Physiological Levels of Glutamine Reduces ICAM-1 Expression in Endothelial Cell Activated by Preeclamptic Plasma

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Running title: Glutamine effect on CAM expression in preeclampsia

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Synopsis

Women with preeclampsia had lower glutamine levels than normal pregnant women. Administering GLN at physiologic levels reduces endothelial cell ICAM-1 expression induced by preeclamptic plasma.

Abstract

OBJECTIVE: The purpose of our study was intended to investigate whether plasma glutamine (GLN) concentration would be depleted in pregnant women with preeclampsia, and whether administering GLN comparable to physiologic levels would decrease cellular adhesion molecule expression in human umbilical vein endothelial cells (HUVEC) induced by plasma from preeclamptic women.

STUDY DESIGN: We assessed plasma GLN levels from blood samples collected from 20 women with preeclampsia and 10 normal pregnant women. HUVEC were cultured in medium-199 containing fetal calf serum, antibiotics, growth factor and with different concentrations (0, 300, 500 uM) of GLN for 24 hr. We stimulated those cells for 1.5-6 hr with sera from patients with preeclampsia, and then determined the expression of intercellular cell adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 on endothelial cells by flow cytometry.

RESULTS: Women with preeclampsia had significantly lower plasma GLN concentrations as compared with the normal pregnant women. We found that there were no differences in VCAM-1 expression in HUVEC among various GLN concentrations at each time point. However, ICAM-1 expression in HUVEC were significantly lower in 500 uM GLN group than 0 and 300uM groups at 3, 4.5, and 6hr.

CONCLUSION: This study showed that plasma from women with preeclampsia had significantly lower GLN levels than those from normal pregnant women, and that administering GLN at physiologic levels reduces HUVEC ICAM-1 expression induced by preeclamptic plasma.

Keywords: glutamine; preeclampsia; human umbilical vein endothelial cells; adhesion molecules.

Introduction

Preeclampsia is a pregnancy-specific syndrome in a previously healthy women characterized by increased blood pressure, proteinuria and/or pathologic edema appearing after 20 weeks of gestation.[1] The mechanisms leading to this syndrome have not been clarified. Recent studies suggest that endothelial activation/dysfunction is a central pathogenic feature in women with preeclampsia.[2,3] Previous reports revealed that circulating levels of intracellular adhesion molecules (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 are increased in women with preeclampsia.[4-6] Soluble CAM levels were associated with the severity of the disease, and have been thought to be markers of endothelial activation in this disease.[4-6] ICAM-1 and VCAM-1 which belong to adhesion molecules of immunoglobin gene superfamily are expressed by activated endothelium, and are important in the adhesion of monocytes, lymphocytes and neutrophils to activated endothelium.[7,8] Previous reports showed that ICAM-1 and VCAM-1 expressions in human umbilical endothelial cells (HUVEC) was induced by plasma from preeclamptic patients.[6,9]

Glutamine (GLN) is the most abundant free amino acid in plasma and tissue pool. It is (1) substrate for protein synthesis and precursors for nucleic acid biosynthesis, (2) an important carrier of nitrogen and carbon, (3) a precursor for gluconeogenesis in the liver, (4) a role in the acid-base balance homeostasis in the kidney and the liver. Being synthesized by body cells, GLN is formerly classified as a nonessential amino acid.[10-12] Over the past 15 years, GLN has been found to have great interest because of its roles in being a critical substrate for enterocytes and rapid proliferating immune cells, and having immuno-enhancing properties.[15-18] Previous reports showed that a relatively GLN-deficient state occurs during catabolic process, and can be corrected following GLN supplementation.[19,20] GLN is considered as an essential amino acid if the patients have certain catabolic, inflammatory and infectious conditions.[21-23] A study done by Fukatsu et al.[24] showed that compared with conventional total parenteral nutrition, GLN-supplemented parenteral nutrition reduced ICAM-1 expression in intestinal homogenates. Another study done by Arndt et al.[25] demonstrated that administering GLN reduced leukocyte adhesion and transmigration in indomethacin-induced intestinal inflammation in rats.

To our best knowledge, the GLN effect on CAM expression in preeclampsia has not been investigated. In this study, we are aiming to test the hypotheses that a chronic inflammation during preeclampsia may deplete plasma GLN, and that administering GLN comparable to physiological levels may decrease CAM expression in HUVEC induced by preeclamptic plasma.

