Excess LIGHT Contributes to Placental Impairment, Increased Secretion of Vasoactive Factors, Hypertension, and Proteinuria in Preeclampsia
Wei Wang, Nicholas F. Parchim, Takayuki Iriyama, Renna Luo, Cheng Zhao, Chen Liu, Roxanna A. Irani, Weiru Zhang, Chen Ning, Yujin Zhang, Sean C. Blackwell, Lieping Chen, Lijian Tao, M. John Hicks, Rodney E. Kellems and Yang Xia

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Excess LIGHT Contributes to Placental Impairment, Increased Secretion of Vasoactive Factors, Hypertension, and Proteinuria in Preeclampsia

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Abstract—Preeclampsia, a prevalent hypertensive disorder of pregnancy, is believed to be secondary to uteroplacental ischemia. Accumulating evidence indicates that hypoxia-independent mediators, including inflammatory cytokines and growth factors, are associated with preeclampsia, but it is unclear whether these signals directly contribute to placental damage and disease development in vivo. We report that LIGHT, a novel tumor necrosis factor superfamily member, is significantly elevated in the circulation and placentas of preeclamptic women compared with normotensive pregnant women. Injection of LIGHT into pregnant mice induced placental apoptosis, small fetuses, and key features of preeclampsia, hypertension and proteinuria. Mechanistically, using neutralizing antibodies specific for LIGHT receptors, we found that LIGHT receptors herpes virus entry mediator and lymphotoxin β receptor are required for LIGHT-induced placental impairment, small fetuses, and preeclampsia features in pregnant mice. Accordingly, we further revealed that LIGHT functions through these 2 receptors to induce secretion of soluble fms-like tyrosine kinase-1 and endothelin-1, 2 well-accepted pathogenic factors in preeclampsia, and thereby plays an important role in hypertension and proteinuria in pregnant mice. Lastly, we extended our animal findings to human studies and demonstrated that activation of LIGHT receptors resulted in increased apoptosis and elevation of soluble fms-like tyrosine kinase-1 secretion in human placental villous explants. Overall, our human and mouse studies show that LIGHT signaling is a previously unrecognized pathway responsible for placental apoptosis, elevated secretion of vasoactive factors, and subsequent maternal features of preeclampsia, and reveal new therapeutic opportunities for the management of the disease. (Hypertension. 2014;63:595-606.) ● Online Data Supplement

Key Words: endothelin-1 ▪ herpes virus entry mediator ▪ lymphotoxin-beta receptor ▪ preeclampsia ▪ receptors, tumor necrosis factor, member 14 ▪ tumor necrosis factor ligand superfamily member 14

Preeclampsia is a life-threatening pregnancy complication that affects ≈7% of first pregnancies and accounts for >50,000 maternal deaths worldwide each year.1 The major features of preeclampsia are hypertension, proteinuria, and placenta and kidney damage.1,2 It is also a leading cause of intrauterine growth restriction, a life-threatening condition that puts the fetus at risk for many long-term cardiovascular disorders.2,3 Thus, preeclampsia is a leading cause of maternal and neonatal mortality and morbidity and has acute and long-term impact on both mothers and babies. Despite intense research efforts, the underlying molecular mechanisms of preeclampsia are poorly understood, and the clinical management of preeclampsia has been unsatisfactory, resulting from the lack of presymptomatic screening, reliable diagnostic tests, and effective therapy. Thus, the identification of specific factors and signaling pathways involved in the pathogenesis of preeclampsia will facilitate the development of specific presymptomatic tests and effective mechanism-based preventative and therapeutic strategies for the disease.

Disease symptoms generally abate after delivery, suggesting that the placenta plays a central role in this disease. It is widely accepted that hypoxia is an initial trigger to induce placental abnormalities, elevated secretion of vasoactive factors, and subsequent maternal features.2 This concept is strongly supported by animal studies showing that experimentally reduced uterine perfusion pressure in pregnant rats results in abnormal placentas, increased secretion of antiangiogenic factors, and key preeclamptic features including hypertension and kidney damage.2 However, recent studies have provided compelling evidence that various factors other than uteroplacental hypoxia also promote...
placental abnormalities and disease progression; these include inflammatory cytokines, growth factors, components of the complement cascade, and autoantibodies. A growing body of evidence indicates that an increased inflammatory response is associated with preeclampsia and may contribute to disease. For example, previous in vitro studies showed that tumor necrosis factor (TNF)-α and interferon-γ induce trophoblast apoptosis and that interferon-γ and interleukin-12 inhibit angiogenesis. Moreover, early in vitro studies indicated that TNF-α contributes to elevated secretion of the antiangiogenic factor, soluble fms-like tyrosine kinase-1 (sFlt-1), from cultured human villous explants. Elevated sFlt-1 likely leads to impaired placentation, decreased uteroplacental perfusion, and subsequent hypertension and kidney injury. Despite these accumulating data, the specific hypoxia-independent mediators and mechanisms underlying placental apoptosis, enhanced secretion of vasoactive factors, and progression of the disease have not been fully determined in vivo. Therefore, this study aimed to identify the pathological role of novel hypoxia-independent mediators in preeclampsia and the underlying mechanisms of disease pathogenesis.

