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Pro-inflammatory mediators and signaling proteins in the decidua of pre-eclampsia

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Abstract. – OBJECTIVE: To evaluate the role of CD68+ macrophages and inflammatory/signaling proteins in the decidua of singleton pregnancies with late-onset pre-eclampsia.

PATIENTS AND METHODS: This study was designed as a prospective case-control study. Decidual tissue samples were obtained from twenty healthy pregnant women as a control group and twenty pregnant women with late-onset pre-eclampsia showing severe symptoms as the study group. We examined the abundance of CD68+ macrophages in both groups using flow cytometry. Protein and mRNA expression levels of inflammatory/signaling proteins, including inducible nitric oxide synthase, nuclear factor-κB inhibitor α, cyclooxygenase-2, and phosphorylated c-Jun N-terminal kinase, in the decidua of both groups were measured using Western blotting and Reverse Transcription-Polymerase Chain Reaction, respectively. Student's t-tests were performed for statistical analysis.

RESULTS: The numbers of CD68+ macrophages were similar in the study and control groups (p=0.47). However, the levels of inducible nitric oxide synthase, nuclear factor-κB, cyclooxygenase-2, and phosphorylated c-Jun N-terminal kinase were significantly increased in the study group. Therefore, pro-inflammatory mediators and signaling proteins in the decidua during pre-eclampsia may be related to the pathogenesis of pre-eclampsia.

CONCLUSIONS: Pre-eclampsia-induced alterations in the expression of inflammatory/signaling proteins in the decidua during singleton pregnancies may play a critical role in the pathogenesis of pre-eclampsia.

Key Words:
Pre-eclampsia, Decidua, Inflammatory/signaling proteins, Macrophage, NF-κB, JNK/MAPK, iNOS, COX-2.

Abbreviations

CCL, C-C motif chemokine ligand; CD, cluster of differentiation; CXCL, C-X-C motif chemokine ligand; COX, cyclooxygenase; FACS, fluorescence-activated cell sorting; FITC, fluorescein isothiocyanate; IκBα, NF-κB inhibitor α; IL, interleukin; iNOS, inducible nitric oxide synthase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor κB; NO, nitric oxide; NOS, nitric oxide synthase; RT-PCR, reverse-transcription polymerase chain reaction; p-JNK, phosphorylated JNK; SD, standard deviation.

Introduction

Pre-eclampsia, a pregnancy complication, is a major cause of maternal and perinatal mortality and morbidity. It typically begins after 20 weeks of pregnancy with clinical symptoms of high blood pressure, edema, and proteinuria, accompanied by platelet activation and systemic endothelial damage to multiple organs. Despite substantial research into the condition, the specific underlying mechanisms and etiology of pre-eclampsia remain unclear. A lack of spiral artery remodeling with shallow invasion of trophoblasts during placentation has been suggested to be the initial process in the progress of pre-eclampsia[1,2]. Pre-eclampsia results in placental ischemia/hypoxia and consequently in increased production of placental factors involved in immune system alteration, inflammation, oxidative stress generation, and anti-angiogenesis. Therefore, research has focused on elucidating the roles of factors involved in implantation, trophoblast invasion, inflammation, angiogenesis, oxidative stress, and immune system alterations associated with abnormal placentation during the development of pre-eclampsia[3-5].

Macrophages are differentiated from monocytes and are thought to play critical roles during implantation and placentation by eliciting innate and adaptive immune responses[3,6]. Abnormal macrophage infiltration in the decidua, suppression of trophoblastic invasion, and

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pro-inflammatory stimuli have been observed to contribute to the development of pre-eclampsia[7]. However, reports on the numbers of decidual macrophages found in pre-eclampsia patients are conflicting. While some studies reported that $CD68^+$ macrophages in the decidua were increased in pre-eclampsia patients compared to healthy controls[8-11], others have reported a decrease in $CD68^+$ macrophages in the decidua of pre-eclamptic placenta[12-14]. In pre-eclampsia, pro-inflammatory macrophages remain active during pregnancy, and the levels of interferon-γ, tumor necrosis factor-α, and interleukin (IL)-6 are elevated, whereas the levels of anti-inflammatory cytokines, such as IL-4 and IL-10 are reduced[15,16]. These findings suggest that an appropriate balance between pro-inflammatory and anti-inflammatory macrophage subsets in the placenta is important for achieving good perinatal outcomes in normal pregnancy.