MATERIALS AND METHODS

Patients and sample information

The pregnant women were recruited from Taipei Medical University-Wan Fang Medical Center. Women with single pregnancies but without chronic diseases were included in this study. Normal pregnant controls (n = 10) were one without hypertensive and proteinuric conditions, gave birth at between 37-42 weeks and their pregnancy was not complicated with fetal growth retardation or other fetal or maternal problems. Preeclampsia (n = 20) was diagnosed if a patient had: (1) the reading of blood pressure was > 140/90 mmHg on at least two occasions occurring after the 20th week of gestation, and (2) more than 300 mg protein in a 24 hr urine collection or 1+ proteinuria detected on reagent strip on two occasions more than 4 h apart.[5] The pregnant controls had mean gestational age of 37.6 ± 0.9 weeks (range 36-41 weeks) and preeclamptic women had 37.3 ± 1.9 weeks (range 34-40 weeks). Of those 20 preeclamptic patients, we collected 5 plasma samples for in vitro study from patients with severe preeclampsia, which was defined as blood pressure being higher than 160/110 mmHg at two occasions 6 h apart and having proteinuria corresponding to > 2+ detected on reagent strip on two occasions more than 4 h apart.[5] The protocol was approved by the TMU-WFMC Ethical Committee, and all subjects provided written informed consent prior to their participation in this study.

HUVEC isolation and culture

HUVECs were isolation as described elsewhere.[26] Briefly, we cannulated and washed the umbilical vein with PBS and then perfused with PBS containing 0.1% collagenase for 10 min at 37℃ in 5% CO_2. Primary cells were collected and plated into 75 cm^2 tissue culture flasks with medium-199 (M-199) containing 20% fetal calf serum (FBS), 20mM $NaHCO_3$, 25mM HEPES, antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin), 10 IU/ml heparin sodium and 15mg/L endothelial cell growth factor at 37℃ in 5% CO_2 /95% humidity. Cells were serially passaged 2-3 for experimental assay. HUVEC (1×10^5 cells/well) from second subcultures were grown on 24-well plate. Every 48-72 h the medium was replaced until the cells reached confluence and were detached with typsin-ethylenediamenetetra-acetic acid (EDTA). When the cells reached a confluent monolayer, they were then incubated in M-199 (with 20% FBS) with different concentrations of GLN (0, 300, 500 uM) for 24 hr. Subsequently, the cells were washed twice with PBS and cultured with various concentrations of GLN (without FBS) and stimulated with 100uL plasma from severe preeclamptic women. Then, we analyzed ICAM-1 and VCAM-1 expression on HUVEC by flow cytometry.

Measurements and analytical procedures

Plasma amino acid analysis

We took blood samples from the healthy pregnant and preeclamptic women at the time they came to the clinic for obstetric care between 34-36 weeks gestational age. We also collected 6 age-matched nonpregnant women as normal control. Antecubital venous blood was drawn from fasting women into EDTA tubes. Blood was centrifuged at 300g for 10 min at 4°C, and plasma was stored at –70°C until assay. We analyzed plasma amino acids with standard ninhydrin technology (Beckman Instruments, model 6300, Palo Alto, CA), after deproteinization of the plasma with 5% salicylic acid.[27]

ICAM-1 and VCAM-1 expression of HUVECs

HUVECs surface expression of ICAM-1 and VCAM-1 were measured after stimulated by preeclamptic plasma for 1.5, 3, 4.5 and 6 hr, because the CAM expression reached a peak at 6 hr after stimulation. After removing supernatant, we washed the cells twice with PBS, resuspended them in 100 ul of M-199 (FBS free and containing 5mM EDTA), and incubated for a further 30min at 4℃ with fluorescein-conjugated mouse anti-human VCAM-1(CD 106) and phycoerythrin conjugated mouse anti-human ICAM-1(CD 54). The cells suspension was collected into tube and suspended in 500uL of PBS (containing 0.3 ml of 350mM formaldehyde). The fluorescence intensity of 5000 cells population was counted and analyzed with flow cytometry (Coulter, Miami, FL, USA).

Measurement of IL-8 and NO in culture medium

We determined IL-8 concentrations by enzyme-linked immunosorbent assay. The human IL-8 enzyme-linked immunosorbent assay kit was purchased from BioSource International (Camarillo, CA, USA). NO concentrations were determined with a commercial kit (R&D Systems Inc., Minneapolis, MN, USA). NO is a highly unstable molecule, and is converted to stable nitrite and nitrate ions in aqueous solution. After conversion of nitrate to nitrite using nitrate reductase, nitrite concentrations were measured using the Griess reagent. Procedures are described in the manufacturer's instruction.