LIGHT, TNF superfamily member 14, is a type II transmembrane protein containing a C-terminal TNF homology domain that folds into a β-sheet sandwich and assembles into a homotrimer. It is found on immature dendritic cells and activated T-cells, and, similar to many TNF superfamly ligands, LIGHT is not only anchored on the cell surface but is also secreted from cells. In both humans and rodents, LIGHT is an immune signaling molecule functioning via 2 specific cellular receptors, lymphotoxin β receptor (LTβR) and herpes virus entry mediator (HVEM). Additionally, decoy receptor 3 (DcR3) is a soluble form of LIGHT receptor, present in humans but not in mice. Numerous studies indicated that DcR3 functions as an immunesuppressor in cancer, inflammation, and transplantation. Importantly, LIGHT has emerged as a key factor involved in multiple conditions, including hepatitis, asthma, atherosclerosis, rheumatoid arthritis, and Crohn disease. Although LIGHT and its receptors are expressed in placentas, especially located in trophoblast cells and endothelial cells, nothing is known about the role of LIGHT in preeclampsia. In the present study, we revealed that LIGHT is substantially elevated in the circulation and placentas of preeclamptic women, and elevated LIGHT signaling via its receptors, LTβR and HVEM, directly triggers placental apoptosis, secretion of vasoactive factors including sFlt-1 and endothelin-1 (ET-1), subsequent maternal symptom development, and small fetuses in vivo. Additionally, we provide evidence that LIGHT-mediated activation of its receptors directly induces apoptosis and secretion of sFlt-1 from cultured human placental villous explants. These results reveal a previously unrecognized role of LIGHT signaling in pathogenesis of preeclampsia and immediately suggest novel therapeutic possibilities for the disease.

Materials and Methods

For additional Materials and Methods, see the online-only Data Supplement.

Patients

Patients admitted to Memorial Hermann Hospital were identified by the obstetrics faculty of the University of Texas Medical School at Houston. Preeclamptic patients (n=36) were diagnosed with severe disease on the basis of the definition set by the National High Blood Pressure Education Program Working Group Report. The criteria of inclusion, including no previous history of hypertension, have been reported previously and include presence of blood pressure ≥160/110 mm Hg and urinary protein ≥300 mg in a 24-hour period or a dipstick value of 2+. Other criteria include presence of persistent headache, visual disturbances, epigastric pain, or hemolysis, elevated liver enzymes, and low platelets syndrome in women with blood pressure ≥140/90 mm Hg. Control pregnant women were selected on the basis of having an uncomplicated, normotensive pregnancy with a normal term delivery (n=34). The research protocol was approved by the Institutional Committee for the Protection of Human Subjects. Detailed information of human subjects is summarized in Table 1.

Introduction of Recombinant Mouse LIGHT and Neutralizing Antibodies Against LTβR or HVEM Into Pregnant and Nonpregnant Mice

Pregnant C57BL/6J mice (Harlan) were anesthetized with isoflurane, and recombinant mouse LIGHT (2 ng; R&D Systems) or the same volume of saline was introduced into pregnant mice by retro-orbital sinus injection on embryonic day (E) 13.5 and E14.5 (LIGHT, n=10; saline, n=8). For neutralization experiments, either LTβR mAb (100 μg; n=6) or HVEM mAb (100 μg; n=6) was simultaneously coinjected with LIGHT. Some of the mice (n=5) were injected with IgG from rat as control. This dosage was adapted from Anand et al. Moreover, blood pressure and proteinuria of some of the pregnant mice with saline or LIGHT injection were further monitored on postpartum day 5 and day 10 (n=5 or 6). The same amount of LIGHT and saline was also injected into nonpregnant mice (LIGHT, n=8; saline, n=6). Some mice injected with LIGHT were coinjected with LTβR mAb (100 μg; n=4) or HVEM mAb (100 μg; n=4) as with pregnant mice. IgG from rat was also used as control (n=4). All protocols involving animal studies were reviewed and approved by the Institutional Animal Welfare Committee of the University of Texas Health Science Center at Houston.

Human Placental Villous Explant Collection and Culture

Human placentas were obtained from normotensive patients who underwent an elective term cesarean section at Memorial Hermann Hospital in Houston. The explant culture system was conducted as described previously. On delivery, the placentas were placed on

Table 1. Clinical Characteristic Features of Human Subjects

<table>
<thead>
<tr>
<th>Clinical Characteristics</th>
<th>NT</th>
<th>Severe preeclampsia</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>34</td>
<td>36</td>
</tr>
<tr>
<td>Age, y</td>
<td>29.6±5.8</td>
<td>28.7±8.6</td>
</tr>
<tr>
<td>Race, %</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Black</td>
<td>43</td>
<td>48</td>
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<tr>
<td>White</td>
<td>27</td>
<td>22</td>
</tr>
<tr>
<td>Hispanic</td>
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<td>28</td>
</tr>
<tr>
<td>Other</td>
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<td>2</td>
</tr>
<tr>
<td>Gravity</td>
<td>2.3±1.4</td>
<td>2.6±1.5</td>
</tr>
<tr>
<td>Body mass index</td>
<td>32.8±4.7</td>
<td>36.6±8.6</td>
</tr>
<tr>
<td>Weeks gestational age</td>
<td>37.2±2.8</td>
<td>31.9±6.4</td>
</tr>
<tr>
<td>Systolic BP, mm Hg</td>
<td>115.7±6</td>
<td>174.2±7</td>
</tr>
<tr>
<td>Diastolic BP, mm Hg</td>
<td>72.8±5.7</td>
<td>98.8±10.7</td>
</tr>
<tr>
<td>Proteinuria, mg/24 h</td>
<td>ND</td>
<td>1809±465</td>
</tr>
</tbody>
</table>

The table illustrates that blood pressure (BP) and proteinuria are elevated in severe preeclamptic women compared with normotensive (NT) pregnant women. The category mean is indicated (±SEM, where applicable). ND indicates not determined.

