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Chymotrypsin-Like Protease (Chymase) Mediates Endothelial Activation by Factors Derived From Preeclamptic Placentas

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Abstract

Endothelial cells (EC) activation is an important inflammatory phenotypic change in the vascular system in women with preeclampsia (PE). In PE, maternal vessel chymotrypsin-like protease (CLP)/chymase expression was increased. Chymase is an inflammatory protease. In this study, we specifically examined whether placental-derived CLP could induce EC activation and whether EC activation is associated with increased cellular protease expression. Human uterine microvascular endothelial cells (UtMVECs) were used. Endothelial activation was determined by endothelial adhesion molecule P-selectin, E-selectin, inter-cellular adhesion molecule (ICAM), and vascular cell adhesion molecule (VCAM) expressions and by extracellular regulated kinase (ERK) activity. Activation of endogenous CLP/chymase associated with ERK phosphorylation was further examined by CLP/chymase short interfering RNA (siRNA). Our results showed that cells treated with PE placental conditioned medium revealed increased P-selectin, E-selectin, and VCAM-1 expressions and increased ERK phosphorylation. Increased endothelial adhesion molecule expression and phosphorylated ERK (pERK) induction could be attenuated or abolished by depletion of CLP in the conditioned medium or by transfecting ECs with CLP/chymase siRNA. These observations suggest that placental-derived CLP/chymase is responsible for inducing endothelial inflammatory phenotypic changes possibly by upregulation of cell adhesion molecule expressions, activation of cellular protease, and induction of ERK phosphorylation. We speculate that activation of endothelial CLP/chymase may directly relate to the increased inflammatory phenotypic changes in the vascular system in women with PE.

Keywords

Endothelial activation; adhesion molecules; CLP/chymase; preeclampsia

INTRODUCTION

Endothelial conversion toward an excessive inflammatory phenotype is a central pathophysiological event of the maternal vascular system in women with preeclampsia (PE).^{1,2} The concept of endothelial activation with inflammatory changes is supported by the findings of increased circulating levels of endothelial adhesion molecules^{3,4} and inflammatory cytokines.² Endothelial activation/dysfunction in PE is also evidenced by increased endothelial permeability,^{5,6} decreased endothelial-derived vasodilator

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prostacyclin,^{7,8} increased cellular fibronectin release, and increased neutrophil and platelet interaction and adhesion to the vessel endothelium.^{9,10}

How endothelial cells become activated/dysfunctioned in PE remains poorly understood. Many believe that the placenta is a source of blood-borne toxic factors that alter endothelial, platelet, and leukocyte functions during pregnancy.^{11,12} However, identifying circulating factors released from the placenta is a great challenge. A recent study by Germain et al showed that circulating syncytiotrophoblast microparticles (STBMs) could bind to monocytes and stimulate the production of inflammatory cytokines.¹³ They concluded that STBMs are potential contributors to the altered systemic inflammatory responsiveness in pregnancy and PE. We previously reported that factors released from preeclamptic placentas could promote neutrophil-endothelial adhesion, and upregulate CD11b and downregulate CD62L expressions on neutrophils.^{14,15} Using our cell coculture model (coculture of endothelial cells with placenta trophoblasts), we found that chymotrypsin-like protease/chymase (CLP) produced by placental trophoblasts could specifically promote endothelial selectin (P-selectin and E-selectin) expressions,¹⁶ suggesting that placental-derived CLP could be a candidate agent that induces endothelial activation in PE.

In endothelial cells, CLP/chymase immunoreactivity was found to be localized in Weibel-Palade bodies along with adhesion molecule P-selectin, cytokine interleukin 8 (IL-8), and vasoconstrictor endothelin.^{17,18} It is well known that P-selectin is upregulated and mobilized to the cell surface and/or shed into extracellular space when endothelial cells are activated. Activated endothelial cells release IL-8. Both of P-selectin and IL-8 play roles in the increased inflammatory response in women with PE.^{19,20} Recently, we found that CLP immunoreactivity was increased in systemic vessel endothelium in women with PE.²¹ The co-localization of CLP with inflammatory agents of P-selectin and IL-8 supports the notion that CLP/chymase could mediate increased inflammatory response in the maternal vessel endothelium in women with PE. In this study, we tested our hypothesis that factors derived from preeclamptic placentas could induce endothelial CLP activation and play a role in converting endothelial cells to the inflammatory phenotypic changes relevant to PE.