Nitric oxide (NO) is synthesized by NO synthase (NOS) in the endothelium and platelets. The level of inducible NOS (iNOS) increases during platelet aggregation, under oxidative stress, and in macrophage infiltration by pro-inflammatory molecules[17,18]. iNOS is also expressed in the placenta of patients with hypertensive or pre-eclamptic pregnancies[19,20]. Thus, pro-inflammatory cytokines may be key contributors to endothelial activation in the pathogenesis of pre-eclampsia[21,22].

Nuclear factor κB (NF-κB) promotes pro-inflammatory cytokine production by regulating inflammatory gene expression and is highly activated in some inflammatory diseases. In pre-eclampsia, increased activation of NF-κB by oxidative stress inducers such as reactive oxygen species and the reduced form of nicotinamide adenine dinucleotide phosphate oxidase may be responsible for inducing inflammation and endothelial dysfunction[23-26]. The production of macrophage-recruiting chemokines and pro-inflammatory cytokines by pro-inflammatory stimuli is mediated *via* the activation of NF-κB and c-Jun N-terminal kinase (JNK)/mitogen-activated protein kinases (MAPKs) in human placental explants in response to oxidative stress[23,27]. Signaling by extracellular signal-regulated kinases and the serine/threonine-specific protein kinase cascade is also involved in the pathogenesis of pre-eclampsia[28]. Although numerous signal molecules have been suggested to be important mediators of the development and progression of pre-eclampsia, in view of the diverse clinical manifestations, further studies are needed to clarify the mechanisms of action of these signal molecules in the disease.

In this research, we evaluated the levels of $CD68^+$ macrophages and analyzed protein expression, activation, and transcript levels of iNOS, NF-κB, JNK, and COX-2 in healthy pregnant women compared to pregnant women with late-onset pre-eclampsia, with the aim of providing a better understanding of the pathogenesis of this disorder during singleton pregnancy.

Patients and Methods

Patients

This study was designed as a prospective case-control study. Decidual tissues were collected from the placental bed of twenty healthy pregnant women, enrolled as the control group, and twenty pregnant women with late-onset pre-eclampsia showing severe symptoms enrolled as the study group. To minimize the influences of other diseases on the current pregnancy in patients with a history of medico-surgical disease, patients with simultaneous pre-eclampsia and autoimmune diseases, as identified by autoimmune screening tests, were excluded.

Cesarean section was performed in cases with indications of breech presentation or a history of cesarean birth, to minimize the effects of labor and inflammation. Surgery was performed at the Department of Obstetrics and Gynecology, Ajou University Medical Center.

The control group was selected to have no history of medico-surgical disease or obstetric complications and was matched with the study group by maternal age, parity, and gestational age at delivery. Severe pre-eclampsia was defined as a blood pressure of 160/110 mmHg or higher after the 20^{th} week of gestation, measured at least twice with a 6-h interval; 2^{++} proteinuria determined twice via a dipstick test; and any other findings such as increased serum creatinine level (>1.2 mg/dL), decreased platelet count (<100,000 cells/μL), or elevated hepatic enzyme activity[29].

Decidual samples were collected immediately after cesarean section, following a previously reported method[30]. Parts of the tissue samples were used for preparing single-cell suspensions for flow-cytometric analysis. Parts of the specimens were snap-frozen in liquid nitrogen for Western blotting and Reverse-Transcription Polymerase Chain Reaction (RT-PCR) analyses.

Antibodies

The following antibodies were used in this study: anti-iNOS (Upstate, New York, NY, USA), anti-pJNK (Sigma, St. Louis, MO, USA), anti-IKBα (Santa Cruz Biotechnology, Dallas, TX, USA), anti-COX-2 (Sigma, St. Louis, MO, USA), anti-β-actin (Sigma, St. Louis, MO, USA), and anti-CD68 (Abcam, Cambridge, UK).

