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Placenta-derived Chymotrypsin-like Protease (CLP) Disturbs Endothelial Junctional Structure in Preeclampsia

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Abstract

Placenta-derived chymotrypsin-like protease may contribute to endothelial activation in preeclampsia. In this study, we determined if placenta-derived chymotrypsin-like protease could disturb endothelial junctional integrity to promote endothelial permeability in preeclampsia. Confluent endothelial cells were cocultured with placental trophoblasts or treated with preeclampsia placenta-conditioned medium. Endothelial junction protein vascular endothelial cadherin expression and distribution were examined by fluorescent staining of endothelial cells with or without depletion of chymotrypsin. The association of endothelial cell junction protein complex VE-cadherin/β-catenin/p120 was examined by a combined immuno-precipitation and immuno-blotting assay. Our results showed that endothelial cells cocultured with preeclampsia trophoblasts or exposed to preeclampsia placental conditioned medium exhibited a discontinuous distribution and reduced expression of vascular endothelial cadherin at cell contact regions. Vascular endothelial cadherin and p120 were expressed in control endothelial cells, but reduced or lost in endothelial cells exposed to preeclampsia placental conditioned medium, suggesting that the junctional protein complex of VE-cadherin/β-catenin/p120 was disrupted in endothelial cells exposed to preeclampsia placental conditioned medium. We also observed that removal of trophoblasts from the coculture system and depletion of the protease from the preeclampsia placental conditioned medium could restore the dysregulated endothelial junction protein expression and distribution. Chymotrypsin also induced a dose dependent increase in endothelial monolayer permeability. We conclude that chymotrypsin-like protease released by the placenta is at least one important mediator responsible for disrupting endothelial cell integrity and inducing endothelial permeability in preeclampsia.

Keywords

CLP; endothelial permeability; VE-cadherin; preeclampsia

INTRODUCTION

Increased vascular permeability is an important event of microvascular dysfunction in pregnancies complicated by preeclampsia (PE), a hypertensive and multiple system disorder during human pregnancy. In vivo evidence of increased endothelial permeability in PE was reported by Campbell & Campbell[1] and Brown et al.[2] Both studies observed a higher rate of disappearance of Evans Blue labeled albumin from the vascular space of preeclamptic

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patients compared to normal pregnant controls, indicating increased endothelial permeability in the systemic vasculature of women with PE. Likewise, an ex vivo study conducted by Svedas et al also observed increased Evans Blue albumin flux in maternal vessels from women with PE compared with those from normal pregnant controls, using vessels obtained during cesarean section deliveries.[3] These studies confirmed that vascular permeability changes indeed occur in preeclampsia. Furthermore, our in vitro cell culture findings of disorganized endothelial junction protein distribution from PE[4] agree with the scanning electron microscopic observations by Svedas et al which showed disrupted and enlarged intercellular gaps in the maternal vessel endothelium.[5] Taken together, these findings argue that increased endothelial permeability is likely due to dysregulated endothelial junction integrity of the maternal vascular system in PE.

An important endothelial function in the vascular system is to create and maintain a restrictive barrier to solute movement between the intravascular and interstitial compartments. The maintenance of this barrier is critically dependent on the integration of the adhesion and tight junctions between the adjacent endothelial cells. Vascular endothelial cadherin (VE-cadherin) is an endothelial-specific adhesion protein located at the endothelial adhesion junctions.[6] Vascular endothelial cadherin plays a critical role in maintaining endothelial monolayer integrity by linking apposed endothelial cells to form intracellular junction complexes with catenins and p120. Importantly, VE-cadherin binding interactions appear to be a prerequisite for the establishment of tight junctions and distributions.

It has long been assumed that the placenta releases blood-borne factors into the maternal circulation during pregnancy, and toxic placenta-derived factors can induce endothelial injury and dysfunction in PE. Our previous published work has shown that placental trophoblast-derived factors diminish endothelial barrier function by altering endothelial junction molecule VE-cadherin and occludin expression and distribution to reduce endothelial electrical resistance.[7] However, the placental factors that alter endothelial barrier function have not been identified. The placenta releases serine protease such as chymotrypsin-like protease/chymase (CLP),[8] and recently, we found elevated CLP activity in the maternal plasma and enhanced CLP immuno-reactivity in the endothelium of systemic vessels from women with PE.[9] It is well accepted that cell junction proteins are sensitive to protease.[10] Thus, the objective of the present study was to determine if placenta-derived CLP contributes to the altered endothelial barrier function in preeclampsia.