*P<0.001 vs NT pregnant women.
ice and submerged in phenol red–free DMEM containing 10% BSA and 1.0% antibiotics. Five to 7 chorionic villous explant fragments were carefully dissected from the placenta and transferred to 24-well plates at 37°C and 5% CO2. All of the initial processing occurred <30 minutes following delivery. The explants were incubated with recombinant human LIGHT (100 pg/mL; R&D Systems) and cotreated with recombinant human DcR3 (4 μg/mL; R&D Systems) or human IgG-Fc (4 μg/mL; R&D Systems). After 24 hours, the collection medium was siphoned and stored at −80°C, and the villous explants were lysed or fixed overnight in 10% formalin for embedding in paraffin wax for further analysis.

Statistical Analysis
Results are expressed as mean±SEM. All data were subjected to statistical analysis using 1-way ANOVA followed by the Newman–Keuls post hoc test or Student t test to determine the significance of differences among groups. Statistical programs were run by GraphPad Prism 5 statistical software (GraphPad, San Diego, CA). Statistical significance was set at P<0.05.

Results
Circulating LIGHT Levels and Expression Profiles of Its Receptors in Placental Tissue of Preeclamptic Patients and Normotensive Pregnant Women
To determine the levels of LIGHT in the circulation of normotensive pregnant women and those with preeclampsia, we used a sensitive ELISA. We found that circulating LIGHT levels were increased 36-fold in women with preeclampsia compared with controls (Figure 1A). Additionally, we investigated the expression of LIGHT in human placental tissue using immunohistochemistry and detected LIGHT in trophoblast cells and endothelial cells, 2 major functional cell types of the placenta (Figure 1B). Quantitative RT-PCR showed that LIGHT mRNA was significantly elevated in the placentas from preeclampsia patients (Figure S1 in the online-only Data Supplement), and Western blotting analysis indicated that LIGHT protein is more abundant in placentas of preeclamptic patients compared with controls (Figure 1C). Overall, we demonstrated that the placental mRNA and protein of LIGHT and circulating LIGHT were elevated in preeclamptic patients.

Next, we examined the localization and expression profiles of all 3 receptors for LIGHT. Similar to LIGHT, we found that all of these 3 receptors were expressed in human placenta trophoblast cells and endothelial cells (Figure 1B). The protein levels of LTβR and HVEM were significantly increased in placental tissue from preeclampsia patients compared with normotensive pregnant women (Figure 1C). In contrast, the level of DcR3, a decoy receptor for LIGHT, was decreased in placenta tissue from preeclampsia patients relative to placenta tissue from normotensive pregnant women (Figure 1C).

LIGHT Induces Placental Tissue Apoptosis and Reduced Fetal Weight in Pregnant Mice Signaling Via LTβR and HVEM
To evaluate the pathogenic role of elevated LIGHT in placental apoptosis, we injected recombinant mouse LIGHT into C57/BL6 pregnant mice on E13.5 and E14.5 to achieve an increase in blood concentrations of LIGHT similar to the increase seen in preeclamptic women. At the end of pregnancy, we evaluated the circulating levels of LIGHT, placental size, and analyzed the histology of the placentas in the LIGHT-injected and saline-injected pregnant mice. We used an ELISA to accurately measure circulating LIGHT levels in the pregnant mice on E18.5 after injection with LIGHT. We found that the circulating level of LIGHT was ≈150 pg/mL in LIGHT-injected pregnant mice versus 90 pg/mL in saline-injected pregnant mice on E18.5 (Figure S2).

Next, we found that the placentas from pregnant mice injected with LIGHT were significantly smaller (0.0832±0.0026 g) than placentas from control pregnant mice injected with the same volume of saline (0.1016±0.0018 g; Table 2). Placenta H&E staining showed that LIGHT-injected pregnant mice had significant tissue damage, including increased calcification (Figure 2A, 2B, and 2F). To determine whether increased...
placental tissue apoptosis is a potential underlying mechanism of LIGHT-induced small placental size and placental tissue damage, we performed terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling to detect apoptotic cells and demonstrated significantly increased apoptosis in the labyrinth zone of placentas from LIGHT-injected pregnant mice compared with placentas from control mice (Figure 2B and 2G). Additionally, increased placental apoptosis in LIGHT-injected pregnant mice was accompanied by an elevated level of Bak, a proapoptotic marker, and a decreased level of Bel-2, an antiapoptotic marker (Figure 2I and 2J).

Because DcR3 is not expressed in rodents, we investigated the importance of LTβR and HVEM for LIGHT-induced placental damage featured with apoptosis in pregnant mice by injecting neutralizing monoclonal antibodies to each receptor into LIGHT-injected pregnant mice. We found that blocking either LTβR or HVEM by specific neutralizing antibodies restored placental size to 0.091±0.0014 g and 0.0907±0.0025 g, respectively (Table 2). In addition, cotreatment of LIGHT-injected mice with either anti-LTβR mAb or anti-HVEM mAb significantly attenuated LIGHT-induced placental tissue damage, apoptosis, and elevation of Bak and reduction of Bel-2 (Figure 2A, 2B, 2F, 2I, and 2J).

Fetal development is dependent on normal placental formation; thus, we were not surprised to find that infusion of LIGHT into pregnant mice resulted in reduced fetal weight and that coinjection of neutralizing antibodies to HVEM or LTβR improved fetal weight (Table 2). Taken together, these findings provide in vivo evidence that elevated LIGHT is a previously unrecognized factor underlying placental apoptosis and reduced fetal weight in pregnant mice by activation of its membrane receptors.

**Injection of LIGHT Into Pregnant Mice Induces Maternal Features of Preeclampsia Via HVEM and LTβR Signaling**

To evaluate the pathogenic role of elevated LIGHT in maternal disease development, we injected recombinant mouse LIGHT into pregnant C57/BL6 mice on E13.5 and E14.5 as described above. Blood pressure was monitored daily, and 24-hour urine was collected for urinary protein measurement before we euthanized the mice on E18.5. We found that systolic blood pressure increased significantly in mice injected with LIGHT (161±3 mm Hg) relative to control mice injected with saline (125±2 mm Hg; P<0.001; Figure 3A). Additionally, we examined the injected mice for proteinuria, another major clinical feature of preeclampsia. The ratio of urinary albumin to creatinine was significantly increased in pregnant mice injected with LIGHT (68±9.9 μg albumin per milligram of creatinine) compared with control mice (17±2.4 μg albumin per milligram of creatinine; P<0.01; Figure 3B).