RNA Isolation and RT-PCR Analysis

Thirty milligrams of decidual tissue samples were homogenized. The homogenates were used for RNA isolation using the RNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol, with minor modifications. cDNA was synthesized using oligo-dT, Moloney murine leukemia virus reverse transcriptase, and deoxynucleoside triphosphate mix in reverse transcriptase buffer. RT-PCRs were run using a commercial kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol, in a Mx3000P instrument (Stratagene, La Jolla, CA, USA). The reaction mixtures contained cDNA, PCR buffer, dNTP mix, Taq polymerase, and gene-specific forward and reverse primers for amplification of the *iNOS, COX-2, JNK-1, NF-κB,* and β-*actin* genes. Thirty thermal cycles were used. The primer sets are listed in Table I.

Western Blot Analysis

Tissue samples were washed with PBS and lysed in 2× Laemmli buffer. Proteins were separated on 8-15% sodium dodecyl sulfate-polyacrylamide gels and then transferred to Immobilon membranes (Millipore, Billerica, MA, USA). The membranes were blocked in 5% non-fat milk in TBS/Tween-20 (0.05% v/v) at 20-25°C for 2 h. The membranes were then incubated with antibodies against target proteins at 20-25°C for 1 h, followed by incubation with horseradish peroxidase-conjugated secondary antibodies at 20-25°C for 1 h. Protein bands were visualized using enhanced chemiluminescence (GE Healthcare, Piscataway, NJ, USA).

Flow Cytometry

Non-homogeneous single cells obtained from decidua were dispersed by passing them through a nylon filter (mesh opening, 53 mm; Spectrum Labs, St. Paul, MN, USA). To analyze $CD68^+$ macrophages, single-cell suspensions (1×10^6 cells/tube) were incubated with a mouse anti-CD68 antibody for 15 min and washed twice with 2% FBS/PBS. A single-cell pellet was generated by centrifugation at $1000 \times g$ and was incubated with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG for 15 min. After two washes with 2% FBS/PBS, the stained cells were analyzed by fluorescence-activated cell sorting (FACS) on a FACSVantage system (BD Biosciences, Franklin Lakes, NJ, USA).

Statistical Analysis

Numerical data were presented as mean values and standard deviations (SDs), whereas qualitative variables were presented as percentages. Means were compared using two-tailed Student's *t*-tests at http://www.physics.csbsju.edu/stats/. $p<0.05$ was considered significant.

Results

Clinical Characteristics of the Patients

There were no significant differences in maternal age, parity, or gestational age at deliv-

Table I. Sequences of the primer pairs used for RT-PCR.

Target gene	Primer sequences	Forward (5′-3′) / Reverse (3′-5′)	Primer length (nucleotides)
β-actin	CCCCAGGCACCAGGGCGTGAT		21
	GGTCATCTTCTCGCGGTTGGCCTTG		25
NF-κB (p50)	CTGGAAGCACGAATGACAGA		20
	TTTCAAGTTGGATGCATTGG		20
JNK	AGAACCAAGAATGGAGTTATACGG		24
	GTCTTCAATGTCAACAGATCCGA		23
COX-2	TTCAAATGAGATTGTGGGAAAATTGCT		27
	AGATCATCTCTGCCTGAGTATCTT		24
iNOS	TGGATGCAACCCCATTGTC		19
	CCCGCTGCCCCAGTTT		16

ery between the study and control groups, because subjects were matched during enrollment to lessen any possible effects of risk factors. Pre-pregnancy body mass index (BMI) did not significantly differ between the two groups. Post-pregnancy BMI was significantly higher in the pre-eclampsia group than in the control group (30.2±4.7 *vs*. 24.7±4.0, $p<0.05$). Both systolic and diastolic blood pressure at admission were significantly higher in the pre-eclampsia group than in the control group (173.4±5.2 *vs*. 113.4±2.6, $p<0.0001$; 102.5±3.7 *vs*. 75.6±4.2, $p<0.0001$; respectively). There were significant differences in the levels of hemoglobin and hematocrit between the pre-eclampsia and control groups (13.5±2.1 *vs*. 11.6±0.4, $p<0.05$; 37.1±3.7 *vs*. 30.2±1.8, $p<0.05$; respectively). Maternal platelet counts and serum creatinine levels were not significantly different. The levels of alanine aminotransferase and aspartate aminotransferase were significantly higher in the pre-eclampsia group than in the control group (123.7±28.5 *vs*. 23.9±10.2, $p<0.05$; 101.2±17.6 *vs*. 15.9±6.3, $p<0.05$; respectively). Neonatal birth weight was significantly lower in the pre-eclampsia group than in the control group (2269±371 g *vs*. 3089±576 g, $p<0.05$). Neonates with intrauterine growth restriction were excluded from the control group. The percentage of neonates with intrauterine growth restriction in the pre-eclampsia group was 50% (Table II).

