SHORT COMMUNICATION

Placental Overexpression of Soluble CORIN in Preeclampsia

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Preeclampsia (PE) is a hypertensive disease of pregnancy associated with substantial maternal and fetal morbidity and mortality. CORIN is a transmembrane type II serine protease expressed in cardiomyocytes that converts pro-atrial natriuretic peptide into atrial natriuretic peptide, a cardiac hormone that regulates blood pressure. High levels of soluble CORIN have been reported in PE and are supposed to be cardiac in origin. We hypothesized that during pregnancy soluble CORIN is released by the syncytiotrophoblast and that increased levels of soluble CORIN in preeclampsia originate from placenta. A total of 375 patients (181 PE patients and 194 controls) were analyzed. High levels of soluble CORIN were confirmed in maternal blood from preeclamptic pregnancies compared with controls. Differentiated primary villous cytotrophoblasts showed that CORIN was expressed (mRNA and protein levels) and secreted by trophoblastic cells, mostly by the syncytiotrophoblast. Finally, placental explants showed a significant increase in CORIN production and secretion in PE cases compared with controls. This study showed that CORIN is secreted by trophoblastic cells and that high levels of soluble CORIN in preeclampsia have a placental origin. (*Am J Pathol* 2020, 190: 970–976; <https://doi.org/10.1016/j.ajpath.2019.12.012>)

Preeclampsia (PE) is a pregnancy complication that affects 3% to 5% of pregnant women and is associated with severe maternal and perinatal morbidity.¹ Despite extensive research, the etiopathogenic mechanisms of this disease remain poorly understood. It currently is accepted that, very early in pregnancy, placentation is impaired with a notable defect of vascular remodeling and invasion of the upper third of the myometrium by extravillous trophoblastic cells.² This phenomenon results in placental dysfunction associated with hypoxia and hypoxia/reoxygenation mechanisms. This placental dysfunction causes the release of various factors into the maternal circulation (cytokines, proinflammatory factors, anti-angiogenic factors, oxidized syncytiotrophoblastic debris)^{3–5} responsible for the maternal endothelial damage that characterizes preeclampsia.

CORIN is a transmembrane type II serine protease expressed primarily in cardiomyocytes, where it cleaves pro-atrial natriuretic peptide (ANP) into active ANP, a cardiac hormone that plays a key role in maintaining cardiac function and normal blood pressure.^{6,7} CORIN also is known to be expressed in other tissues, such as the bone, kidney, uterus, and brain.⁸ CORIN can be shed into the circulation through autocleavage and metalloproteinase-mediated hydrolysis processes of the membrane form of the protein.⁹ It also has been shown that circulating soluble CORIN has the same activity as membrane-bound CORIN.¹⁰

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CORIN has been shown to be involved in placental development and in the pathophysiology of preeclampsia. Pregnant *CORIN* knockout mice develop a significant increase of systolic blood pressure associated with late-gestational proteinuria, mimicking the human preeclampsia phenotype.¹¹ These corin-deficient mice also have smaller uterine spiral arteries than wild-type mice, pointing to impaired trophoblast invasion and spiral artery remodeling.¹² In preeclamptic patients, uterine levels of *CORIN* mRNA and protein are significantly lower than those in normal pregnant women.¹² Thus, a reduction of CORIN at the uteroplacental interface is considered to play an important role in the pathophysiology of PE.¹² Paradoxically, levels of soluble CORIN have been shown to be increased in the maternal circulation during PE.^{12,13} Because plasma CORIN levels did not reflect that in tissues, it was suggested that plasma CORIN was derived from the heart, where CORIN expression is increased in response to high blood volume and pressure in pregnancy.¹²

Because most of the molecules increased in maternal circulation during pregnancy are released from the placenta [soluble fms-like tyrosine kinase-1 (sFlt-1), human chorionic gonadotropin (hCG), soluble endoglin (s-endoglin), vascular cell adhesion molecule (VCAM), leptin¹⁴], this study hypothesized that CORIN is secreted by the syncytiotrophoblast and that the high levels of soluble CORIN in preeclampsia are related to placental dysfunction.