MATERIALS AND METHODS

Sample Collection

Placentas and umbilical cords were collected immediately after delivery from normal pregnant women and from women with PE at the Main Hospital, Louisiana State University Health Sciences Center in Shreveport (LSUHSC-S), LA. Fourteen placentas (6 form normal and 8 from preeclampsia) were used either for trophoblast cell isolation or for placental conditioned medium (CM) preparation. Umbilical cords from normal placentas were used for endothelial cell isolation. Normal pregnancy is defined as a pregnancy in which the mother has normal blood pressure (≤ 140/90 mm Hg) without medical and obstetrical complications. The criteria for preeclampsia follow the Williams Obstetrics guideline.[11] Diagnosis of mild preeclampsia was defined as follows: sustained systolic blood pressure of ≥140 mm Hg or a sustained diastolic blood pressure of ≥90 mm Hg on 2 separate readings; proteinuria measurement of 1+ or more on dipstick, or 24-hour urine protein collection with ≥300 mg in specimen. Preeclampsia was defined severe if 1 or more of the following criteria is present: maternal blood pressure ≥160/110 mm Hg; proteinuria >3+ or >5 g/24 h; oliguria of less than 500 mL in 24 hours; intrauterine growth restriction, and presence of persistent headache or visual disturbances. In this study, only placentas delivered from severe PE were

used. Table 1 shows demographic characteristics for normal and preeclamptic pregnant women from which placentas were used. None of the study participants had prolonged rupture of membranes or signs of infection. Smokers were excluded. This study was approved by the Institutional Review Board (IRB) for Human Research at LSUHSC-S, LA.

Endothelial Cell Isolation

Human umbilical vein endothelial cells were isolated by collagenase digestion from normal placentas and cultured with endothelial cell growth medium (EGM; Lonza, Walkersville, Md) as previously described.[12] The purity of isolated endothelial cells was >98%. Cells for immunostaining were grown on glass cover ships in 24-well/plates. Cells used for protein expression were grown in 25 cm^2 culture flasks. Only the first passage cells (P1) were used in the study. All assays were performed when cells were confluent in culture.

Trophoblast Isolation and Placental Conditioned Medium Preparation

Placental trophoblast cells were isolated by Dispase digestion and purified by Percoll density gradient centrifugation as previously described.[13,14] The purity of isolated trophoblasts was >95%.[12] Freshly isolated trophoblasts were cocultured with endothelial cells (see below). Placental CM was prepared by culture of villous tissue with serum-free Dulbecco's Modified Eagle's Medium (DMEM, Sigma, St. Louis, Mo) as previously described.[14] Conditioned medium was stored at −80°C freezer until used for the assay.

Coculture of Endothelial Cells With Trophoblast Cells

Coculture of endothelial cells with trophoblast cells was carried out for studying endothelial cell VE-cadherin expression and distribution. Briefly, freshly isolated trophoblasts were placed into 24-well/8 μm pore size transwell inserts with a density of 1×10^5 cells per insert and then cocultured with confluent endothelial cells grown on glass cover slips. After 48 hours of coculture, trophoblast filters were removed and endothelial cover slips were fixed with 95% ethanol, permeabilized with acetone and then stained for the endothelial junction protein VE-cadherin.

Endothelial cells were also treated with placental conditioned medium with or without depletion of protease chymotrypsin. Depletion of chymotrypsin was performed by immunoprecipitation using protein-G immunoprecipitation kit (Sigma) according to the manufacturer's instruction. Briefly, monoclonal antibody specific for human chymotrypsin was reacted with protein-G agarose for 1 hour, then placental conditioned medium was added to the antibody/protein G mixture and incubated with constant head-over-tail mixing at 4°C overnight. This procedure allows chymotrypsin in the CM bind to antibody/protein-G complex. The eluded CM medium (without chymotrypsin) was used to treat ECs and CM without depletion was used as controls. The depletion of chymotrypsin was confirmed by immunoblot of antibody/protein-G complex samples. After immunoprecipitation, the antibody/protein-G complex was mixed with protein loading buffer. The samples were then run on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose, which was probed with anti-chymotrypsin antibody, the same antibody used for immunoprecipitation.

