the study. Q.G. and Z.X. participated in analysis of the data and drafted the manuscript. J.C. carried out the MTT analysis. All authors took part in the interpretation of data, revision of the manuscript, and in the final approval of the version to be published.

Conflicts of interest

There are no conflicts of interest.

Keywords

apoptosis; G1 arrest; P53; preeclampsia; umbilical vein endothelial cells

INTRODUCTION

Vascular endothelial cell is a type of epithelium that lines the interior surface of blood vessels, forming an interface between circulating blood and vessel wall. Endothelial cells are involved in many aspects of vascular biology, including barrier function, blood clotting, formation of new blood vessels (angiogenesis), and regulating vascular tone. Therefore, conditions of endothelial cells could reflect blood vessels' health or diseases. In fact, in recent years, endothelial wounding and injury or loss of proper endothelial functions (endothelial dysfunction) are often regarded as a hallmark for many vascular diseases, including hypertension [1], coronary artery disease [2], atherosclerotic diseases [3], and preeclampsia [4].

Preeclampsia is a pregnancy-specific disorder and long-term cardiovascular risk factor for the mother and possibly the offspring too [5]. To date, although the potential mechanisms underlying the pathogenesis of preeclampsia remain unclear, endothelial cellular injury has become a major research focus of the pathogenetic processing implicated in preeclampsia [6,7]. About the pathogenesis of preeclampsia, current research has aimed at the imbalance between vasodilator and vasoconstrictor substances produced by the endothelium [8,9]. However, information is limited regarding the vascular endothelial cells in placental–umbilical cord circulation in preeclampsia, including cellular proliferation, migration, and formation of capillary-like tube structures. Therefore, the present study used umbilical blood vessels collected from both healthy and preeclampsia women to investigate whether and how endothelial cells would be affected under conditions of preeclampsia.

In the 1990s, a few studies reported that human umbilical cord vein endothelial cells (HUVECs) proliferation was suppressed by the plasma from the women with preeclampsia, suggesting that there was a blood-borne endothelial cell suppressive factor in preeclampsia that may be derived from the placenta [10,11]. Subsequent research had validated that the inhibitory effect of the plasma from women with preeclampsia on endothelial cells proliferation was closely linked to placental syncytiotrophoblast microvillous membranes fragments [12,13]. Those studies suggested that potentially harmful factors have been existing in the maternal and fetal circulation in preeclampsia, which could influence vascular endothelial development and functions. However, there lacks studies on assessing certain endothelial cell damages in preeclampsia, including apoptosis and cell-cycle arrest, which may prevent proliferation of stressed or damaged cells.

In the present study, we isolated and cultured HUVECs from normal and preeclampsia pregnancies *in vitro*, and found that the extent of G1 arrest and apoptosis were significantly increased in the cultured HUVECs from preeclampsia pregnancies. We used 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay to measure cell growth and determined cell-cycle distribution by flow cytometric analysis. Annexin V-fluorescein isothiocyanate/PI double staining was employed for cell apoptosis experiments. The

apoptosis- associated gene expression and cell-cycle-associated gene expression were determined. Quantitative real-time PCR and western blot were performed to determine expression levels of the key mRNA and protein in the pathways that may underlie possible cellular changes in the HUVECs from preeclampsia. In addition, knockdown of P53 by small interfering RNA (siRNA) and co-immunoprecipitation (co-IP) assay were performed to assess possible mechanisms or pathways involved. The new data gained provided interesting information on the development of umbilical endothelial cells in human, as well as the influence of preeclampsia on those cell growth and proliferation.

MATERIALS AND METHODS

Sample collection

Healthy normal pregnant ($n = 30$) and preeclampsia women ($n = 23$) were recruited from the local hospitals, Suzhou, China. Written informed consent was obtained from all patients. All procedures used in this study were approved by the Institute's Ethics Committee. The clinical characteristics of all participants are summarized in Table 1.