Materials and Methods

Patients

This study used the samples from the Search for an Association between CX3CR1 and V249I Polymorphisms, Preeclampsia and Endothelial Injury study, a multicenter case-control study. The patients were recruited from six French university hospitals between May 2003 and October 2007, as previously reported.¹⁵ The cases were pregnant women with PE at the time of diagnosis. The controls were pregnant women without PE. PE was defined as blood pressure higher than 140/90 mm Hg occurring after 20 weeks of gestation with previously normal blood pressure, associated with proteinuria greater than 0.3 g in a 24-hour urine specimen. The PE group was subdivided into early onset PE (delivery <34 gestational week) and late-onset PE (delivery ≥34 gestational week). Written informed consent was obtained from each woman before enrollment and blood sampling. Venous blood was collected in EDTA solution at the time of enrollment. The samples were stored at -80°C until testing. The study was approved by the Ethics Committee (Comité de Protection des Personnes dans la Recherche Biomédicale) of the Bichat-Claude Bernard Hospital in Paris.

Villous Cytotrophoblast and Chorionic Villous Explant Cultures

Term placental tissues from women with an uncomplicated pregnancy undergoing a normal Cesarean section and PE placental tissues [$n = 2$ early onset (32 to 33 gestational weeks) and $n = 2$ late-onset (36 gestational weeks)] were obtained from the Cochin Port-Royal Maternity Unit (Paris, France). These biological samples were obtained with the patients' written informed consent and the study protocol was approved by the local ethics committee (Comité de Protection des Personnes 2015-mai-13909).

Villous cytotrophoblasts (VCTs) were isolated from human term placentas as previously described.^{16,17} After taking off the basal layer, pieces of cotyledon were cut and then rinsed in $1 \times$ Hanks' balanced salt solution. Next, the chorionic villi were isolated. A part of the chorionic villi was used for VCT isolation and the other part was used for explant cultures. For VCT isolation, the chorionic villi were incubated for the first 30 minutes at 37°C without agitation in 5 mL/g of tissue in digestion buffer containing 1% trypsin, 100 μ mol/L $MgSO_4$, 100 μ mol/L $CaCl_2$, 25 IU DNase, and 7% semiskim milk. After the first incubation, chorionic villi then were incubated in the same trypsin solution for 10 minutes at 37°C until the dissociated cells emerged. The supernatants containing VCT were collected after tissue sedimentation, filtered (40 μ m), and incubated on ice with 10% fetal calf serum (v/v) to stop trypsin activity. Cell suspensions then were centrifuged at 1200 rpm for 10 minutes at room temperature. Cell pellets were suspended in Dulbecco's modified Eagle's medium, pooled, layered on the top of a preformed Percoll (GE Healthcare, Fisher Scientific, Illkirch, France) gradient (60%, 50%, 45%, 35%, 30%, 20%, and 10%), and centrifuged at 2500 rpm at room temperature without braking for 20 minutes. The layer between the 45% and 35% of Percoll-containing trophoblast cells was collected, suspended in Dulbecco's modified Eagle's medium, and centrifuged at 1200 rpm at room temperature for 10 minutes. The resulting cell pellet was suspended in complete Dulbecco's modified Eagle's medium (1% glutamine, 1% penicillin-streptomycin, and 10% fetal calf serum) and the cells were counted using a TC20 Automated Cell Counter (Bio-Rad, Marnes-La-Coquette, France). The cells were seeded at a density of 150,000 cells/cm² on 35-mm or 60-mm Techno Plastic Products tissue culture dishes (TPP, Trasadingen, Switzerland) and incubated overnight in 5% CO₂ at 37°C. After 24 hours (VCT) and 72 hours [syncytiotrophoblasts (ST)] of culture, cells were snap-frozen and stored at -80°C until use. After 72 hours of culture, ST supernatants were centrifuged to remove cellular debris, transferred into new tubes, and stored at -80°C until use. For explants, chorionic villi were cultured as previously described.^{18,19} Three villi were placed in a 24-well plate using a folded pin and cultured in 2 mL of complete Dulbecco's modified Eagle's medium (1% glutamine, 1% penicillin-streptomycin, and 10% fetal calf serum). After 24 hours of culture, explant

supernatants were centrifuged to remove cellular debris, transferred into new tubes, and stored at -80°C until use.