Statistical analysis

Data were presented with mean ± SD. Differences among groups were analyzed by ANOVA using Duncan's test. The differences were considered significant if p value was smaller than 0.05.

RESULTS

Plasma GLN levels

Plasma GLN levels in normal pregnant group were significantly higher than those in the preeclampsia groups (normal pregnant 509.5 ± 52.1 vs. preeclamptsia 349.8 ± 55.1 umol/L, $p < 0.05$), but were lower than the normal non-pregnant women (570.5 ± 46.4 umol/L, $p < 0.05$).

CAM expression in HUVEC induced by plasma from preeclamptic women

As shown in Fig. 1, ICAM-1 expression in HUVEC were significantly lower at 500 uM than at 0, 300 uM GLN after incubation for 3, 4.5, and 6 hr ($p < 0.05$, power > 80%). There were no differences among various GLN concentrations at 0 and 1.5 hr after incubation. No differences were observed in HUVEC VCAM-1 expression among various GLN concentrations at each time point (Fig. 2).

IL-8 and NO production from endothelial cells

No differences were found in IL-8 production among various GLN levels at different time points after incubation (Fig. 3). No difference in NO levels were also observed among various GLN concentrations at different time points after incubation (Fig. 4).

Discussion

In this study, we observed that pregnant women had lower plasma GLN levels than non-pregnant controls, possibly result from hemodilution during the pregnancy. The finding of this study also showed that plasma from women with preeclampsia had significantly lower GLN levels than that from normal pregnant women. This finding was compatible with the previous reports that plasma GLN was reduced during a catabolic conditions such as inflammatory, infection, and injury.[17,21-23] To understand whether physiologic levels of GLN may reduce CAM expression on endothelial cell stimulated by preeclamptic plasma, we pretreatment the endothelial cells with or without GLN including: 0 uM, 300 uM (similar to preeclamptic patients) and 500 uM (similar to normal pregnant women). The findings showed that administering GLN at levels similar to physiological condition reduced HUVEC ICAM-1 expression, but that VCAM-1 did not alter when same concentration of GLN was administered. Since the ICAM-1 and VCAM-1 receptors expressed on immune cells differ,[28] the GLN effect on different CAM expression may vary. ICAM-1is the ligand counterpart of integrins on polymorphonuclear neutrophils, reduced ICAM-1 expression may decrease polymorphonuclear neutrophil-endothelium interaction and thus attenuate tissue injury.[7]

Preeclampsia is a multiple system disorder. Redman et al. [29] suggest that preeclampsia is an excessive maternal inflammatory response to pregnancy, and that the endothelial dysfunction is a part of a more generalized intravascular inflammatory reaction. Cytokines which are peptides produced by cells of the immune system that act as mediators of the immune response and the response to inflammation. Previous studies have shown that some cytokines were associated with the initiation of preeclampsia.[30,31] IL-8 which is a potent chemo-attractant for leukocytes to implicate inflammation, initiates the acute inflammatory cascade and is considered as an early marker of the inflammatory process.[32] Previous report revealed that an increased IL-8 level can increase permeability in endothelial cells from preeclampsia.[31] Study by Fowler et al.[33] showed that exogenous NO suppresses IL-8 gene expression in activated endothelial cells by inhibiting NF-kB binding to DNA. Since ICAM-1 is regulated transcriptionally by NF-kB, inhibition of NF-kB may result in reduced ICAM-1 expression.[9] An in vitro study by Huang et al.[34] showed that GLN decreases LPS-induced IL-8 production in Caco-2 cells. In this study, we found that pretreatment with 500 uM GLN reduced ICM-1 expression in vascular endothelial cells 3, 4.5 and 6 hr after stimulation with preeclamptic plasma. However, no differences were observed in IL-8 and NO levels between various GLN concentrations at different time points. Wang et al.[35] showed that maternal plasma from pregnancies with umbilical placental vascular disease did not affect endothelial cell expression of NO synthase. A recent study by Wang et al.[31] revealed that no difference in IL-8 production was found between endothelial cells from normal and preeclamptic pregnancies. Those studies showed that endothelial cell IL-8 and NO production may not responsible for endothelial cells activation in preeclampsia. Based on the results of this study, we found that GLN did not influence endothelial cell IL-8 and NO production, and that the GLN effect to reduce endothelial ICAM-1 expression was independent of IL-8 and NO production.