Macrophage Numbers in the Decidua

$CD68^+$ macrophages in the decidua were analyzed using flow cytometry of single cells freshly isolated from decidual tissue samples, and anti-human CD68 mouse IgG and FITC-conjugated anti-mouse IgG. Flow-cytometric analysis of the stained cells revealed that the percentages of $CD68^+$ macrophages were 12.5±0.1% and 11.9±0.1% in the pre-eclampsia and control groups, respectively ($p=0.47$, Figure 1). Thus, the number of $CD68^+$ macrophages in the decidua did not significantly differ between patients and controls.

Protein and Transcript Levels of Pro-Inflammatory Mediators and Signal Molecules

The protein levels of pro-inflammatory mediators and signal molecules present in decidual cell lysates from the pre-eclampsia and control groups were compared using Western blot analysis. Relative protein levels were calculated as densitometric ratios of target protein to β-actin (Figure 2). The level of iNOS was increased in the pre-eclampsia group compared to the control group (0.7±0.6 *vs*. 0.2±0.2, $p<0.0005$). NF-κB activation was detected by measuring the IκBα protein level, which decreases upon NF-κB activation. The IκBα level was decreased in the pre-eclampsia group compared to the control group (0.1±0.1 *vs*. 1.2±0.9, $p<0.0005$), indicating

Table II. Clinical characteristics of the study subjects.

Characteristics	Control group (n = 20)	Pre-eclampsia group (n = 20)	p
Age (years)	30.6 ± 3.2	30.9 ± 4.9	NS
Primiparity, no. (%)	12 (60%)	12 (60%)	NS
Pre-pregnancy BMI ($kg/m^{2)}$	23.14 ± 2.76	24.05 ± 3.18	NS
Post-pregnancy BMI (kg/m^2)	24.72 ± 3.95	30.21 ± 4.69	< 0.05
Systolic BP at admission (mmHg)	113.4 ± 2.6	173.4 ± 5.2	< 0.0001
Diastolic BP at admission (mmHg)	75.6 ± 4.2	102.5 ± 3.7	< 0.0001
Hemoglobin (g/dL)	11.6 ± 0.4	13.5 ± 2.1	< 0.05
Hematocrit (%)	30.2 ± 1.8	37.1 ± 3.7	< 0.05
Platelet count (cells/μL)	201000 ± 57000	195000 ± 91000	NS
Serum creatinine (mg/dL)	0.6 ± 0.2	1.1 ± 0.5	NS
ALT (U/L)	23.9 ± 10.2	123.7 ± 28.5	< 0.05
AST (U/L)	15.9 ± 6.3	101.2 ± 17.6	< 0.05
Proteinuria (mg/day)	0	2341 ± 505.7	–
GA at delivery (weeks)	37.6 ± 1.2	35.2 ± 3.1	NS
Neonatal birth weight (g)	3089 ± 576	2269 ± 371	< 0.05
No. of intrauterine growth restrictions, N (%)	0	10 (50%)	–

Values are presented as mean ± SD, Student's *t*-test, BP: blood pressure, BMI: body mass index, ALT: alanine aminotransferase, AST: aspartate aminotransferase, GA: gestational age, NS: not significant.