Immunofluorescent Staining

Immunofluorescent staining of junction protein VE-cadherin was performed as previously described.[4] Monoclonal mouse anti-human VE-cadherin (Immunotech, France) and Cy3 labeled secondary donkey anti-mouse IgG (H + L) were used (Jackson Immunoresearch laboratories Inc. Westgrove, Pa). After staining, cover slips were mounted on glass slides and examined by fluorescent microscopy (Olympus, Tokyo, Japan). For quantitative

measurement of VE-cadherin expression, 5 to 6 images were captured randomly per slide by a digital camera with PictureFrame computer software (Uptronics Inc., Sunnyvale, Calif) and recorded to a microscope-linked PC computer. All images were captured at × 100 magnification. The image covers an area of 10 800 μm^2 with 8 to 10 cells per image. The relative density for VE-cadherin expression was measured by computer software NIH Image J and the data was expressed as pixels/10 800 μm^2.

VE-cadherin or p120 Expression

For immunoprecipitation, after ECs were treated with preeclamptic conditioned medium, total cellular protein was extracted by lysis buffer and then immunoprecipitated with Protein-A immunoprecipitation kit (Sigma). The immunoprecipitation procedure was performed according to the manufacturer's instruction. An aliquot of 500 μg of total cellular protein per sample was used to precipitate with mAb against β-catenin (Santa Cruz, San Diego, Calif). The precipitated protein was then run on SDS-PAGE and transferred to nitrocellulose, and probed with antibody against VE-cadherin or p120 (Cell Signaling Technology, Inc. Beverly, Mass). The secondary antibody was either a horseradish peroxidase (HRP)-linked anti-mouse antibody or a HRP-linked anti-rabbit antibody. Samples were visualized with an enhanced chemiluminescent detection kit (Amersham Corp, Arlington Heights, Ill).

Endothelial Permeability Assay

Endothelial permeability was determined by measuring the passage of HRP in cells exposed to protease chymotrypsin through confluent endothelial monolayer grown on polycarbonate cell culture inserts as previously described.[4] The HRP enzymatic activity was measured by a spectrophotometer with the wavelength at 470 nm (Ultraspec 3000, Pharmacia Biotech). Data were calculated as OD470 nm sample-OD470 nm blank, and expressed as OD470 nm for permeation of HRP across transwell filters.

Data Analysis

Data are expressed as mean ± SE and analyzed by analysis of variances (ANOVA) or paired *t*-test using StatView computer software (Cary, NC). Student-Newman-Keuls test was used as a post hoc test. A probability level of less than .05 was considered statistically significant.

RESULTS

VE-cadherin Distribution in Endothelial Cells is Altered by Coculture With Trophoblasts From Preeclamptic Placentas

Figure 1 shows representative images of VE-cadherin expression and distribution in endothelial cells cocultured with trophoblasts from normal and preeclamptic placentas. Vascular endothelial cadherin staining is localized at cell contact regions in cells with or without coculture. The zipper-like structure is clearly seen around the cell borders and continuously expressed in control cells (Figure 1A) and cells cocultured with normal placental trophoblasts (Figure 1B), but reduced or became linear in cells cocultured with trophoblasts from preeclamptic placentas (Figure 1C). In cells cocultured with preeclamptic trophoblasts, multimorphology changes were observed. Cells became elongated and cell shape was irregular (C2, C3) compared to the control cells and cells cocultured with normal trophoblast cells. Pore formation was also observed in cells cocultured with preeclamptic trophoblasts (C1 and C3). Figure 1D shows VE-cadherin staining after recovery in cells cocultured with preeclamptic trophoblasts, in which the trophoblast cell filter was removed from the coculture chamber after 48 hours of coculture. Fresh endothelial growth medium was replaced and cells were continuously incubated for 24 hours before staining of VE-

cadherin. The recovered cells exhibit similar morphology, cell shape, size, and zipper-like structure around the cell borders. These results clearly show that factors released from preeclamptic trophoblasts disrupt endothelial junction structure and through a disturbance of VE-cadherin protein distribution at cell contact regions. This suggests that the morphology and integrity of the monolayer endothelial cells are sensitive to the factors produced by trophoblasts from preeclamptic placentas.