Isolation and culture for umbilical vein endothelial cells

Umbilical cords (about 20 cm in length) were excised from the placenta immediately after vaginal spontaneous or cesarean section delivery and placed into cold sterile phosphate-buffered saline (PBS). Endothelial cells were isolated from umbilical veins as described previously [14] and cultured in DMEM (HyClone) containing 20% fetal bovine serum at 37°C with 5% CO₂ and 95% air humidified incubator. Cells were checked under a microscope system.

Measurement of cell growth by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

Five thousand endothelial cells were plated onto 96 wells plate. Incubated the each well with 10 µl of 5 mg/ml MTT for 4 h at 37°C and carefully removed without disturbing the cells. Then 100 µl dimethyl sulfoxide was added into each well to dissolve the crystals. Absorbance was read at 590 nm with a reference filter of 620 nm.

Cell-cycle distribution

Endothelial cells were seeded at 6-cm dishes and allowed to grow for 24 h. Cells were harvested and fixed at least 1 h with 70% ice-cold alcohol at 4°C. After washing in cold PBS three times, cells were resuspended in 300 µl PBS containing 50 µg/ml propidium iodide and 100 µg/ml ribonuclease A, and incubated for 30 min in the dark at room temperature, then analyzed with a flow cytometer (BD Biosciences; San Jose, California, USA). The fractions of cells in the G1, S, and G2/M phases of the cell cycle were analyzed using dedicated software. Each condition was repeated in triplicate.

Annexin V-fluorescein isothiocyanate/propidium iodide double staining

Endothelial cells were seeded at 6-cm dishes and plated onto 24 wells plate, respectively. After growing for 24 h, the cells were stained using Annexin V- fluorescein isothiocyanate

(FITC)–propidium iodide Apoptosis Detection Kit (Beyotime Biotechnology, Jiangsu, China) according to the manufacturer's instruction. Samples from 6-cm dishes were analyzed with a flow cytometer within 1 h after the staining. For 24 well plates, after staining, the cells were quickly fixed with 4% paraformaldehyde and then incubated with 4', 6-diamidino-2-phenylindole, as previously described [15]. Images were acquired with a microscope system.

Quantitative real-time polymerase chain reaction

Total RNA was isolated from cells by using Trizol reagent (Invitrogen) according to the manufacturer's protocol. First-strand cDNA synthesis was performed using reverse transcription kit (Toyobo, Japan) and quantitative real-time PCR (qRT-PCR) was performed with SYBR Green Supermix Taq Kit (Takara, Dalian, China). All reactions were carried out in triplicate. Relative gene expression was calculated by using C_t method. All the qRT-PCR primers used are listed in Table 2.

Western blot

The protein abundance of P21, P27, P53, BIRC5, Bcl-2, Bax, cyclin E, and Caspase 7 in HUVECs were assessed by western blot normalized to β -actin. Antibodies against P53, BIRC5, Bcl-2, Bax, Caspase 7, and β -actin were from Santa Cruz Biotechnology (Santa Cruz, California, USA). P21, cyclin E, and P27 antibodies were from Beyotime Biotechnology. Western blot analyses were performed as previously described [16].

Knockdown of P53 by siRNA

The siRNAs against human P53 were synthesized and purified by Shanghai GenePharma Co., Ltd. The sequences of three P53 siRNAs are as follows: 5'-CUACUUCUGAAAACAACGTT-3 (siP53–270), 5'-UGGUUCACUGAAGACCCAGTT-3 (siP53–354), and 5'-GACUCCAGUGGUAUAUCUACTT-3 (siP53–972). P53 knockdown in HUVECs for expression profiling was carried out using siP53–354 according to the manufacturer's instructions. The sequence of control siRNA is 5'-UUCUCCGAACGUGUCACGUTT-3.