Western Blot

Proteins from cells and chorionic villi were extracted with a lysis buffer to which a cocktail of protease/phosphatase inhibitors was added. The samples were ground and centrifuged to recover the protein lysates. The assay was performed using the Pierce Micro kit BCA Protein assay kit (Fisher Scientific, Illkirch, France) according to the manufacturer's instructions. An EnSpire microplate reader (Perkin Elmer, Villebon-sur-Yvette, France) was used for reading. Proteins were loaded on a 7.5% Mini-Protean gel TGX (Bio-Rad), transferred to a nitrocellulose membrane, and then immunoblotted with antibodies to CORIN (1 $\mu\text{g}/\text{mL}$, PAG316Hu03; Cloud-Clone Corp., Euromedex, Souffelweyersheim, France) and β -ACTIN (ACTB) (0.2 $\mu\text{g}/\text{mL}$, A5441; Sigma-Aldrich, Saint Quentin Fallavier, France). After incubation with an appropriate Alexa Fluor-conjugated secondary antibody (680 or 800 conjugate; Molecular Probes, Fisher Scientific, Illkirch, France), blots were shown using the Odyssey infrared fluorescent system (Li-Cor, Bad Homburg, Germany). Signal intensity was quantified using ImageJ software version 1.48v (NIH, Bethesda, MD; <http://imagej.nih.gov/ij>). The arbitrary pixel densities of each protein were normalized to ACTB for cells or loading control for supernatants.

Immunohistochemical Analysis of Placental Tissues

Placenta samples ($n = 3$ controls, $n = 3$ PE) were processed as previously described.²⁰ Briefly, samples were fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) for 4 hours at 4°C , dehydrated, and embedded in Paraplast (Leica Biosystems, Fisher Scientific, Illkirch, France). Placental tissue sections (5 μm) were deparaffinized in SafeSolv (Labonord, Templemars, France) and rehydrated in ethanol/water. Antigens were retrieved by treatment in citrate buffer, pH 6.1 (Dako, Trappes, France) for 40 minutes at 90°C in a water bath. Sections were permeabilized in 0.2% Triton X-100 (Molecular Probes, Fisher Scientific) for 30 minutes, and then washed 3 times in phosphate-buffered saline for 5 minutes. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 30 minutes and nonspecific binding by 1-hour incubation in 3% bovine serum albumin and 22 $\mu\text{g}/\text{mL}$ human IgG (Jackson ImmunoResearch Laboratories, Interchim, Montluçon, France) solution at room temperature. Immunostaining was performed with a universal streptavidin-peroxidase immunostaining kit (Dako) using specific antibody against CORIN (2 $\mu\text{g}/\text{mL}$, PAG316Hu03; Cloud-Clone Corp.) or nonspecific rabbit IgG as control. Finally, tissue sections were mounted in an aqueous mounting medium (Dako) and slides were scanned using a Lamina multilabel slide scanner (Perkin

Elmer) using brightfield imaging at a magnification of $\times 40$. Quantification was performed on five random 650×650 pixel images per sample ($n = 3$ controls, $n = 3$ PE, resulting in a total of 30 images) using the ImageJ software version 1.48 thresholding analysis as previously described.²¹

RNA Isolation and Real-Time Quantitative PCR

Total RNA from cells was purified on RNeasy columns (Qiagen, Courtaboeuf, France) according to the manufacturer's protocol and quantified using a Nanodrop spectrophotometer (Thermo Scientific, Fisher Scientific, Illkirch, France). Reverse transcription (250 ng total RNA) was performed using Superscript III (Invitrogen, Fisher Scientific, Illkirch, France) with random hexamers. cDNA was prepared in duplicate and pooled to perform real-time quantitative PCR. As previously described,^{18,20} real-time quantitative RT-PCR was performed in triplicate with real-time quantitative PCR MasterMix Plus for SYBR Green (Eurogentec, Angers, France) using an ABI Prism 7900 Sequence Detection System (Applied Biosystems, Villebon sur Yvette, France) under the following conditions: an initial denaturation step (95°C for 10 minutes); 40 cycles of 95°C for 15 seconds, then 60°C for 60 seconds; followed by a three-step dissociation phase of 15 seconds at 95°C , 30 seconds at 60°C , and 15 seconds at 95°C to eliminate possible artifacts such as oligonucleotide dimers. The primer sequences were as follows: *CORIN* (NM_006587) forward primer: 5'-ATTGCAATCCCACAACAGAGC-3', reverse primer: 5'-ACATTGAAACGTGCTGGGGA-3'; *18S* (NR_145820) forward primer: 5'-TCCCCCAACTTCTTA-GAGG-3', reverse primer: 5'-CTTATGACCCGCACT-TACTG-3'; *HPRT1* (NM_000194) forward primer:

Figure 1 Concentrations of soluble CORIN in maternal plasma of pregnant women with preeclampsia (PE) or normal pregnancy. Data are expressed as means $\pm$ SD. $n = 181$ PE ($n = 81$ early-onset PE and $n = 100$ late-onset PE); $n = 194$ control. *** $P < 0.001$ (paired t -test).