MATERIALS AND METHODS

Endothelial Cell Culture

Uterine myometic vascular endothelial cell (UtMVECs) were purchased from Cambrex (Charles City, IA). UtMVECs were cultured with endothelial cell growth medium (BioWhittaker, Inc, Walkersville, MD). Cells used for adhesion molecule expression studies were grown in 48-well cluster cell culture plates and for protein expressions were grown in 25-cm² flasks. Human umbilical cord vein endothelial cells (HUVECs) were isolated from normal placentas and cultured as previously described.²²

Placental Conditioned Medium Preparation

Placentas from normal and preeclamptic pregnant women were obtained immediately after delivery and processed for preparation of placental conditioned media. As we previously described,¹⁴ placental villous tissues were incubated with serum-free Dulbecco's Modified Eagles Medium (DMEM; Sigma, St. Louis, MO) for 48 hours. Medium samples collected at the end of incubation were stored at -80°C and used as conditioned medium for the study. The study was approved by the Institutional Review Board (IRB) for Human Research at Louisiana State University Health Sciences Center in Shreveport (LSUHSC-S), LA.

Surface Molecule Expression Assay

Confluent endothelial cells were treated with placental conditioned medium or maternal serum with or without depletion of protease chymotrypsin. Depletion of chymotrypsin in conditioned media and in sera was performed by immunoprecipitation (IP) using Protein-G immunoprecipitation kit (Sigma). The immunoprecipitation was performed according to the manufacturer's instruction as previously described.²³ Briefly, monoclonal antibody specific for human chymotrypsin was reacted with protein-G agarose for 1 hour, and then placental conditioned medium was added to the antibody/protein G mixture. For serum samples, the antibody was reacted with protein-G agarose for 4 hours before 20% serum was added to the antibody/protein G mixture. The sample/antibody/protein G mixtures were then incubated with constant mixing at 4°C overnight. This procedure allows chymotrypsin in the sample to bind to antibody/protein-G complex. The eluted conditioned medium or serum (without chymotrypsin) and non-IP conditioned medium or 20% serum samples (with chymotrypsin) were used to treat endothelial cells. Assays of endothelial surface adhesion molecule expressions for P-selectin, E-selectin, inter-cellular adhesion molecule (ICAM), and vascular cell adhesion molecule (VCAM) were carried out as previously described.²² Primary antibodies (mouse anti-human) to P-selectin, E-selectin, ICAM-1 (CD54), and VCAM-1 were purchased from Southern Biotechnology Associates, Birmingham, AL. The secondary antibody horseradish peroxidase (HRP)-goat anti-mouse immunoglobulin G (IgG) was purchased from Sigma. All cultures were treated in triplicate; untreated cells reacted only with secondary antibody served as background control.

Immunofluorescent Staining

Confluent endothelial cells grown on glass coverslips were treated with normal or preeclamptic placental conditioned medium for 4 hours. The cells were fixed with 95% ethanol, permeabilized with 50% acetone, and then stained with monoclonal antibody against human chymotrypsin (Abcam, Cambridge, MA). Cy3 labeled donkey anti-mouse IgG (H+L) was used as the secondary antibody (Jackson Immunoresearch laboratories Inc, Westgrove, PA). Stained cells were examined by fluorescent microscope (Olympus IX71). Fluorescent images were recorded by a digital camera linked to a computer with Picture-Frame software (Optronics Inc, Sunnyvale, CA).

Western Blot Analysis

Total cellular protein, 10 µg per sample, was subject to electrophoresis using a Mini-cell protein-3 gel running system (Bio-Rad, Hercules, CA). Antibodies against extracellular regulated kinase (ERK) and phosphorylated ERK 44/42 (pERK) were purchased from Cell Signaling Technology, Inc (Beverly, MA). β-actin expression was used as an internal control for each sample. Membranes were stripped, blocked, and then re-probed with different primary and secondary antibodies. To confirm the success of chymotrypsin depletion from conditioned medium or serum, immunoblot was also performed for immunoprecipitated samples. Both eluted (without chymotrypsin) and protein G mixture (with chymotrypsin) samples (10 µg/sample) were run on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). The membranes were hybridized with antibody against chymotrypsin.