Co-immunoprecipitation assay

To prepare whole cell extracts, HUVECs from two 10-cm dishes were washed with cold sterile PBS three times and lysed with ice-cold lysis buffer [20 mM Tris–HCl (pH 8.0), 150 mmol/l sodium chloride (NaCl), 1% nonidet P-40, 2 mmol/l ethylenediamine tetraacetic acid (EDTA), 2 mmol/l phenylmethanesulfonyl fluoride, and protease inhibitor cocktail from Sigma-Aldrich] for 1 h on ice. Then, the lysates were centrifuged at 12 000 rpm for 20 min at 4°C to remove cell debris. Immunoprecipitation was performed with protein G-agarose beads (Sigma-Aldrich) or (and) CDK2 antibody, as previously described [17]. Western blot was performed using CDK2 and cyclin E antibodies, as described above.

Data analysis and statistics

Data were expressed as the mean $\pm$ SEM. Statistical analysis was performed with *t* test or two-way analysis of variance using SigmaStat 3.1.0 analysis software (Systat Software Inc., San Jose, California, USA). Statistical significance was accepted at *P* less than 0.05.

RESULTS

Cellular growth and cell-cycle distributions of human umbilical cord vein endothelial cells

Although *in vitro* cultured HUVECs from normal and preeclampsia pregnancies appeared similar in morphology (Fig. 1a), the growth of HUVECs from preeclampsia pregnancies was obviously slower than that of normal pregnant group. MTT assay revealed that cell number in preeclampsia group was significantly reduced from the second day ($P < 0.05$) (Fig. 1b). Cell-cycle distribution assay showed that the percentage of G1 phase was increased by 10.03% ($P < 0.001$) compared with normal pregnant group; the percentages of S phase and G2 phase in preeclampsia group were decreased by 7.61% ($P < 0.01$) and 2.42% ($P > 0.05$), respectively (Fig. 1c and d). These results indicated that cell cycle was arrested in G1 phase (G1 arrest) in cultured HUVECs from preeclampsia pregnancies.

Expression of the regulatory proteins related to G1 phase progression and G1/S transition

To determine the mechanisms on G1 arrest in cultured HUVECs from preeclampsia pregnancies, we detected the expression of the regulatory proteins related to cell-cycle G1 phase progression and G1/S transition. The mRNA and protein levels of P21, but not P16 and P27, were markedly increased in HUVECs from preeclampsia pregnancies (Fig. 2a and b). In contrast, the mRNA and protein levels of cyclin E were significantly decreased, whereas CDK2 showed no significant difference between normal pregnant and preeclampsia groups (Fig. 2c). Then we examined the CDK2–cyclin E interaction in cultured HUVECs using co-IP assay. As shown in Fig. 2d, co-IP with cyclin E could be detected at a reduced level in cultured HUVECs from preeclampsia pregnancies in comparison with that of normal pregnant group.

Endothelial cell apoptosis

Apoptosis in cultured HUVECs was determined using flow cytometry and fluorescence microscopy via Annexin V-FITC/propidium iodide double staining. Fig. 3a and b revealed a significant increase in the percentage of apoptotic cells in cultured HUVECs from preeclampsia pregnancies (9.65%) compared with the normal pregnant group (1.97%). As shown in Fig. 3c, an increase in the red (propidium iodide)/green (Annexin V) fluorescence intensities of cultured HUVECs from preeclampsia pregnancies was observed.

Expression of apoptosis-associated proteins

The expression of apoptosis-associated gene was tested in cultured HUVECs from normal pregnant and preeclampsia pregnancies. Compared with normal pregnant group, the expression of BIRC5, not BIRC4, was downregulated significantly. Although no statistical significance was attained, Bcl-2 expression was also downregulated in cultured HUVECs from preeclampsia pregnancies. By contrast, the expressions of Bax and caspase 7, not

caspace 3, were markedly upregulated in cultured HUVECs from preeclampsia pregnancies (Fig. 4a and c). Consistently, the altered mRNA expression was associated with the corresponding changes in protein abundance (Fig. 4d). In addition, although there was no statistical significance in Bcl-2 expression between normal and preeclampsia pregnancies, the ratio of Bax to Bcl-2 was significantly upregulated in cultured HUVECs from preeclampsia pregnancies (Fig. 4b).