GLN which is considered as the main fuel for rapid proliferating cells, can modulate immune response and has antioxidant effect under metabolic stress. Hong et al.[36] found that GLN supplemented nutrition protects the liver during hepatic injury by preserving glutathione (GSH) stores. An in vitro study by Babu et al.[37] also found that GLN can prevent liver from damage possibly mediated via GSH synthesis. GSH is a major antioxidant and acts as a vital component in host defense. GLN was found to be rate limiting for GSH synthesis, and the availability of GLN is critical to generate GSH stores.[38] Previous study showed that preeclampsia increased oxidative stress, and that plasma from women with preeclampsia had significantly higher malondialdehyde and lipid peroxide levels than those of normal pregnant women.[9] Lipid peroxide and oxidative stress up-regulate endothelial NF-kB activity and ICAM-1 expression.[9] Based on the findings of this study, we suggest that the GLN antioxidant property may be partly implicated in reducing HUVEC ICAM-1 expression. Whether other mechanisms are also involved in the favorable effect of GLN on preclamtic plasma-induced CAM expression requires further investigation to clarify.

In summary, our study revealed that plasma from women with preeclampsia had significantly lower GLN levels than normal pregnant women. Administering GLN at levels similar to physiological condition reduces HUVEC ICAM-1 expression stimulated by preeclamptic plasma.

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Figure legends

Fig. 1. Intercellular adhesion molecule-1 (ICAM-1) expression (%) in HUVECs with various GLN concentrations at different incubation times. ICAM-1 expression was significantly lower at 500 uM than at 0 and 300 uM GLN after incubation for 3, 4.5, and 6 h. * $p < 0.05$ compared with 0, 300 uM GLN at the same time point.

Fig. 2. Vascular cellular adhesion molecule-1 (VCAM-1) expression (%) in HUVECs with various GLN concentrations at different incubation times. No differences were found among various GLN concentrations at each time point.

Fig. 3. Effect of pretreatment with different concentrations of GLN on IL-8 production from HUVECs after stimulation with preeclamptic plasma. There were no differences among various GLN concentrations at each time point.

Fig. 4. Effect of pretreatment with different concentrations of GLN on nitric oxide (NO) released from HUVECs after stimulation with preeclamptic plasma. There were no differences among various GLN concentrations at each time point.