Short Interfering RNA Preparation and siRNA Transfection

Chymotrypsin-like protease/chymase specific siRNA was used to knock down CLP induced endothelial inflammatory response. Specific short interfering RNA (siRNA) duplex sequences were designed according to Promega siRNA target designer program (http://www.promega.com/SiRNA_Designer). The DNA oligos (sense: 5'-GGA TCC TAA TAC GAC TCA CTA TAG AGG AAG AAG ACA CAT GGC-3' and anti-sense: 5'-GGA

TCC TAA TAC GAC TCA CTA TAG GAG GAT CTC CTT AAA TGC-3') were synthesized by Integrated DNA Technologies, Inc (IDT, Coralville, IA). The selected sequence regions were verified, and no match was found with any other human gene (using the NCBI standard nucleotide-nucleotide BLAST program). The 5' primer contains a 23bp T7 promoter sequence. The oligos produced a 550 base pairs (bp) DNA fragment. Double-stranded RNA (dsRNA) and siRNA were prepared by using a silencer siRNA cocktail kit (RNase III; Ambion Inc, Austin, TX). Control siRNA was purchased from Santa Cruz (San Diego, CA). Short interfering RNA transfection was carried out when cells were approximately 70% confluent with siPORT Lipid Transfection Agent (Ambion Inc, Austin, TX) according to the manufacturer's instructions. P-selectin and E-selectin expressions were assayed 24 hours after transfection.

Data Analysis

Data are expressed as mean $\pm$ SE and analyzed by analysis of variance (ANOVA). Computer software StatView (SAS Institute, Inc, Cary, NC) was used. A *P* level less than .05 was considered statistically significant.

RESULTS

Upregulation of Endothelial Adhesion Molecule Expression

To determine whether placental-derived CLP could induce endothelial P-selectin, E-selectin, ICAM, and VCAM expressions, endothelial cells were treated with placental conditioned medium with or without depletion of CLP. We found no difference for P-selectin, E-selectin, ICAM-1, and VCAM-1 expressions in cells treated with normal placental conditioned medium with or without depletion of CLP. In contrast, increased P-selectin, E-selectin, and VCAM-1 expressions induced by preeclamptic conditioned medium were abolished when CLP-depleted medium was used, $P < .01$ (Figure 1A). These results indicate that CLP produced by preeclamptic placentas could promote endothelial activation by upregulating endothelial adhesion molecule expression. Figure 1B shows a representative immunoblot for chymotrypsin in normal and preeclamptic conditioned media after immunoprecipitation. The result confirms the success of depleting CLP from conditioned media. The data also shows that much less CLP was present in the normal conditioned medium than in the preeclamptic conditioned medium.

Endothelial CLP Expression

Chymotrypsin-like protease/chymase has been considered as an inflammatory protease. To determine if placental-derived factors could upregulate CLP expression in endothelial cells, cells were exposed to normal and preeclamptic placental conditioned medium for 1 hour and then chymotrypsin expression was examined by immunostaining. Our results showed that positive protease expression was observed in cells treated with preeclamptic placental conditioned medium (Figure 2B). In contrast, the protease expression was not seen in control cells (Figure 2A) or in cells treated with normal placental conditioned medium (data not shown). These results suggest that factors derived from preeclamptic, but not from normal, placentas could induce endothelial protease activation.

Protease Activation Is Responsible for Upregulation of Adhesion Molecule Expression

We next examined if preeclamptic conditioned medium induced upregulation of adhesion molecule expressions is a consequence of cellular protease activation in endothelial cells. In this experiment, cells were transfected with CLP siRNA or control siRNA prior to exposure of placental conditioned medium. Endothelial adhesion molecule P-selectin and E-selectin expressions were then examined. As shown in Figure 3, CLP siRNA (Figure 3A), but not

control siRNA (Figure 3B), could attenuate the upregulation of P-selectin and E-selectin expressions induced by preeclamptic conditioned medium. These data demonstrated that upregulation of P-selectin and E-selectin expressions could be a consequence of the protease activation in endothelial cells.

Induction of ERK Phosphorylation

Numerous studies have shown that induction of ERK phosphorylation is a step of endothelial activation. To determine if upregulation of cellular protease is accompanied by ERK phosphorylation, ERK and pERK expressions were determined in cells treated with preeclamptic conditioned medium for 15 minutes, 30 minutes, and 2 hours. Our data showed that there was no change for nonphosphorylated ERK expression, but pERK was intensively expressed in cells after exposure to preeclamptic conditioned medium (Figure 4). To further evaluate if placenta-derived CLP or activation of endothelial protease by placenta derived CLP is responsible for inducing ERK phosphorylation, 2 experiments were performed: (1) cells were treated with conditioned medium after depleted with CLP; and (2) cells were transfected with CLP/chymase siRNA prior to conditioned medium stimulation, and then ERK expression was determined. As we expected, pERK expression induced by preeclamptic conditioned medium was not seen in cells treated with the CLP-depleted medium (Figure 5A). Consistently, pERK expression induced by preeclamptic conditioned medium could be attenuated in cells transfected with CLP/chymase siRNA (Figure 5B).