Expression of P53

As a key regulator in multicellular organisms, the protein P53 plays a crucial role in regulating cell apoptosis and cell-cycle arrest by activating or repressing various downstream target genes [24]. In this study, mRNA and protein abundance of P53 were determined in cultured HUVECs from normal pregnant and preeclampsia pregnancies using qRT-PCR and western blot. As shown in Fig. 5a and b, the expression of P53 was significantly increased in cultured HUVECs from preeclampsia pregnancies. To further confirm that the upregulation of P53 was involved in the increasing of cell apoptosis and G1 arrest in HUVECs from preeclampsia pregnancies, we used three different siRNAs (siRNA270, siRNA354, and siRNA972) that targeted P53 mRNA to knock down the endogenous P53 and then performed rescue experiments. Endogenous P53 was significantly depleted by transfection of siRNA354, as demonstrated by qRT-PCR and western blot (Fig. 5c and d).

P53-induced cell apoptosis and G1 arrest in cultured human umbilical cord vein endothelial cells from preeclampsia pregnancies

We transfected HUVECs from preeclampsia pregnancies with siRNA354 against P53 and quantitatively measured several known P53 target genes involved in apoptosis and G1 arrest by qRT-PCR and western blot. P53 mRNA and protein were specifically depleted by its corresponding siRNA354 (Fig. 6a and b). qRT-PCR and western blot analysis showed that knockdown of P53 in HUVECs from preeclampsia pregnancies led to a reduced expression of Bax, caspase 7, and P21; by contrast, knockdown of P53 resulted in increased expression of BIRC5 and cyclin E (Fig. 6a and b). In addition, co-IP with cyclin E could be detected at an increased level when knockdown of P53 was observed in HUVECs from preeclampsia pregnancies (Fig. 6c). Together, these data demonstrated that, in cultured HUVECs from preeclampsia pregnancies, the upregulation of P53 was involved in inducing G1 arrest and apoptosis, followed by the regulation of expression of P21 and apoptosis-associated proteins.

DISCUSSION

In the present study, HUVECs from normal and preeclampsia pregnancies were isolated and cultured *in vitro*. To the best of our knowledge, this was the first report to demonstrate various signaling pathways-mediated apoptosis and suppressed cell proliferation in HUVECs between healthy and preeclampsia women. The present study showed that the growth of HUVECs from preeclampsia pregnancies was obviously slow, which was due to the increased extent of G1 arrest and apoptosis. In the determination of possible underlying mechanisms, the present study provided new evidence that upregulation of P53 induced the G1 arrest, followed by upregulating P21 expression and downregulating cyclin E expression, which caused a decrease of CDK2–cyclin E complexes. Upregulation of P53 also activated

or repressed apoptosis-associated genes, including *BIRC5*, *Bax*, and *Bcl-2*, and then increased the Bax/Bcl-2 ratio, and subsequently activated caspase cascade, resulting in the initiation of the apoptotic machinery.