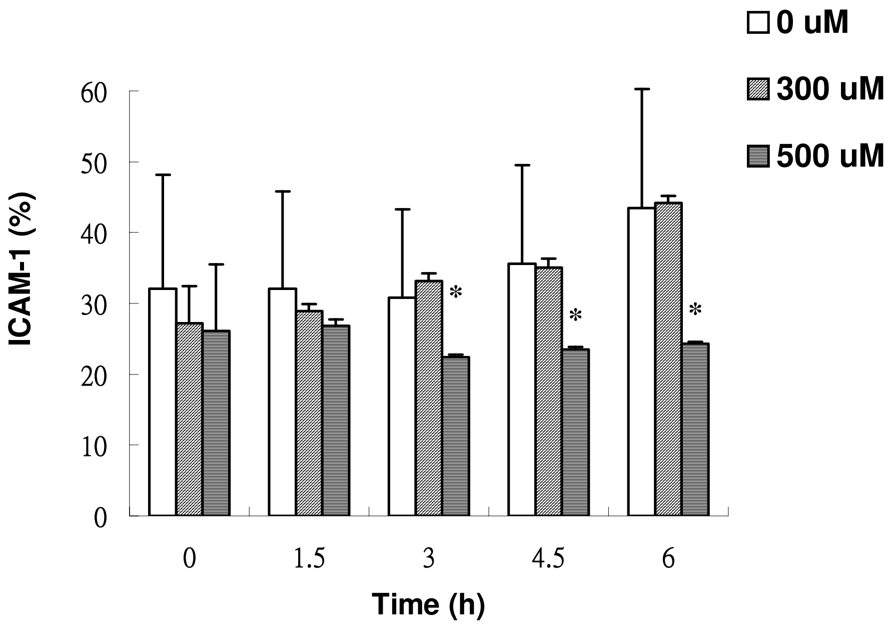

Fig. 1

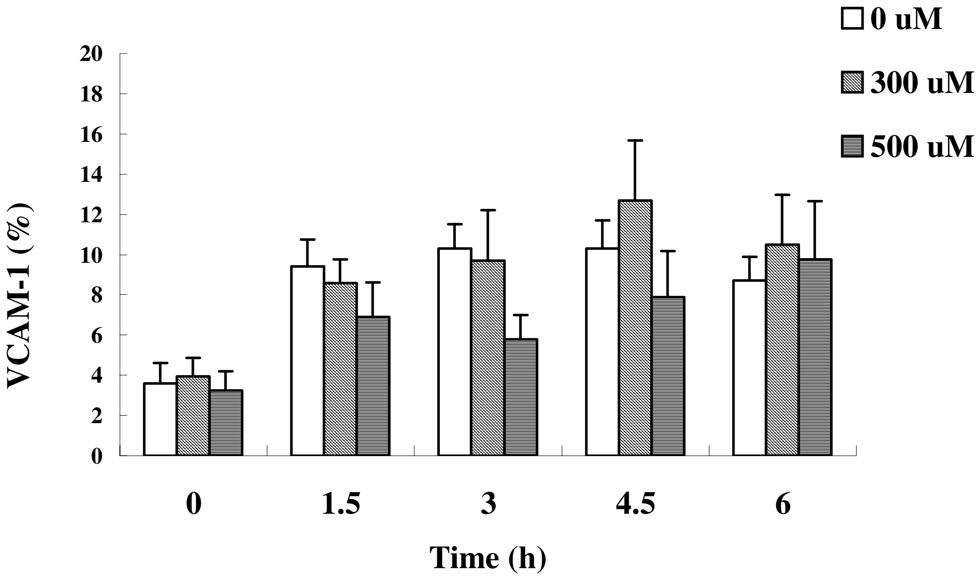

Fig.2

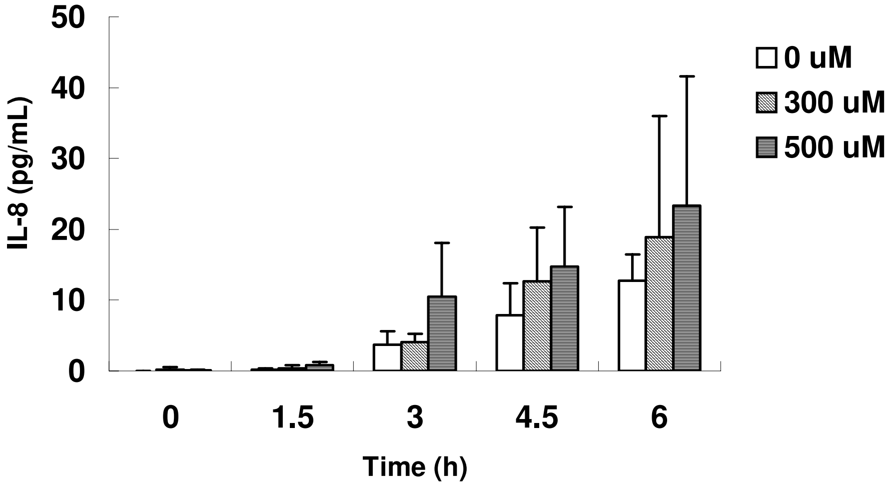

Fig. 3

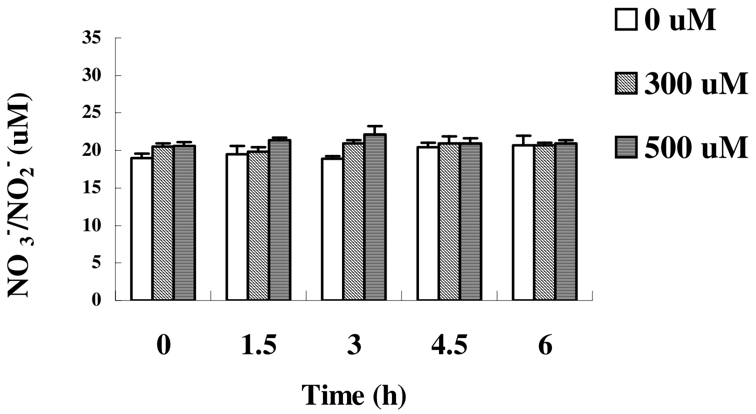

Fig. 4