To evaluate the pathogenic role of elevated LIGHT in maternal disease development, we injected recombinant mouse LIGHT into pregnant C57/BL6 mice on E13.5 and E14.5 as described above. Blood pressure was monitored daily, and 24-hour urine was collected for urinary protein measurement before we euthanized the mice on E18.5. We found

<table>
<thead>
<tr>
<th>Variable</th>
<th>Placental weight, g</th>
<th>Fetal weight, g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>0.101±0.0018</td>
<td>1.02±0.02</td>
</tr>
<tr>
<td>LIGHT</td>
<td>0.0832±0.0026*</td>
<td>0.85±0.02*</td>
</tr>
<tr>
<td>LIGHT plus control IgG</td>
<td>0.0823±0.0024*</td>
<td>0.86±0.01*</td>
</tr>
<tr>
<td>LIGHT plus anti-LTβR</td>
<td>0.0914±0.0024†</td>
<td>0.92±0.04†</td>
</tr>
<tr>
<td>LIGHT plus anti-HVEM</td>
<td>0.0907±0.0025†</td>
<td>0.96±0.06†</td>
</tr>
</tbody>
</table>

The table illustrates that injection of mouse recombinant LIGHT resulted in placentas and fetuses weighing less compared with pregnant mice injected with saline. Only pups born in litters of 6 to 8 were analyzed, and those depicted in Figure 3 were selected from the center of the uterine horn (n=51–60, collected and analyzed in 4 independent experiments). HVEM indicates herpes virus entry mediator; and LTβR, lymphotixin β receptor.

*P<0.05 vs saline treatment.
†P<0.05 vs LIGHT treatment.
Figure 2.
To validate the kidney injury, we conducted electron microscopic studies. Electron microscopic studies revealed that kidney tissue from pregnant mice injected with LIGHT displayed typical endotheliosis alterations as seen in pre-eclampsia patients, including markedly decreased luminal space of capillary loops accompanied by enlarged and swollen endothelial cells. Thus, the narrowed capillaries tended to be engorged with erythrocytes. However, the basement membranes of the capillaries were not significantly thickened. Additionally, the overlying podocyte foot processes were partially effaced and attenuated. There was no mesangial cell or matrix hyperplasia (Figure 2E). In contrast, the kidney tissue from mice injected with LIGHT and anti-LTβR, or LIGHT and anti-HVEM, showed similar ultrastructural features as the saline control group with unremarkable glomerular structure. Bland endothelial cells lined the capillary loops with basement membranes of appropriate thickness. The capillary loops were open and not engorged with erythrocytes. The podocytes showed typical foot processes without effacement and showed no evidence of hyperplasia. The mesangial cells and matrix were not increased (Figure 2E). Overall, we showed here that LIGHT signaling via both HVEM and LTβR contributes to hypertension, proteinuria, and kidney injury in pregnant mice as seen in pre-eclampsia patients.

**LIGHT Signaling Via LTβR and HVEM Induces Hypertension But Not Proteinuria in Nonpregnant Mice**

To determine whether pregnancy is required for LIGHT-induced features of preeclampsia, we injected LIGHT on 2 consecutive days into nonpregnant female mice. The results (Figure 3C) show that LIGHT induced high blood pressure in nonpregnant mice (153±3 mmHg) compared with saline-injected control mice (122±8 mmHg). Moreover, to validate blood pressure measurement by tailcuff method, we determined intracarotid mean arterial pressure31 (Figure S4). Additionally, we coinjected anti-LTβR mAb or anti-HVEM mAb separately into nonpregnant mice that were injected with LIGHT. As with pregnant mice, blocking LTβR mAb or anti-HVEM mAb separately into nonpregnant mice that were injected with LIGHT. As with pregnant mice, blocking LTβR (131±6 mmHg) or HVEM (134±9 mmHg) by neutralizing antibody significantly reduced LIGHT-induced hypertension in nonpregnant mice (Figure 3C). However, urinary protein levels in nonpregnant mice were not significantly affected by LIGHT injection (Figure 3D), with or without coinjection with neutralizing antibody to LTβR or HVEM. These findings indicate that pregnancy is required for LIGHT-induced proteinuria. However, LIGHT-induced hypertension is independent of pregnancy.

**Circulating sFlt-1 Is a Downstream Mediator Induced by LIGHT Via HVEM and LTβR Activation in Pregnant Mice But Not Nonpregnant Mice**

In an effort to identify intracellular mediators functioning downstream of HVEM and LTβR that contribute to LIGHT-induced preeclampsia in pregnant mice, we measured circulating sFlt-1, a well-accepted pathogenic factor thought to play an important role in the pathogenesis of preeclampsia.32 We found that circulating sFlt-1 levels were significantly increased in LIGHT-injected pregnant mice (57.20±10.46 ng/mL) compared with control group (29.93±2.73 ng/mL). Moreover, we observed that elevated sFlt-1 levels in the circulation of LIGHT-injected pregnant mice were significantly downregulated by cotreatment with anti-LTβR mAb (43.53±3.15 ng/mL) or anti-HVEM mAb (38.57±6.68 ng/mL; Figure 3E). In contrast, there were no significant differences in the low levels of circulating sFlt-1 between the LIGHT-injected nonpregnant mice with or without coinjection of anti-LTβR and anti-HVEM (Figure 3F). These findings indicate that LIGHT-mediated sFlt-1 induction is pregnancy-dependent and suggest that sFlt-1 induction by LIGHT signaling via LTβR and HVEM contributes to proteinuria as seen in pregnant mice but not in nonpregnant mice.