P53 and G1 phase arrest

The cell cycle is driven forward by cyclin-dependent kinases (CDKs; including CDK1–CDK9) that form heterodimer complexes with cyclins (cyclin A–T) [18]. The cyclin/CDK complexes orchestrate the progression of the cell through different phases of its growth cycle [18]. For example, in the middle to late G1 phase, CDK2 forms complexes with cyclin E, which specifically target and phosphorylate retinoblastoma protein, required for the transition from G1 to S phase of the cell cycle [19]. Thus, it can be speculated that decreased intracellular cyclin E/CDK2 complexes may result in cell-cycle arrest in the G1 phase. The P21 protein, belonging to the family of CDK inhibitors, can bind to and inhibit the activity of cyclin E/CDK complexes and thus prevents cells from going through the G1/S phase checkpoint, allowing accumulation of the number of cells in the G1 phase and cell-cycle arrest in the G1 phase [20,21]. In addition, P21 can also lead to the cell-cycle arrest in the G1 phase by suppressing the expression of CDK2 and cyclin E [22,23]. In the present study, the number of cells in the G1 phase was significantly increased in HUVECs from preeclampsia pregnancies. Subsequently, upregulation of P21, down-regulation of cyclin E, and a reduced level of CDK2/cyclin E complexes were detected in cultured HUVECs from preeclampsia pregnancies. These results indicated that upregulation of P21 was connected with the depression of CDK2 and cyclin E in cultured HUVECs from preeclampsia pregnancies, ultimately leading to arrest of the cell cycle and reduction of the proliferation of cells. In cells, the expression of P21 is tightly controlled by P53 [24]. Our study found that the protein and gene of *P53* showed significantly higher expression in the preeclampsia group, and knockdown P53 in cultured HUVECs from preeclampsia pregnancies was able to down-regulate the expression of P21. These results suggested that the increasing P53 upregulated P21 expression and downregulated the interaction of CDK2 and cyclin E and subsequently induced G1 phase arrest in cultured HUVECs from preeclampsia pregnancies.

P53 and apoptosis

Apoptosis is the process of programmed cell death that occurs when cells are exposed to physiological, pathogenic, or cytotoxic stimuli. Many well known proteins are closely implicated in the apoptotic process, such as Bcl-2 family, caspases members, and inhibitors of apoptosis (IAP) family [25–29]. The Bcl-2 family mainly consists of antiapoptotic (including Bcl-2 and Bcl-xl) and proapoptotic (including Bax and Bad) members. Bcl-2 is specifically considered as an important antiapoptotic protein and has been shown to prevent apoptosis by blocking cytochrome C release from mitochondria [30,31]. Another well known Bcl-2 family member Bax, presents high homology to the Bcl-2 protein, enabling it to heterodimerize with Bcl-2 and to display its proapoptotic functions [32]. The apoptotic process is the consequence of a series of precisely regulated events and is tightly regulated by the balance between proapoptotic and antiapoptotic proteins [26,30]. An imbalance between proapoptotic and antiapoptotic factors contributes to the activation of caspases cascade and initiation of the apoptotic machinery [33]. The caspase family of cysteine proteases, that plays an indispensable and integral role in the apoptotic process, is

categorized into initiator caspases (e.g. caspase 2, 8, 9, and 10) and effector caspases (e.g. caspase 3, 6, and 7) [34]. XIAP (BIRC4) and survivin (BIRC5) are important IAP family members, which can bind caspase-3 and caspase-7, thereby inhibiting their activation and preventing apoptosis [29]. In the present study, the percentage of apoptotic cells was increased in HUVECs from preeclampsia pregnancies, accompanied by the significantly upregulating expressions of Bax and caspase 7, whereas the BIRC5 expression was markedly downregulated. In addition, the ratio of Bax to Bcl-2 was significantly upregulated in cultured HUVECs from preeclampsia pregnancies. These results suggested that in cultured HUVECs from preeclampsia pregnancies, antiapoptotic, and (or) suppressor genes were reduced whereas proapoptotic genes increased, ultimately leading to the increased extent of apoptosis. P53 also plays a crucial role in regulating cell apoptosis [24]. P53 can directly activate the expression of Bax [35], whereas it inhibits Bcl-2 and BIRC5 expressions at the transcriptional level [36,37]. Interestingly, RNA interference experiments in the present study revealed that knockdown P53 in cultured HUVECs from preeclampsia pregnancies was able to rescue the expressions of Bax, caspase 7, and BIRC5. These new results demonstrated that upregulation of P53 was involved in triggering cell apoptosis in cultured HUVECs from preeclampsia pregnancies by activating *Bax* gene and repressing *Bcl-2* and *BIRC5* genes.