Placenta-derived Factors Dissociate Endothelial Membrane Junction Protein Complex Interaction

The cytoplasmic tail of VE-cadherin molecule directly binds to β-catenin and p120 forming an intracellular junction complex (Figure 2).[15] To determine if placenta-derived factors could disrupt endothelial membrane junction protein complex interaction, combined immunoprecipitation and immunoblotting experiments were performed. After ECs were treated with preeclamptic placental CM for 6 hours, total cellular protein was immunoprecipitated with antibody against β-catenin. Then the precipitated protein was immunoblotted with antibody against VE-cadherin and p120. Our results showed that both VE-cadherin and p120 expressions were detected in a complex in control cells when cellular protein was precipitated with β-catenin (Figure 3, lane 1), consistent with binding interaction between VE-cadherin, p120 and β-catenin in ECs (Figure 2). However, the complex was dissociated in cells treated with preeclamptic CM, as demonstrated by loss of VE-cadherin and p120 expression (Figure 3, lane 2). These data demonstrate that factors derived from preeclamptic placentas disrupt endothelial VE-cadherin/β-catenin/p120 complex, leading to disassembly of adheren junction.

Reduced Endothelial VE-cadherin Expression by Placental-Derived CLP in PE

Our previous study showed that placental-derived CLP could induce endothelial inflammatory responses by upregulating endothelial adhesion molecules, eg, P- and E-selectin.[16] Vascular endothelial cadherin is sensitive to proteolysis.[17] To investigate if placental-derived CLP could reduce endothelial integrity and alter junction structure of monolayer endothelial cells, VE-cadherin distribution was examined in cells treated with preeclamptic placental CM with or without depletion of CLP. Again, VE-cadherin distribution was disrupted, and the intensity of VE-cadherin expression at cell junctions was reduced when cells were exposed to preeclamptic CM (Figure 4B) compared to the control cells (Figure 4A). Separation of intercellular VE-cadherin was also observed between adjacent cells (Figure 4B 1 and 2). However, these morphological changes and altered VE-cadherin distribution at cell junctions were not observed in cells treated with preeclamptic CM after CLP was depleted (Figure 4C). Figure 4 (lower panel) shows the relative intensity of VE-cadherin expression at cell junctions in control cells, cells treated with preeclamptic conditioned medium, and preeclamptic CM after depletion of chymotrypsin. These results argue strongly that placenta-derived CLP disturbs VE-cadherin distribution and expression at endothelial junctions through a proteolysis-dependent mechanism.

Protease Chymotrypsin Increases Endothelial Solute Permeability and Downregulates Endothelial VE-cadherin and Occludin Expressions

To determine whether chymotrypsin could recapitulate these effects on endothelial permeability and downregulation of endothelial junction protein expression, chymotrypsin was applied to EC cultures. Endothelial solute permeability was evaluated by measuring horseradish peroxidase (HRP) flux and correlated with VE-cadherin and occludin expressions determined by Western blot. Our results showed that chymotrypsin induced a dose-dependent increase in endothelial monolayer permeability (measured by HRP leakage; Figure 5A), which is accompanied by reduced VE-cadherin and occludin expressions (Figure 5B).

DISCUSSION

The vascular endothelium is a functionally complex tissue that forms an active boundary between the bloodstream and the underlying tissues. The integrity of the vascular endothelium is an essential requirement for limiting transvascular solute flux and for protecting the vessel wall accumulating leukocytes, excessive platelet deposition and thrombus formation. It is well known that interstitial fluid volume is markedly expanded in most women with severe preeclampsia.[18] This phenomenon reflects endothelial dysfunction or injury leading to capillary leakage and circulating fluid extravasation into the extracellular space. Electron microscopic studies by Svedas et al provided convincing evidence that the increased vascular permeability in PE may be directly related to the morphological changes and enlarged endothelial junctions.[3,5]