**Circulating ET-1 Is a Common Downstream Mediator Induced by LIGHT Via HVEM and LTβR Activation in Pregnant and Nonpregnant Mice**

Several studies suggest that increased levels of ET-1 contribute to the hypertensive features of preeclampsia.33,34 To determine whether LIGHT-induced hypertension is associated with elevated ET-1, pregnant and nonpregnant mice were injected with LIGHT in the presence or absence of neutralizing antibody to LTβR or HVEM and circulating ET-1 levels determined. The results (Figure 3G) show that LIGHT injection resulted in elevated circulating ET-1 in pregnant (2.37±0.54 pg/mL) and nonpregnant mice (1.54±0.15 pg/mL) compared with saline-injected pregnant (0.42±0.13 pg/mL) and nonpregnant mice.
nonpregnant mice $0.54\pm 0.23$ pg/mL). Coinjection with anti-LTβR (pregnant mice, $1.11\pm 0.13$ pg/mL; nonpregnant mice, $1.04\pm 0.19$ pg/mL) or anti-HVEM (pregnant mice, $0.84\pm 0.19$ pg/mL; nonpregnant mice, $1.05\pm 0.29$ pg/mL) significantly inhibited LIGHT-mediated induction of ET-1. These results indicate that LIGHT-mediated induction of ET-1 is independent of pregnancy and likely an important factor responsible for LIGHT-induced hypertension in both pregnant and nonpregnant mice.

**DcR3 Inhibits LIGHT-Induced sFlt-1 Secretion and Placental Apoptosis in Human Villous Explants**

Mice and rats do not have a DcR3 gene. Thus, to explore the biological function of soluble receptor DcR3 in LIGHT-induced...
features of preeclampsia, we incubated human placental villous explants from healthy term pregnancies with recombinant human LIGHT in the presence or absence of recombinant human DcR3. We measured the supernatant sFlt-1 levels by ELISA and demonstrated a significant increase in sFlt-1 secretion by placenta villous explants incubated with LIGHT (0.31±0.02 ng/mL per milligram) compared with placenta villous explants incubated with saline (0.16±0.05 ng/mL per milligram). However, elevated sFlt-1 secretion induced by LIGHT was significantly inhibited by cotreatment with DcR3 (0.23±0.02 ng/mL per milligram; Figure 4B).

To determine whether placental tissue apoptosis is a downstream consequence of LIGHT treatment, we examined human placental tissue explants for apoptotic cells by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay after incubation with LIGHT. Our results (Figure 4A and 4C) demonstrate a significant increase in the abundance of apoptotic cells in LIGHT-treated human placental tissue. Additionally, we found that LIGHT treatment increased Bax expression and reduced Bcl-2 expression in human placental explants (Figure 4D and 4E). Furthermore, increased placenta trophoblast cell apoptosis induced by LIGHT was significantly attenuated by cotreatment with DcR3 (Figure 4). Altogether, our results indicate that DcR3 can prevent LIGHT-induced sFlt-1 secretion and placental trophoblast cell apoptosis in human placenta explants.

Discussion

As a TNF superfamily member, LIGHT was initially discovered to be expressed in mature immune cells and involved in multiple inflammatory diseases and autoimmune conditions. Although LIGHT and its receptors were subsequently shown to be expressed in trophoblasts and endothelial cells of human placentas and that an elevated inflammatory response is a recognized feature of preeclampsia, nothing was known about the role of LIGHT signaling in preeclampsia. Here, we provide both in vivo animal studies and in vitro human evidence that elevated LIGHT is a key contributor to hypertension, proteinuria, placental apoptosis, and small fetuses in pregnant mice. Mechanistically, we determined that elevated LIGHT signaling via 2 membrane-anchored receptors, HVEM and LTβR, contributes to placental apoptosis and disease development. We further revealed that LIGHT signaling is a previously unrecognized immune cascade responsible not only for the induction of the vasoconstrictive peptide, ET-1, but also for the induction of the antiangiogenic factor, sFlt-1, and thereby plays an important role in hypertension and proteinuria in pregnant mice. Overall, both mouse and human studies reported here provide strong evidence that LIGHT-mediated receptor activation is an underlying mechanism for placental damage, increased secretion of ET-1 and sFlt-1, and subsequent maternal clinical manifestations and suggest that these signaling pathways are novel therapeutic targets for disease management (Figure 4F).

LIGHT, as well as other TNF superfamily members, triggers both cell death and survival signaling pathways. The functions of LIGHT are performed by the activation of 2 receptors, HVEM and LTβR, expressed on the surface of specific target cells. Biological activities of LIGHT–LTβR signaling include the induction of apoptosis, generation and activation of various cytokines, developmental organogenesis of lymph nodes, and restoration of secondary lymphoid structure and function. LIGHT signaling through HVEM is an important costimulatory signal for activation and proliferation of T-cells. LIGHT stimulates T-cell proliferation and induces interferon-γ production, which can be inhibited by neutralizing antibodies to HVEM. Although most studies indicate that HVEM works principally as a prosurvival receptor for T-cells and LTβR activation contributes to LIGHT-induced apoptosis, there is also evidence that HVEM, but not LTβR, plays a key role in LIGHT-induced tumor cell death in lymphoid malignancy. Although LIGHT and its receptors are known to be expressed in the first trimester and term placental tissues, the biological function of LIGHT in normal pregnancy and pregnancy-related diseases has not been extensively explored. Our study is the first to show that LIGHT is significantly increased (>36-fold) in the circulation of preeclamptic women compared with normotensive pregnant women. In vivo experiments using pregnant mice and in vitro experiments using cultured human villous explants demonstrated that LIGHT triggers placental damage characterized with placental apoptosis. These findings are strongly supported by early in vitro studies showing that LIGHT can induce human placental trophoblast cell apoptosis. Mechanistically, we demonstrated that both LTβR and HVEM are responsible for LIGHT-induced placental cell apoptosis by increasing Bax expression and reducing Bcl-2 levels in LIGHT-injected pregnant mice. Similarly, we provide human evidence that the soluble receptor, DcR3, inhibits LIGHT-induced sFlt-1 secretion and apoptosis in human placental tissue explants, indicating a potentially protective role for DcR3 in the initiation and progression of preeclampsia. Altogether, we revealed that elevated LIGHT signaling via its receptors is responsible for placental damage featured with placental apoptosis, an important mechanism in the pathogenesis of preeclampsia.