Endothelial cell injury and preeclampsia

As we know, vascular endothelial cell is necessary for formation and development of the fetal vascular system by vasculogenesis and angiogenesis processes [38]. As part of blood vessels, endothelial cells also have unique functions such as fluid filtration, homeostasis, hormone trafficking, and regulating vascular tone. Consequently, during pregnancy, the damage of endothelial cells plays key roles in the pathogenesis of vascular diseases, including preeclampsia [4]. In the present study, we demonstrated that the upregulation of P53 induced apoptosis and suppressed endothelial cell proliferation in preeclamptic pregnancy. This condition of endothelial cell in the maternal and fetal circulation may be one of the causes for the pathogenesis of preeclampsia. For the fetus, this condition of endothelial cell could also have adverse effect on the development of fetal vascular system. On the other hand, under conditions of preeclampsia, apoptosis and suppressed proliferation of endothelial cells could result in a number of functional alterations in the maternal and fetal vascular endothelium, such as impaired regulation of vasodilation and vasoconstriction, and angiogenesis, which could ultimately aggravate preeclampsia disorders. The present study provides novel information on endothelial cell injury that is beneficial to better understanding the pathogenetic mechanisms implicated in preeclampsia.

Vascular endothelial growth factor and endothelial cell survival

In addition, vascular endothelial growth factor (VEGF), specifically acting on endothelial cells, plays a central role in the regulation of cell survival via its endothelial-specific receptors, including VEGFR1, VEGFR2, and VEGFR3 [39]. VEGF can promote endothelial cell growth via activation of the VEGFR2 [40] and inhibit apoptosis by inducing expression of Bcl-2 [41]. According to recent studies, circulating levels of VEGF were decreased in women with severe preeclampsia comparing with normotensive pregnant [42,43]. These previous studies suggested that VEGF was potentially involved in the reduced growth of the

cultured HUVECs from preeclampsia pregnancies. Therefore, we previously conceived that the expressions of VEGF and (or) its endothelial-specific receptors would be possible to be decreased in cultured HUVECs from preeclampsia pregnancies (Supplementary data Fig. 1, <http://links.lww.com/HJH/A614>). However, in analysis of the expressions of VEGF and its endothelial-specific receptors, we did not find significant differences in the expressions of VEGF, VEGFR1, VEGFR2, and VEGFR3 in cultured HUVECs between the normal and preeclampsia pregnancies. These negative results could indicate that the depressed growth of the cultured HUVECs from preeclampsia pregnancies might not be caused via the VEGF pathway. Taken together, the results suggest that P53 pathways may play critical roles in an increased cellular death and suppressed cellular proliferation in umbilical endothelial cells from preeclampsia.

In conclusion, the present study demonstrated that the upregulation of P53 induced G1 phase arrest and apoptosis by upregulating P21 and Bax expressions; conversely, downregulating Bcl-2 and BIRC5 levels ultimately led to the suppressed growth of the HUVECs from preeclampsia pregnancies. These results indicated that in preeclampsia, vascular endothelial cells could be damaged and cellular proliferation could be affected via certain signaling pathways. The novel information gained on endothelial cellular injury is beneficial to better understanding the pathogenetic mechanisms implicated in preeclampsia. This interesting finding also raised important questions, including what are possible functional consequences from the suppressed cellular proliferation in umbilical endothelial cells, which is worth further investigation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank all of the volunteers and staff involved in this research.

The work was supported partly by Grants 2013BAI04B05 and 2012CB947600; National Nature & Science Foundation of China (81320108006 and 81401244); Jiangsu Natural Science Foundation (BK20140292); and Suzhou Natural Science Foundation (SYS201451).