Several published works have shown that toxic factors are present in the maternal circulation that could induce vascular permeability in women with PE.[19–22] For example, Anim-Nyame et al. studied the relationship between filtration capacity and plasma levels of vascular endothelial growth factor (VEGF), tumor necrosis factor α (TNF-α), and leptin in women with PE.[20] They found increased filtration capacity was correlated to the increased plasma TNF-α, but not VEGF and leptin, levels in women with PE.[20] An in vitro study reported by our group showed that serum from PE women could reduce endothelial electrical resistance and increased HRP leakage and the increased endothelial permeability was related to increased serum levels of interleukin 8 (IL-8) and lipid peroxides.[21] Haller et al found that increased endothelial permeability induced by preeclamptic serum could be attenuated by protein kinase C inhibitors Goe 6976 and staurosporine, indicating altered endothelial protein kinase C isoforms alpha and epsilon function was involved.[19] An interesting study conducted by Neal et al showed significant increase in hydraulic conductivity and reduction in oncotic reflection coefficient in frog mesenteric microvessels perfused with plasma from women with preeclampsia, suggesting that increase in permeability was due to an increase in pore size in perfused vessels.[22] All of these studies demonstrate that toxic factors are present in the maternal circulation, which play a significant role in promoting endothelial permeability in PE.

Our previous in vitro studies have shown that increased endothelial permeability is associated with altered distribution of the endothelial junction protein VE-cadherin and tight junction protein occludin in cells from preeclampsia. [4] We have also demonstrated that factors derived from preeclamptic placentas disturb endothelial junction organization,[7] consistent with the hypothesis that factors released by the placenta contribute to the increased endothelial permeability in PE through disorganization of endothelial junctional proteins.

Placental-derived CLP was responsible for inducing endothelial inflammatory response in preeclampsia.[16] In the present study, we specifically investigated the role of placental-derived CLP in altering endothelial junction protein distribution and expression pertaining to preeclampsia. Our results showed that placental factors not only downregulate VE-cadherin expression and disorganize VE-cadherin distribution at the cell junction, but also dissociate the homophilic binding of VE-cadherin between the adjacent cells, through disruption of VE-cadherin/β-catenin/p120 complexes. The preeclamptic placenta releases proteases such as chymotrypsin.[8] Depletion of chymotrypsin from the placental conditioned medium prevented the placental factor-induced downregulation of VE-cadherin expression and dissociation of the homophilic binding of VE-cadherin at cell contacts, and maintained the association of VE-cadherin complexes. These results clearly indicate that CLP is a candidate agent derived from the placenta that induces endothelial injury and promotes endothelial permeability in PE.

The present study also demonstrated that endothelial junctional structure and function is highly sensitive to the trophoblast-derived CLP. This concept is supported by the observation of reversible changes in VE-cadherin expression and distribution at cell junctions in cells cocultured with preeclamptic trophoblasts or treated with preeclamptic conditioned medium, that is, removal of trophoblasts from the coculture system or depletion of the CLP from the preeclamptic placental conditioned medium restored endothelial VE-cadherin expression and distribution. These findings provide important links between the microvasculature and the trophoblast pathophysiology of PE and support the concept that placenta-derived factors produce endothelial dysfunction in preeclampsia.

The harmful effects of CLP on endothelial barrier function were further confirmed by treating the cells with chymotrypsin as determined by HRP leakage and reduced expressions of both adhesion junction protein VE-cadherin and tight junction protein occludin.

The significance of placenta-derived CLP-induced endothelial dysfunction has several implications for studies on the pathophysiology of PE. First, placenta-derived CLP induces endothelial inflammatory responses (demonstrated by upregulation of endothelial selectins),[16] supporting the idea that CLP/chymase is an inflammatory protease; Second, placenta-derived CLP was able to mobilize angiotensin II, a powerful systemic vasoconstrictor, generation by endothelial cells, because CLP/chymase is a potent non-ACE angiotensin II generating enzyme[23,24]; lastly, our present study shows that CLP-derived from preeclamptic placenta disrupts endothelial junction protein integrity leading to increased endothelial permeability. All of these findings show that in PE, ECs are an important target for CLP, and increased CLP activity leads to the pathophysiology of preeclampsia, that is, exaggerated inflammatory response, augmented vasoconstriction, and increased vascular permeability. Therefore, we believe that CLP-derived from the placenta is a candidate agent that contributes to the endothelial dysfunction in maternal vascular system in PE.