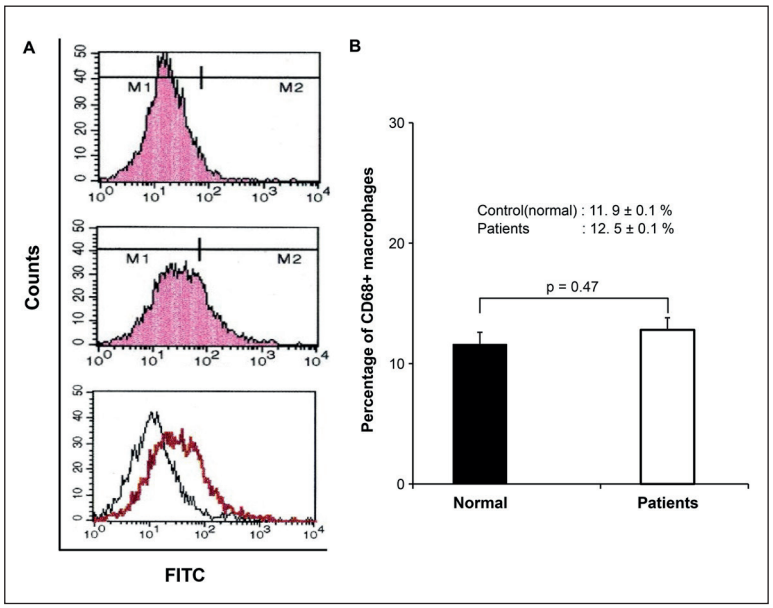

Figure 1. Flow-cytometric analysis of FITC-labeled CD68$^+$ macrophages in the decidua. (**A**) Representative histograms for the negative control (secondary IgG only, upper panel), CD68$^+$ macrophages (middle panel), and the overlap of the negative control and CD68$^+$ macrophages (the lower panel). (**B**) Average percentages of CD68$^+$ macrophages in the control and pre-eclampsia groups. Single cells isolated from placental decidua were stained with anti-human CD68 mouse IgG followed by FITC-conjugated anti-mouse IgG. Stained cells were analyzed by FACS Aria III and DIVA. The experiments were repeated three to four times. FITC, fluorescein isothiocyanate; FACS, fluorescence-activated cell sorter.

NF-κB activation in the pre-eclampsia group. JNK-1/MAPK activation was analyzed by measuring p-JNK. The p-JNK level was elevated in the pre-eclampsia group compared with that in the control group (1.4±0.8 *vs.* 0.1±0.1, $p<0.0005$). For unknown reasons, COX-2 could not be detected by Western blotting.

Relative mRNA levels of *iNOS*, *NF-κB*, *COX-2*, and *JNK-1* in the pre-eclampsia and control groups were compared (Figure 3). Relative mRNA levels were calculated as densitometric ratios of the target to β-*actin*. Consistent with the protein levels, the *iNOS* mRNA level was higher in the pre-eclampsia group than in the control group