Abbreviations

PE	preeclampsia
DAPI	4',6-diamidino-2-phenylindole
DMSO	dimethyl sulphoxide
HUVECs	human umbilical cord vein endothelial cells
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NP	normal pregnant
PI	propidium iodide

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Reviewers' Summary Evaluations

Referee 1

This study was performed in human umbilical vein endothelial cells (HUVECs) isolated from human subjects with preeclampsia and normotensive control subjects, and provides key information on mechanisms of endothelial cell damage associated with preeclampsia, namely cell cycle arrest and apoptosis. It would be of interest to determine whether altered endothelial mechanisms described in this study involve oxidative stress. Oxidative stress is important in the pathophysiology of hypertension, thus an investigation of whether elevated NADPH oxidase expression and activity is associated with endothelial damage in HUVECs from women with preeclampsia is warranted.

Referee 2

The article by Gao *et al.* focuses on endothelial dysfunction in preeclampsia and analyses cell-cycle regulators on human vein endothelial cells (HUVECs). Strengths: The manuscript highlights novel mechanisms of altered cell cycle control in HUVECs from preeclamptic patients, specifically the role of the tumor suppressor p53 in regulating expression of key players of cell cycle progression.

Weaknesses: This comprehensive analysis would have benefit from the identification of fetal sex since recent studies show sex differences in HUVECs cells and correlations between fetal sex and the preeclampsia syndrome.

FIGURE 1.

Cellular growth and cell-cycle distributions of human umbilical vein endothelial cells from normal pregnant and preeclampsia pregnancies. (a) Cell morphology was observed under a microscope by magnifying 4 or 40 diameters (4 or 40 $\times$). (b) Cell proliferation was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay once daily for 4 days. (c and d) Cell cycle distribution was performed by the flow cytometric analysis. Cells in G1 phase are depicted in blue; cells in S phase are depicted in red, and cells in G2/M phase are depicted in purple. Error bars showed mean $\pm$ SEM. $N = 8/\text{each group}$. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. N , number of participants.

FIGURE 2.

Expression of the regulatory proteins related to G1 phase progression and G1/S transition in cultured human umbilical cord vein endothelial cells from normal pregnant and preeclampsia pregnancies. (a–c) Expressions of P16, P21, P27, CDK2, and cyclin E were assessed by quantitative real-time PCR (**normal pregnant group, $N = 30$; preeclampsia group, $N = 23$**) and/or western blot analysis ($N = 3/\text{each group}$). (d) CDK2–cyclin E complexes were measured by co-immunoprecipitation assay ($N = 4/\text{each group}$). Error bars showed mean SEM. **, $P < 0.01$. N , number of participants.

FIGURE 3.

The extent of endothelial cell apoptosis was determined by Annexin V-FITC/propidium iodide double staining. (a and b) Endothelial cell apoptosis were assessed by double staining of FITC-Annexin V and propidium iodide with flow cytometry. (c) The fluorescence intensities for both propidium iodide (red) and Annexin V (green) of human umbilical cord vein endothelial cells were measured using a fluorescence spectrometer. Error bars showed mean $\pm$ SEM. $N = 9/\text{each group}$. $*P < 0.05$. N , number of participants.

FIGURE 4.

Expressions of apoptosis-associated protein in cultured human umbilical cord vein endothelial cells. (a and c) mRNA levels of BIRC4, BIRC5, Bcl-2, Bax, Caspase3, and Caspase7 ($N = 21$ for normal pregnant group; $N = 14$ for preeclampsia group). (b) The ratio of Bax to Bcl-2. (d) Protein abundance of BIRC5, Bcl-2, Bax, and Caspase7 ($N = 3$ /each group). Error bars showed mean $\pm$ SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. N , number of participants.

FIGURE 5.