Acknowledgments

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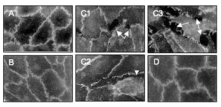

Figure 1.

Vascular endothelial cadherin (VE-cadherin) expression and distribution. A, control endothelial cells (ECs); B, ECs cocultured with normal TCs; C, ECs cocultured with preeclampsia (PE) TCs; and D, EC recovery after cocultured with PE-TCs. Vascular endothelial cadherin was continuously expressed and distributed at region of cell contact in control cells and in cells cocultured with normal TCs (A and B). Multimorphology changes were observed in cells cocultured with preeclamptic TCs (C1–3): elongation, pore formation (arrow), and separation of VE-cadherin expression between adjacent cells (arrowhead). Disorganized VE-cadherin expression and distribution was restored after PE-TCs were removed from the coculture (D).

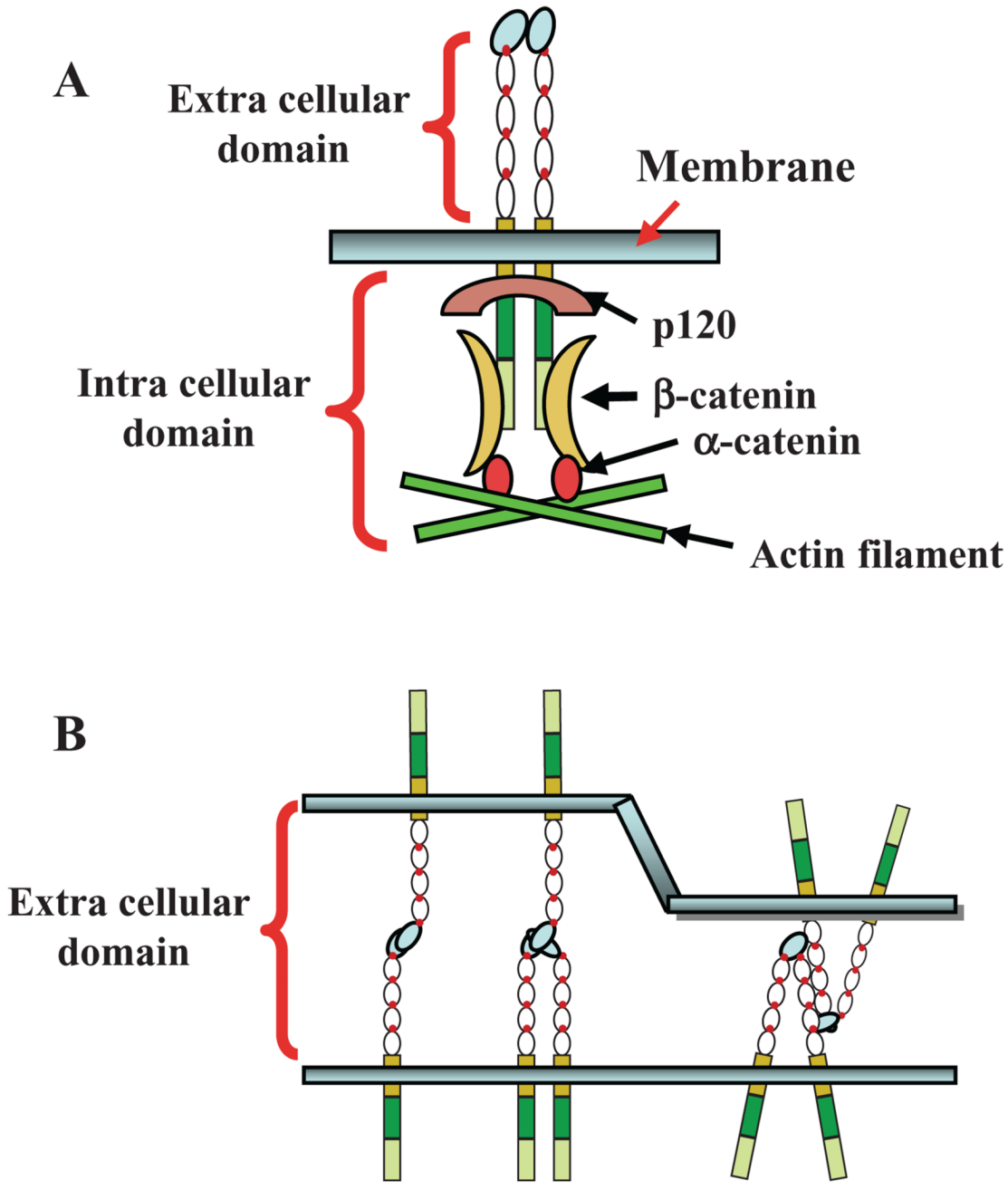

Figure 2.

Schematic drawing of endothelial Vascular endothelial cadherin (VE-cadherin) domain organization and models for VE-cadherin based adhesion between adjacent endothelial cells.[15] A, Endothelial VE-cadherin domains and intracellular protein complex. The cytoplasmic tail of VE-cadherin directly binds to β-catenin and p120 forming an intracellular junction complex. B, Vascular endothelial cadherin intercellular binding models. The dual homophilic binding model of VE-cadherin molecules between cells shows increased strength of cell-to-cell adhesion and binding force between adjacent cells.

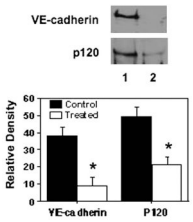

Figure 3.