Effects of Circulating CLP on Endothelial P-Selectin and E-Selectin Expression

If placenta-derived CLP contributes to the maternal CLP levels during pregnancy, then circulating CLP could promote endothelial activation. To answer this question, cells were treated with sera from normal and preeclamptic pregnant women with or without depletion of chymotrypsin and then P-selectin and E-selectin expressions were determined. The result is shown in Figure 6. P-selectin and E-selectin expressions were moderately increased in cells treated with sera from normal pregnant women ($n = 4$), but significantly increased in cells treated with sera from women with PE ($n = 4$), $P < .01$. CLP-depleted sera had less effects to induce endothelial P-selectin and E-selectin expressions.

DISCUSSION

We previously reported that placental-derived CLP was responsible for inducing endothelial inflammatory response as determined by increased endothelial adhesion molecule expressions.¹⁶ In this study, we specifically examined placental-derived CLP-induced endothelial activation. Our results showed that depletion of CLP in the conditioned medium eliminated preeclamptic conditioned medium-induced increases in P-selectin and E-selectin expressions in endothelial cells. These findings are significant pertaining to the increased inflammatory response in PE. It is well known that early phase of endothelial inflammatory response is associated with rapid mobilization of adhesion molecules P-selectin and E-selectin onto the cell surface. These molecules then mediate the tethering and rolling process of leukocytes and platelets on endothelial cells.²⁴ Activated endothelial cells also shed P-selectin and E-selectin into the circulation. Maternal P-selectin and E-selectin levels are elevated in women with PE.^{19,25} Thus, our data support the idea that placental-derived CLP could be a candidate agent derived from the placenta that induces or initiates endothelial activation and inflammatory response in PE.

The notion that CLP mediates endothelial activation is also supported by the observation of increased CLP expression in endothelial cells after stimulated by preeclamptic conditioned medium. Although, it is not known whether endothelial CLP activation is a direct effect upon placental CLP stimulation or an indirect effect or consequence of endothelial

activation in general when exposed to placental-derived stimuli, our data support the notion that CLP/chymase activation is associated with inflammatory response in endothelial cells, because CLP/chymase is considered as an inflammatory protease.²⁶ By siRNA transfection, CLP-mediated endothelial cell activation was further confirmed. CLP/chymase is localized in Weibel-Palade bodies of endothelial cells¹⁸ and could be released when cells were activated.²⁷ Adhesion molecule P-selectin, cytokine interleukin-8, and vasoconstrictor endothelin are also stored in Weibel-Palade bodies of endothelial cells.¹⁷ Therefore, when cells are activated these inflammatory molecules could be released or upregulated and consequently involved in the downstream inflammatory responses in the vascular system.

Endothelial activation induced by placenta-derived CLP was further examined by ERK expression. Activation ERK is a key step in the increased inflammatory response in endothelial cells. Induction of ERK phosphorylation (pERK) was observed in cells treated with preeclamptic conditioned medium. In contrast, depletion of CLP in the conditioned medium or CLP siRNA transfection eliminated the conditioned medium-induced ERK phosphorylation. These observations provide further convincing evidence that CLP mediates endothelial activation. Although in this study we did not specifically examine the downstream signaling cascade after ERK activation induced by CLP, previous published works have shown that serine proteases including trypsin and chymotrypsin could induce ERK activation and increase intracellular Ca^{2+} through G-protein coupled protease-activated receptor-2 (PAR-2).^{28,29}

Maternal CLP activity was increased in PE.²¹ In this study, we found that both normal and preeclamptic sera could induce endothelial P-selectin and E-selectin expressions, but sera from PE had a strong stimulatory effect. We also noticed that depletion of CLP in the sera significantly reduced, but not completely abolished, the stimulatory effect on endothelial adhesion molecule expression. These observations support the concepts: (1) circulating factors, other than CLP, are able to induce endothelial activation; and (2) circulating factors are toxic to vascular endothelial cells in PE.

In summary, our data support the hypothesis that placental-derived CLP/chymase could be a candidate agent to induce endothelial cell activation in PE via promoting cell adhesion molecule expression, upregulating protease expression, and inducing ERK activation. Recently, we reported enhanced endothelial CLP expression in maternal vessels from women with PE.²¹ We also found that placental-derived CLP/chymase may contribute to increased vasoconstriction in PE possibly via regulation of local angiotensin II generation,³⁰ because CLP/chymase is a potent non-ACE angiotensin II generating enzyme in the vascular tissue.³¹ Therefore, we speculate that activation of endothelial CLP may represent a signal amplification mechanism leading to the inflammatory phenotypic changes that occur in the vascular system associated with PE. Thus, further study of CLP/chymase regulation in the vascular tissue may provide an insight into the mechanism of the protease in the pathophysiology of PE.