Expression of P53 in cultured human umbilical cord vein endothelial cells. (a and b) mRNA and protein levels of P53 (**normal pregnant group, $N = 15$; preeclampsia group, $N = 13$**). (c and d) Depletion of P53 by specific siRNAs. Human umbilical cord vein endothelial cells (**preeclampsia group, $N = 3$**) were transfected with siRNA against P53 for 2 days, and the effect on the levels of endogenous P53 was determined by quantitative real-time PCR and western blot. siRNA-con, nonspecific control siRNA. Error bars showed mean $\pm$ SEM. * $P < 0.05$; ** $P < 0.01$. N , number of participants.

FIGURE 6.

P53 induced cell-cycle arrest and apoptosis. (a and b) Human umbilical cord vein endothelial cells from preeclampsia pregnancies were transfected with siRNA-354 for 2 days. The expression of P53, Bcl-2, BIRC5, Bax, caspase 7, P21, and cyclin E were measured by quantitative real-time PCR and western blot. (c) CDK2–cyclin E interaction was measured by co-immunoprecipitation assay. Error bars showed mean $\pm$ SEM. $N = 9$ /each group. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

TABLE 1

Basic characteristics of preeclampsia cases and normotensive controls

Characteristics	NP	PE
Number of study participants	30	23
Maternal age (y)	29.1±4.3	28.7±4.1
Gestational age (weeks)	38.4±2.5	35.2±3.4 *
Birth weight (kg)	3.1±0.4	2.85±0.67 *
SBP (mmHg)	116.9±8.2	160.5±17.1 **
DBP (mmHg)	76.4±8.3	104.6±12.0 **
Proteinuria (g/24 h)	0.12±0.03	10.83±5.1 **
S/D ratio	2.17±0.2	3.48±0.4 **

The data was expressed as mean ± SD. Preeclampsia vs. normal pregnant. S/D ratio, ratio of systolic and diastolic blood flow in the umbilical artery. PE, preeclampsia; NP, normal pregnant

* $P < 0.05$.

** $P < 0.01$.

TABLE 2

Sequence for quantitative real-time PCR primers

Primer/siRNA	Sequence
qRT-PCR primers	
P53 mRNA	5'-CAGTCAGATCCTAGCGTCGA
P53 mRNA	3'-CATTCTGGGAGCTTCATCTG
P16 mRNA	5'-GCAGTAACCA TGCCCGCATAGAT
P16 mRNA	3'-AGTTGTGGCCCTGTAGGACCTT
P21 mRNA	5'-CGGCAGACCAGCATGACAGATTTC
P21 mRNA	3'-GACACACAACTGAGACTAAGGC
P27 mRNA	5'-GACCTGCAAC CGACGATTCTTC
P27 mRNA	3'-CAGGATGTCCATTCCATGAAGTC
CDK2 mRNA	5'-GGCACGTACGGAGTTGTGTACAA
CDK2 mRNA	3'-GAATGCCAGTGAGAGCAGAGGCAT
cyclin E mRNA	5'-CAGGATCCAGATGAAGAAATGGCC
cyclin E mRNA	3'-TGGATGGTGCAATAATCCGAGG
BIRC4 mRNA	5'-GAGGAGTGTCTGGTAAGAAC
BIRC4 mRNA	3'-GCATTCACTAGATCTGCAACCA
BIRC5 mRNA	5'-CACCGCATCTCTACATTCAAG
BIRC5 mRNA	3'-TGTTCTCTCTCGTGATCC
Bcl-2 mRNA	5'-GCAGAAGTCTGGGAATCGATCT
Bcl-2 mRNA	3'-CAGTCTACTTCCTCTGTGATGTTG
Bax mRNA	5'-ATGGAGCTGCAGAGGTGA
Bax mRNA	3'-CTTGAGCACCAGTTTGCTGGC
Caspase 3 mRNA	5'-GTTG ATGATGACATGGCGTGTC
Caspase 3 mRNA	3'-CAAGCTTGTCGGCATACTGT
Caspase 7 mRNA	5'-GATCAGCCTTGTGGGATGGCAGA
Caspase 7 mRNA	3'-GTACTGATATGTAGGCACTCG