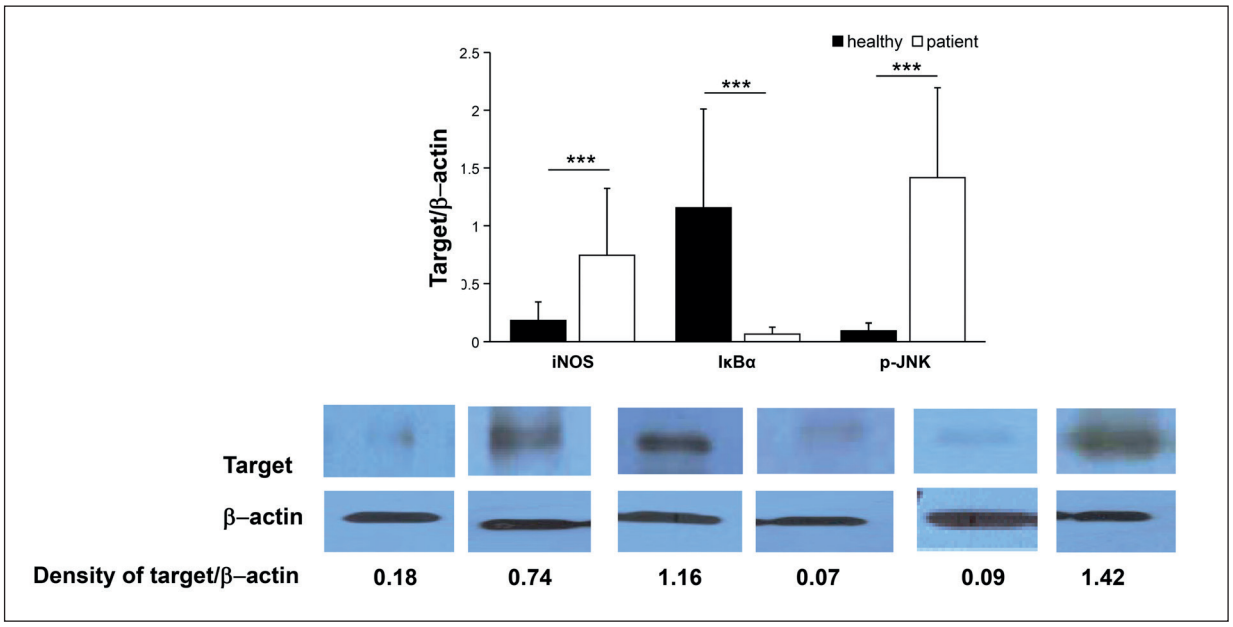

Figure 2. Protein levels in the decidua. Western blot analysis of protein levels of iNOS, IκBα, and p-JNK in decidual cell lysates. Band densities were quantified using densitometry, and target protein levels were normalized to the β-actin level. The assay was repeated five times. ***$p<0.0005$. iNOS, inducible nitric oxide synthase; IκBα, NF-κB inhibitor α; p-JNK, phosphorylated c-Jun N-terminal kinase.

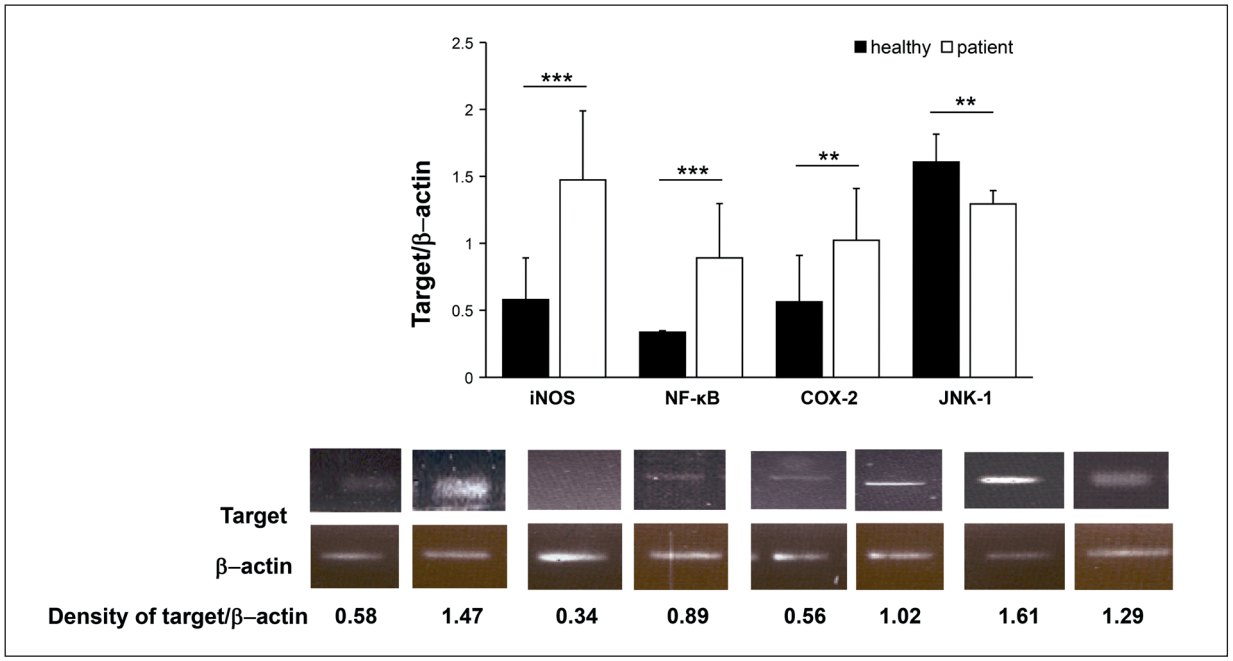

Figure 3. Transcript levels in the decidua. RT-PCR analysis of the relative mRNA levels of *iNOS*, *NF-κB*, *COX-2*, and *JNK* in decidual tissues. The assay was repeated three to five times. ***p*<0.005, ****p*<0.0005. iNOS, inducible nitric oxide synthase; NF-κB, nuclear factor-κB; COX-2, cyclooxygenase-2; JNK, c-Jun N-terminal kinase.

(1.5±0.5 *vs.* 0.6±0.3, *p*<0.0005). The mRNA level of *COX-2*, another pro-inflammatory marker, was higher in the pre-eclampsia group than in the control group (1.0±0.4 *vs.* 0.6±0.3, *p*<0.005). The *NF-κB* mRNA level was also higher in the pre-eclampsia group than in the control group (0.9±0.4 *vs.* 0.3±0.01, *p*<0.0005). Despite the increase in JNK protein phosphorylation in the pre-eclampsia group, their *JNK* mRNA level was decreased (1.3±0.1 *vs.* 1.6±0.2, *p*<0.005), indicating that JNK was phosphorylated and activated without an increase in JNK protein translation.