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Figure 1.

Effects of placental-derived chymotrypsin-like protease (CLP) on endothelial adhesion molecule P-selectin, E-selectin, inter-cellular adhesion molecule 1 (ICAM-1), and vascular cell adhesion molecule 1 (VCAM-1) expressions. A, Endothelial cells were treated with normal or preeclampsia (PE) conditioned medium with or without depletion of chymotrypsin for 2 hours. PE-conditioned medium (PE-CM), but not Nor-CM, could upregulate P-selectin, E-selectin, and VCAM-1 expressions. The PE-CM induced increases in P-selectin, E-selectin, and VCAM-1 expressions were not seen in cells treated with CLP-depleted medium. $**P < .01$: PE-CM vs. control; $\#P < .05$, $##P < .01$: PE-CM vs. Nor-CM; and $\Delta\Delta P < .01$: CLP-depleted PE-CM vs. PE-CM, respectively. Data are means $\pm$ SE from 5 independent experiments. B, A representative immunoblot of chymotrypsin expression in normal-CM and PE-CM after immunoprecipitation (IP), eluted medium (E) without chymotrypsin and IP sample with chymotrypsin, respectively.

Figure 2. Endothelial chymotrypsin-like protease (CLP) expression. A, control cells; B, cells treated with PE placental conditioned medium (CM). CLP expression was upregulated in cells after exposure to PE-CM.

Figure 3.

Effects of chymase short interfering RNA (siRNA) on P-selectin and E-selectin expressions in endothelial cells treated with normal and preeclampsia (PE) placental conditioned medium. CLP siRNA (A), but not control siRNA (B), abolished the upregulation of P-selectin and E-selectin expressions in endothelial cells induced by PE conditioned medium. * $P < .05$ and ** $P < .01$: PE-CM vs. control and Nor-CM; $\Delta\Delta P < .01$: PE-CM + chymase siRN (siChym) vs. PE-CM, respectively. Data are means $\pm$ SE from 3 independent experiments.

Figure 4.

Extracellular regulated kinase (ERK) expression in endothelial cells stimulated with preeclampsia (PE) placental conditioned medium. Endothelial cells (ECs) were exposed to PE-CM for 15 minutes, 30 minutes, and 2 hours. Total cellular protein was extracted. ERK and phosphorylated ERK (pERK) expressions were examined by Western blot. pERK expression was intensively expressed in cells treated with PE-CM. These data indicate that factors derived from PE placentas could induce ERK activation. $^{**}P < .01$: PE-CM treated vs. control. Data are means $\pm$ SE from 3 independent experiments.

Figure 5.

Endothelial extracellular regulated kinase (ERK) expression. A, Endothelial cells (ECs) were stimulated with placental CM with or without depletion of chymotrypsin. Endothelial cells were exposed to conditioned medium for 30 minutes. $*P < .05$ and $**P < .01$: CM treated vs. control; and $###P < .01$: chymotrypsin depleted PE-CM vs. PE-CM, respectively. B, ECs were transfected with CLP/chymase short interfering RNA (siRNA), $**P < .01$: PE-CM treated vs. control; and $##$ siRNA + PE-CM vs. PE-CM, respectively. Data are means $\pm$ SE from 3 independent experiments. The increased phosphorylated ERK (pERK) expression in cells stimulated by PE-CM could be abolished after CLP/chymase was depleted or attenuated by siRNA transfection. These results suggested that activation of ERK signaling pathway is involved in endothelial activation process either induced by placental derived CLP/chymase or by activation of endogenous CLP/chymase in endothelial cells.

Figure 6.

Effects of circulating chymotrypsin-like protease (CLP) on P-selectin and E-selectin expressions. Endothelial cells were treated with 20% serum samples from normal and preeclampsia (PE) pregnant women with or without depletion of chymotrypsin. Both normal and preeclamptic serum samples could promote P-selectin and E-selectin expressions, but sera from PE had significant effects, $P < .01$, respectively. CLP-depleted sera had much less effects on endothelial P-selectin and E-selectin expressions. NS: normal sera ($n = 4$); and PE serum (PS): PE sera ($n = 4$). * $P < .05$: NS vs. controls (C); ** $P < .01$: PS vs. all other groups, respectively.