Disassembly of vascular endothelial cadherin (VE-cadherin) and intracellular protein complex in endothelial cells (ECs) by factors released from preeclampsia (PE) placentas. Lane 1: control cells; Lane 2: cells treated with PE placental conditioned medium (CM). Total cellular protein was precipitated with antibody against β-catenin. The precipitated protein was run on SDS-PAGE and immunoblotted with antibodies against VE-cadherin and p120, respectively. Vascular endothelial cadherin and p120 were expressed in control cells, but not in cells treated with PE CM. These data suggest that dissociation occurred between the interaction of VE-cadherin and intracellular protein p120 and β-catenin in cells exposed to PE placental factors. The blot was representative from 3 independent experiments. The bar graph shows relative density of VE-cadherin and p120 expression, * $P < .05$, respectively.

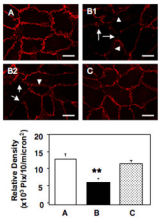

Figure 4.

Effects of placental chymotrypsin-like protease (CLP) on endothelial vascular endothelial cadherin (VE-cadherin) expression and distribution examined by fluorescent staining. A, control cells; B1 & B2: cells treated with preeclampsia (PE) placental conditioned medium (CM); and C, cells treated with PE placental CM after depletion of chymotrypsin. Reduced VE-cadherin expression and separation of VEcadherin expression between adjacent cells were observed in cells treated with PE placental CM, but not in cells treated with PE placental CM after depletion of chymotrypsin. Arrow: pore formation; and arrowhead: separation of VE-cadherin expression between adjacent cells. The bar graph shows a quantitative measure for VE-cadherin expression at endothelial junctions for control cells (A), cells treated with PE placental CM (B): cells treated with PE placental CM after depletion of chymotrypsin (C). Data are means from 5 independent experiments each in triplicate.