Figure 2 Differential expression of CORIN between villous cytotrophoblasts (VCTs) and syncytiotrophoblasts (STs). **A:** Quantitative RT-PCR analysis shows a significant increase of *CORIN* mRNA level in *in vitro*-differentiated STs compared with VCTs. **B:** Western blot analysis on total cell protein lysates. **C:** Semi-quantitative analysis of protein expression indicates a significant reduction in the native 120-kDa CORIN protein and a significant increase in the cleaved 60-kDa CORIN protein during differentiation of VCTs into STs. **D:** Western blot analysis on ST supernatant indicates that the cleaved 60-kDa CORIN protein is secreted. ** $P < 0.01$, *** $P < 0.001$ (paired *t*-test). ACTB, β -ACTIN.

5'-GGCGTCGTGATTAGTGATG-3', reverse primer: 5'-CAGAGGGCTACAATGTGATG-3'; *RPLP0* (NM_001002) forward primer: 5'-AACATCTCCCCCTTCTCCT-3', reverse primer: 5'-ACTCGTTTGTACCCGTTGAT-3'; and *SDHA* (NM_004168) forward primer: 5'-CCACCACTGCATCAAATTCATG-3', reverse primer: 5'-TGGGAACAA-GAGGGCATCTG-3'. The relative RNA expression of each gene was normalized to the geometric mean of four endogenous controls (*18S*, *HPRT*, *RPLP0*, and *SDHA*) using qBasePlus 2 Software (Biogazelle, Gent, Belgium).

Soluble CORIN Levels in Maternal Plasma

Soluble CORIN levels were measured in the plasma of 375 patients (181 PE and 194 controls) using an enzyme-linked immunosorbent assay kit (Quantikine Human CORIN Immunoassay; R&D Systems, Bio-Techne SAS, Rennes, France) according to the manufacturer's instructions.

Statistical Analysis

All measurements were made from at least $n = 3$ samples. The data are expressed as the means $\pm$ SD of the indicated number. Statistical analysis (*t*-test) was performed using GraphPad Prism software version 6.07 (GraphPad, San Diego, CA). Results were considered significant if the *P* value was less than 0.05.

Results

Levels of Soluble CORIN Are Increased in PE Compared with Normal Pregnancy

Plasma levels of soluble CORIN in 375 patients, distributed into 194 controls and 181 PE cases subdivided into two groups (81 early-onset PE and 100 late-onset PE), were measured. CORIN levels were significantly higher in both PE cases (early- and late-onset) than in controls [1671 ± 636 (early PE); 1796 ± 678 (late PE) versus 1096 ± 426 pg/mL]

(Figure 1), and a difference between early- and late-onset was observed (1671 ± 636 versus 1796 ± 678 , respectively).

Native CORIN Is Highly Expressed in VCT whereas Cleaved CORIN Is Highly Expressed and Secreted by ST

This study first investigated the mRNA and protein expression of *CORIN* in VCT and ST obtained after *in vitro* differentiation of VCT. To this end, VCT cells were isolated from human uncomplicated placentas and cultured in serum-containing media to allow spontaneous syncytialization. Real-time quantitative PCR analysis showed a significantly higher *CORIN* mRNA expression in ST compared with VCT (Figure 2A). Western blot analysis on VCT and ST cell lysates showed two bands of CORIN, one at approximately 120 kDa that corresponds to the native CORIN, and another band at approximately 60 kDa that corresponds to a cleaved form of CORIN (Figure 2B). The semiquantitative analysis indicated that, relative to ACTB, the protein level of the native CORIN (approximately 120 kDa) was significantly higher in VCT cell lysates compared with ST cell lysates (Figure 2C), and the cleaved CORIN (approximately 60 kDa) was significantly higher in ST cell lysates compared with VCT cell lysates (Figure 2C). To determine if CORIN is secreted by the ST, Western blot was performed on ST cell supernatant. Only the cleaved form of CORIN (approximately 60 kDa) was found to be secreted (Figure 2D).

CORIN Expression and Secretion Are Significantly Higher in Placental Tissues from PE Pregnancies

Next, this study investigated the expression and secretion of CORIN by placental tissues from uncomplicated pregnancies and pregnancies complicated by PE. Immunohistochemical analysis of placental tissue sections was performed using an antibody that recognizes both the membrane-bound and soluble form of CORIN. The results showed that CORIN was expressed in villous cytotrophoblasts, STs, and endothelial cells of the fetal capillaries in placentas from