Discussion

We found that the levels of pro-inflammatory mediators and signaling proteins iNOS, NF-κB, COX-2, and p-JNK were significantly increased in the decidua of pre-eclampsia patients, without changes in the numbers of $CD68^+$ macrophages.

Kim et al[32] reported no changes in decidual macrophage numbers, which is consistent with our findings. The difference in findings between previous studies[8-14,32] and this study may be explained by differences in the methods and macrophage marker antibodies used. Some studies used immunohistochemistry[8-10,13] and/or immunofluorescence staining[12], whereas other used flow cytometry[14], as we did. Among the different macrophage subpopulations, $CD14^{+}$[(10,13)], $CD14^{+}/CD16^{+}$[(14)], and $CD14^{+}/CD68^{+}$[(32)] have been frequently analyzed. $CD68^+$ macrophages[7,8,10,11] have also been a focus of attention, as they were for us. Macrophage activation as well as increased numbers of macrophages have been reported in pre-eclampsia[33,34]. Our study clearly showed that the number of $CD68^+$ macrophages in the decidua may not be the critical pathogenic factor in the development of pre-eclampsia.

With respect to the pathogenic mechanisms underlying pre-eclampsia, there is evidence that pro-inflammatory mediators[15,16,21,22], oxidative stress[17-20,31], and immune system alterations associated with abnormal placentation [3-5] are major contributors.

Oxidative stress is a potent inducer of the release of pro-inflammatory factors. Pro-inflammatory mediators such as NO[35,36], iNOS[19,20], NF-κB[23-26], JNK[23,27], and COX-2[37] have been reported to be related to the pathogenesis of pre-eclampsia. NF-κB and MAPK are activated by pro-inflammatory stimuli, resulting in the production of macrophage-recruiting chemokines such as CCL2 and CXCL8, and NF-κB promotes the production of pro-inflammatory cytokines in a feed-

back reaction[27]. We observed that the pre-eclampsia group had higher protein and/or transcript levels of iNOS, NF-κB, and COX-2, and stronger activation of JNK and NF-κB than the control group. Other studies have reported similar results and hypothesized that monocytes are more strongly activated in pre-eclampsia patients than in healthy controls, by the release of increased amounts of soluble factors from the stressed placenta of patients[27,34]. We only evaluated the numbers of $CD68^+$ macrophages and the activation of inflammatory/signaling proteins in the decidua. To confirm our findings, further experiments are needed, such as immunohistochemistry analysis and extensive analysis of macrophage subpopulations and numerous cytokines. Future studies should ideally enroll larger patient and control cohorts, although our study cohort was larger than those of previous studies. Despite some limitations, one strong advantage of our study is that we homogenously categorized enrolled cases to minimize potential influences of pre-eclamptic risk factors. We found that rather than increasing in numbers, macrophages in the patient decidua were activated and consequently affected downstream processes, thereby increasing tissue concentrations of pro-inflammatory mediators and activating signal molecules. Our findings can further improve understanding of the pathogenesis of pre-eclampsia.

Conclusions

The results of this study indicate that the expression and/or activation of the pro-inflammatory mediators and signaling proteins iNOS, JNK, COX-2, and NF-κB increases in the decidua during late-onset pre-eclampsia in singleton pregnancies. The findings provide evidence for the importance of inflammation and oxidative stress in pre-eclampsia, and further our understanding of the pathogenesis of pre-eclampsia. Our findings also indicate that the activation of molecules by $CD68^+$ macrophages present in the placental decidua, rather than increased numbers of macrophages, might be a major pathogenic factor in pre-eclampsia. Our results do not support the contention that $CD68^+$ macrophage counts can be used to reliably evaluate the risk of pre-eclampsia. Further large-scale studies will be needed to confirm these findings.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Declaration of Funding Interests

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Ethics Statement

The study, including consent forms, was approved by the Institutional Review Board of Ajou University Medical Center (approval number: AJIRB-CRO-07107). The participants and their family members were fully informed about this study before enrollment and signed written consent forms.

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