We found that both LTβR and HVEM are expressed in human placenta tissue, consistent with preliminary research reported by Gill et al. To our surprise, not only was LIGHT expression but also the levels of LTβR and HVEM were increased in preeclamptic placentas. Thus, the detrimental effects of elevated LIGHT signaling through its receptors is further enhanced by the increased placental expression of LIGHT and its receptors along with increased levels of LIGHT in the circulation. Supporting this model, we used neutralizing antibodies for LIGHT receptors to demonstrate that both HVEM and LTβR function downstream of LIGHT and contribute to LIGHT-induced disease development, including small placentas and fetuses in pregnant mice. It is possible that the combination of neutralizing antibodies would have even greater effect compared with either one individually. Taken together, these studies reveal a new function of LIGHT signaling via HVEM and LTβR in the pathogenesis of preeclampsia and highlight novel therapeutic approaches to prevent LIGHT-induced placental apoptosis and preeclampsia features.

It is a widely held view that increased secretion of vasoactive factors by the placenta into the maternal circulation is responsible for systemic endothelial dysfunction, hypertension, and multiorgan damage. In this regard, numerous recent studies showed that sFlt-1 is significantly elevated in...
Elevated sFlt-1 functions as downstream mediator to further impair placental development. As such, without interference, LIGHT–sFlt-1–placental impairment leads to progression of the disease and symptom development. Thus, these studies support our working model that LIGHT is a detrimental factor contributing to placental damage, increased placental secretion of vasoactive factors, and disease development.

Because preeclampsia is a pregnancy-specific disease, the placenta has been considered to play an indispensable role in the initiation and progression of the disease. To assess the importance of placentas in LIGHT-induced hypertension and proteinuria, we injected LIGHT into nonpregnant as well as pregnant mice. In contrast to pregnant mice, LIGHT only induced hypertension, but not proteinuria, in nonpregnant mice. Decoy receptor 3 can attenuate sFlt-1 secretion and placenta apoptosis stimulated by LIGHT. Thus, LIGHT signaling is a novel pathway contributing to pathogenesis of preeclampsia and serves as a potential therapeutic target for the disease.
was only observed in pregnant animals and was correlated with the induction of sFlt-1 only in pregnant animals. LIGHT-mediated proteinuria in pregnant mice may be attributable to renal damage caused by increased sFlt-1 generation and secretion from placenta. In contrast to our findings with LIGHT-induced hypertension in nonpregnant mice, LaMarca and colleagues found that TNF-α, interleukin-6, and interleukin-17 can induce high blood pressure in pregnant rats, not in nonpregnant rats. However, these cytokine effects are presumably concentration-dependent, and it is possible that lower doses of LIGHT may be unable to induce hypertension in nonpregnant mice but still have effects in pregnant mice. Nevertheless, our studies provide a molecular basis underlying LIGHT-mediated differential effects between pregnant and nonpregnant mice: (1) LIGHT-mediated elevation of circulating ET-1 alone is enough to induce hypertension in nonpregnant mice; (2) the lack of renal pathology in LIGHT-injected nonpregnant mice is correlated with the lack of elevated sFlt-1 in these animals; and (3) a major source of sFlt-1 during pregnancy is the placenta, where LIGHT-mediated induction of sFlt-1 is likely to contribute to renal pathology.

In contrast to other members of the TNF receptor superfamily, the gene encoding DcR3 does not include a transmembrane domain and results in the production of an obligate secreted protein of 300 amino acids. As a soluble receptor, DcR3 is capable of neutralizing the biological effects of 3 members of the TNF superfamily: Fas ligand (FasL/TNFSF6), LIGHT (TNFSF14), and TNF-like molecule 1A (TL1A/TNFSF15). The infusion of DcR3 ameliorates the autoimmune kidney disease. LIGHT-mediated elevation of circulating ET-1 alone is enough to induce hypertension in nonpregnant mice; (2) the lack of renal pathology in LIGHT-injected nonpregnant mice is correlated with the lack of elevated sFlt-1 in these animals; and (3) a major source of sFlt-1 during pregnancy is the placenta, where LIGHT-mediated induction of sFlt-1 is likely to contribute to renal pathology.

Perspectives
The work reported here is the first to show that elevated LIGHT, coupled with enhanced LTβR and HVEM receptor activation, promotes placental damage and triggers release of potent vasoactive factors (sFlt-1 and ET-1) in both human and murine pregnancy, as well as suggest that LIGHT signaling is likely an important mediator of pathogenesis associated with preeclampsia. Therefore, the use of HVEM/LTβR neutralizing antibodies, or the soluble form of LIGHT receptor, DcR3, to blunt the effects of elevated LIGHT may represent a novel treatment for preeclampsia. The current findings significantly advanced our understanding of pathogenesis of preeclampsia by revealing the pathogenic role of LIGHT signaling in preeclampsia and also provided a strong foundation for future translational studies to determine whether circulating LIGHT is elevated before symptoms and in this way serves as a presymptomatic biomarker and therapeutic target for preeclampsia.