Figure 3 Differential expression of CORIN between controls and preeclampsia (PE) placental tissues. **A:** Immunohistochemical staining of CORIN in controls (left panel) and PE (right panel) placental tissues. Higher magnification and negative controls for CORIN immunostaining (isotype) are shown to the right. **B:** Quantitative analysis of CORIN expression by placental tissues from PE and control uncomplicated pregnancies confirm the significantly higher expression of CORIN in PE. **C and D:** Semiquantitative analysis of Western blots on total placental tissue lysates (**C**) and on placental explant supernatants (**D**) indicate a significant increase in native 120-kDa and cleaved/secreted 60-kDa CORIN proteins in PE compared with uncomplicated pregnancies. * $P < 0.05$, *** $P < 0.001$ (paired t -test). Scale bars: 100 μ m (**A** and **B**, main images); 20 μ m (**A** and **B**, enlargements). ACTB, β -ACTIN.

uncomplicated pregnancies as well as from pregnancies complicated by PE (Figure 3A). However, CORIN expression was significantly higher in the placenta from pregnancies complicated by PE (Figure 3B). To validate the immunohistochemical results, semiquantitative analysis of Western blots was performed. Relative to ACTB, the protein levels of the native (approximately 120 kDa) and cleaved (approximately 60 kDa) CORIN were significantly higher in PE placental tissue lysates compared with tissue lysates from uncomplicated pregnancies (Figure 3C). Moreover, the protein level of the cleaved/secreted (approximately 60 kDa) CORIN also was significantly higher in PE placental explant supernatants compared with placental explants from uncomplicated pregnancies (Figure 3D).

Discussion

The majority of previous studies on CORIN, especially the soluble form, investigated its role in cardiovascular and

hypertensive diseases.^{22–24} The current study showed that the maternal plasma soluble CORIN levels were significantly higher in PE cases than in controls. This result was in accordance with previous studies of plasma¹² and serum.^{13,22,23,25}

Based on the literature, *CORIN* mRNA and protein were expressed in heart, bone, kidney, skin, brain, and pregnant uterus.^{7,8,26} One of the main goals of the present study was to identify whether CORIN was expressed and secreted by the syncytiotrophoblast. In their study of immunostaining on placental and decidual tissues, Miyazaki et al¹³ suggested that CORIN was expressed in the placenta. However, secretion of soluble CORIN by the placenta has never been shown. This study is the first to show that CORIN is secreted by the syncytiotrophoblast and is expressed more significantly in the syncytiotrophoblast than in undifferentiated villous cytotrophoblasts. Because these cells are in direct contact with maternal blood, these findings suggest that placental CORIN is a new source of soluble CORIN found in the maternal blood.

Because CORIN has been implicated in hypertensive pregnancy disorders,^{12,13,22} this study further investigated the expression of CORIN in preeclamptic placental tissues. Immunohistochemistry and Western blot analyses showed that CORIN is expressed significantly more in preeclamptic placenta compared with normal pregnancy. Furthermore, this study is the first to show that CORIN is released/secreted significantly more by preeclamptic placental tissues than by controls. These results suggest that soluble CORIN found in maternal blood is of placental origin and that, similar to other markers of PE (eg, sFlt-1, hCG, s-endoglin, VCAM, leptin),¹⁴ the increase of circulating levels of soluble CORIN in preeclamptic patients reflects placental dysfunction.

The potential mechanistic role of soluble CORIN in the pathophysiology of PE still needs to be explored. Physiologically, activation of the CORIN leads to activation of the ANP, resulting in vasodilation and hypotension. CORIN appears to be a protective factor. Therefore, increased soluble CORIN levels could be the result of a compensative mechanism to offset increased vasoconstriction in PE. Another hypothesis could be a paradoxical effect that already has been described,²⁷ and linked to an increase in type C natriuretic peptide receptors and a decrease in type A and B receptors. The type A and B natriuretic peptide receptors mediate the vasodilatory effect of ANP whereas type C natriuretic peptide receptors are involved in the degradation of ANP.²⁷ Gu et al²⁷ observed that pro-ANP/CORIN/natriuretic peptide receptor signaling is altered in PE. Moreover, another explanation would be that the soluble CORIN present in the maternal circulation is not active and therefore unable to degrade pro-ANP to ANP. Therefore, soluble CORIN appears as a marker of placental dysfunction but without necessarily having a direct role in the disease.

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Author Contributions

S.A.D. designed the work, acquired data, analyzed and interpreted the results, and wrote the manuscript; A.C., A.S., J.G., and L.M. acquired data and analyzed and interpreted the results; T.F. provided administrative and technical support; V.T. conceived and designed the work, analyzed and interpreted the results, and revised and edited the manuscript; S.A.D. and V.T. have full access to all data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis, and are the guarantors of this work; all authors approved the final version of the manuscript.

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