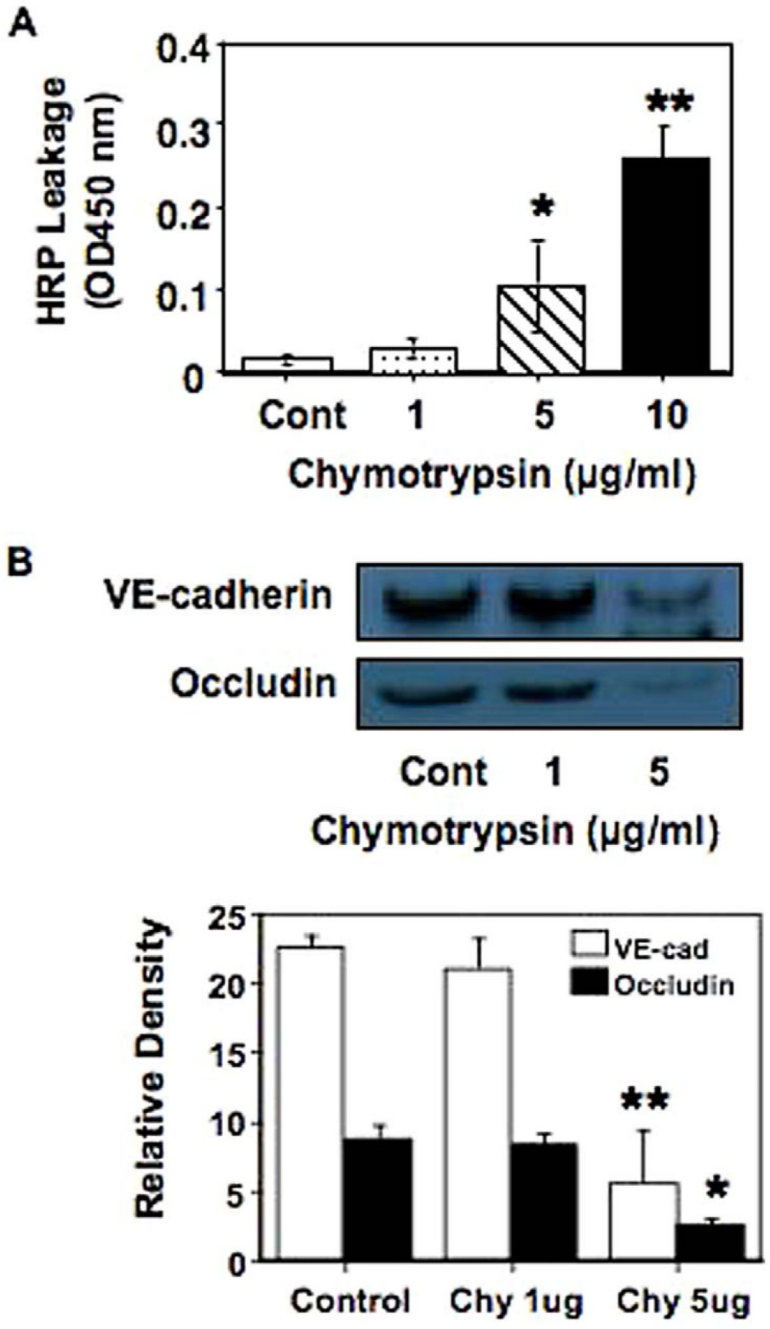

Figure 5.

Chymotrypsin protease induced altered endothelial barrier dysfunction. A: Endothelial permeability measured by horseradish peroxidase (HRP) leakage in ECs treated with chymotrypsin, which induced a dose-dependent increase in HRP leakage, * $P < .05$, ** $P < .01$ compared to control cells, respectively. Data are means ± S.E. from 5 independent experiments each in duplicate. B, VE-cadherin and occludin expression in ECs treated with different concentrations of chymotrypsin. The blot was representative from 3 independent experiments. The bar graph shows relative density of VE-cadherin and occludin expression, chymotrypsin treated vs. control: ** $P < .01$ and * $P < .05$, respectively

Table 1

Demographic Characteristics for Normal and Preeclamptic Pregnancies From Which Placentas Were Used in the Study

Variables	Normal (n = 6)	Preeclampsia (n = 8)	*P* value
Maternal age (years)	22 ± 4	24 ± 7	.412
Racial status			
White	0	1	—
Black	5	7	—
Other	1	0	
Gestational age (weeks)	38 ± 2	33 ± 3	<.01
Gravidity			
Primigravid	0	6	—
Multigravid	6	2	—
Blood pressure (mm Hg)			
Systolic	126 ± 5	172 ± 12	<.0001
Diastolic	74 ± 7	109 ± 8	<.0001
Proteinuria	Negative	>3+	<.0001
Mode of delivery			
Vaginal	6	3	—
Caesarean section	0	5	—
Baby weight (gram)	3064 ± 336	1814 ± 506	<.001

Data presented as mean ± SD.