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Disclosures
None.

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**Novelty and Significance**

**What Is New?**

- The tumor necrosis factor family member LIGHT is substantially elevated in the circulation and placentas of women with preeclampsia.
- LIGHT-mediated activation of its receptors, lymphotoxin β receptor (LTβR) and herpes virus entry mediator (HVEM), induces apoptosis and increases soluble fms-like tyrosine kinase-1 (sFlt-1) secretion by cultured human placental explants.
- The introduction of LIGHT in pregnant mice stimulates placental apoptosis and increases production of sFlt-1 and endothelin-1 (Et-1) and is accompanied with hypertension and proteinuria via activation of its receptors.

**What Is Relevant?**

- Our results reveal a previously unrecognized role of LIGHT signaling in the pathogenesis of preeclampsia and reveal potential therapeutic opportunities.

**Summary**

We report that LIGHT, a novel tumor necrosis factor superfamily member, is significantly elevated in the circulation and placentas of preeclamptic women compared with normotensive pregnant women. A pathological role for LIGHT in preeclampsia is indicated by the results of experiments showing that the infusion of LIGHT into pregnant mice directly induced placental apoptosis, small fetuses, and key features of preeclampsia, hypertension and proteinuria. Mechanistically, using neutralizing antibodies specific for LIGHT receptors, we found that LIGHT transmembrane receptors, HVEM and LTβR, are required for LIGHT-induced placental impairment, small fetuses, and preeclampsia features in pregnant mice. Accordingly, we further revealed that LIGHT functions through these 2 receptors to induce secretion of sFlt-1 and Et-1, 2 well-accepted pathogenic factors in preeclampsia, and thereby plays an important role in hypertension and proteinuria in pregnant mice. Lastly, we extended our animal findings to human studies and demonstrated that activation of LIGHT receptors resulted in increased apoptosis and elevation of sFlt-1 secretion in human placental villous explants. Overall, both human and mouse studies revealed a novel role of LIGHT in pathophysiology of preeclampsia and potential therapeutic targets.
ONLINE SUPPLEMENT

For

Excess LIGHT contributes to placental impairment, secretion of vasoactive factors, hypertension and proteinuria in preeclampsia

by

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Running title: LIGHT Signaling and Preeclampsia

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MATERIALS AND METHODS

Generation and purification of neutralizing antibodies specific for LTβR and HVEM

Hybridoma cells that specifically secrete rat anti–mouse HVEM mAb or rat anti–mouse LTβR mAb were originally prepared and characterized by Dr. Lieping Chen (Yale University). These antibodies effectively blunt activation of these two receptors1, 2. Hybridoma cells were grown in DMEM/F12 supplemented with 10% low-IgG FBS (Life Technologies Inc.) and 25 mM HEPES. Supernatant was harvested and mAb purified using G–Sepharose column chromatography (GE Healthcare)1-3. Rat IgG Abs (Santa Cruz.) were used as controls.

Blood pressure measurement and quantification of proteinuria

The systolic blood pressure was non-invasively measured by determining the tail blood volume with a volume pressure recording sensor and an occlusion tail-cuff (CODA System, Kent Scientific). This method shows good agreement with radiotelemetry measurements of blood pressure5. Blood
pressure was measured at the same time daily (±1 hour) while the mice were kept warm using a warming pad. Twenty-four hour urine was collected for analysis using metabolic cages (Nalgene). Mice were trained in metabolic cages for 2 days prior to urine collection. All of the mice were euthanized on GD18.5 before delivery when their serum and organs, including placetas and kidneys were collected. We quantified urinary albumin by ELISA (Exocell) and measured urinary creatinine by a picric acid colorimetric assay kit (Exocell). We used the ratio of urinary albumin to urinary creatinine as an index of urinary protein. All of the animal protocols were reviewed and approved by the institutional animal welfare committee at the University of Texas at Houston Health Science Center.

**Mean Arterial Pressure Measurement**
Mean arterial pressure (MAP) was monitored by cannulating the right carotid artery with a mouse jugular catheter connected to a pressure transducer and an amplifier unit. The amplifier was linked to a data acquisition module and MAP was recorded on a personal computer by Chart 5 Software (AD Instruments Inc.)

**Hematoxylin and eosin (H&E) staining and quantification in kidneys and placenta.**
Kidneys and placentas were harvested from the mice on E18.5, fixed in 4% formaldehyde, dehydrated, and embedded in paraffin. Sections of 4 μm were cut, stained with H&E using standard techniques and analyzed by light microscopy. The extent of renal damage was assessed by quantifying the glomeruli that showed characteristic features of damage in PE: decreased Bowman’s space and occlusion of capillary loop spaces. To examine those features, the glomeruli were counted in 6–9 fields of randomized and blinded slides (×10 magnification), with each field having at least 16–22 glomeruli. The glomeruli in each field were given a score based on the amount of capillary space evident within the Bowman’s capsule. A highest score of 5 was accorded to glomeruli with a normal amount of capillary space within Bowman’s capsule. A score of 1 was assigned to the glomeruli that showed complete loss of capillary space and an intermediate score of 3 was assigned to the glomeruli that displayed reduced, but not completely obliterated, capillary space. The scores for each field were divided by the number of glomeruli to get an average score per glomerulus for each field. Similarly placental histological quantification was carried by quantifying the number of calcifications/field under ×10 magnification. Placental sections were examined under the microscope and the number of calcifications was counted in each field and then plotted as number of calcifications recorded per field.

**Transmission electron microscopy (TEM)**
Upon sacrifice, kidneys were immediately removed. Tissue samples were cut into 1mm³ cubes and subsequently fixed in 3% glutaraldehyde overnight. The samples were then rinsed and exposed to 1% osmium tetroxide, dehydrated and embedded in an araldite-epox mixture. Semi-thin tissue sections were prepared (0.6mm) and stained with uranyl acetate and lead citrate. The prepared samples were examined with a JEOL 1210 transmission electron microscope (JEOL Corporation).

**Immunostaining in mouse placetas and kidneys and human placetas**
Mouse placetas and kidneys were harvested on GD18.5 and human placentas were isolated
immediately after delivery. Tissues were fixed in 4% formaldehyde, dehydrated, and embedded in paraffin. Sections of 4μm were cut and stained with antibody against LIGHT (1:50, Santa Cruz), LTβR (1:100, Santa Cruz), HVEM (1:100, Santa Cruz), DcR3 (1:200, Abcam) in a humidified chamber at 4 °C overnight. Following the primary antibody incubation, anti-mouse IgG HRP detection kit (cat. no. 551011, BD Pharmingen, San Diego, CA), anti-rabbit ABC Staining System (cat. no. sc-2018, Santa Cruz) or anti-goat ABC Staining System (Santa Cruz) were used to detect the primary antibody. The immunohistochemical staining (brown) was quantified by Image-Pro Plus software (Media Cybernetics, Bethesda, MD).

**RT-PCR to measure LIGHT mRNA in human placenta**

RNA was extracted using total RNA isolation reagent (Invitrogen, Carls-View, CA) from the placentas of normal pregnant women and preeclamptic women. Genomic DNA contamination was eliminated by DNase treatment with RNeasy Micro Kit (Qiagen GmbH, Hidden, Germany). PCR was performed on an ABI Prism 7700 sequence Detector (Applied Biosystems). The condition is: 95°C 10min; 95°C 30s, 60°C 30s, 72°C 1min, 40 cycles; 95°C 1min, 60°C 30s, 95°C 30s. For each gene, the fold changes were calculated as difference in placentas gene expression. P<0.05 is considered significant. LIGHT human forward: 5'CAAGAGCGAAGGTCTCACGAG3'; LIGHT human reverse: 5'TCACACCATGAAAGCCCCGAA3'. β-actin human forward: 5'AAATCTGGCACCACACCTTC3'; β-actin human reverse: 5'GGGGTGTTGAAGGTCTCAAA3'.

**Western blot analysis**

Protein in human and mice placentas was analyzed by western blotting analysis. Frozen tissues were used for protein extraction. Proteins were run on 8% SDS-PAGE gels and transferred the gels to a polyvinylidene fluoride membrane. Membranes were incubated with commercially available antibody to LIGHT (1:50, Santa Cruz, Catalog#: SC-7767), LTβR (1:100, Santa Cruz, Catalog#: SC-8376), HVEM (1:100, Santa Cruz, Catalog#: SC-7766), DcR3 (1:1000, Santa Cruz: SC-23464), Bax (1:100, Santa Cruz) and Bcl-2 (1:1000, Cell Signaling) as the primary antibody.

**Enzyme-linked immunosorbent assays for LIGHT, sFlt-1 and ET-1**

The concentrations of LIGHT in mouse plasma and human plasma were determined using commercial ELISA kits (Uscn Life Science Inc; cat. # SEA827Mu for mouse and SEA827Hu for human). Sensitivity for each kit is approximately 6.3 and 11.5 pg/mL, respectively. The intra- and inter-assay variabilities are less than 10%. The concentrations of sFlt-1 in mouse plasma and human placenta explants supernatant were determined using commercial ELISA kits (R&D Systems; cat. # MVR100 for mouse and DVR100B for human)^6-8. Sensitivity for each kit is approximately 15 pg/mL and the intra- and inter-assay variability is less than 10%. To measure circulating ET-1 levels, plasma was collected from pregnant and non-pregnant mice. Considering high level of other proteins in plasma, sample extraction was required by using Solid Phase Extraction (SPE) column (Octadecyl C18; 500 mg/6 mL; Honeywell Burdick & Jackson) before ELISA measurement. 200μl plasma from each mouse was used for sample extraction. After evaporating extracted samples to dryness using a centrifugal concentrator under vacuum, we reconstituted samples with 200 μL of the assay buffer and measured ET-1 immediately by commercial ELISA Kit (Enzo Life Sciences, cat. # ADI-900-020A). Sensitivity is approximately 0.4 pg/mL.
**TUNEL assay and index.** Human placental tissue collected from the experiments described above were permanently fixed onto a glass slide, deparaffinized, and rehydrated through an alcohol gradient by standard techniques. Tissues were permeabilized using cold, fresh 0.1% Triton X-100 in 0.1% sodium citrate and stained by TUNEL using a commercial kit (Roche) according to the manufacturer’s protocol. TUNEL positive cells were identified by cellular morphology (cell shrinkage, membrane blebbing, and nuclear fragmentation) and positive green staining under 515–565 nm of fluorescent light. Negative control sections were treated in a similar fashion but lacking the terminal deoxynucleotidyl transferase enzyme. To identify healthy cells with normal nuclear morphology, after TUNEL staining was complete, a DAPI nuclear stain was added and visualized as blue when excited at 360 nm of fluorescent light (Vector Laboratories). For each TUNEL and DAPI-stained cell or tissue section, green apoptotic and blue healthy nuclei were blindly counted in 10 random microscopic fields. Quantification of the apoptotic index (the number of apoptotic nuclei per total nuclei × 100) was assessed blindly in 10 random microscopic fields per sample using Image Pro Plus 6.3 software (Media Cybernetics).

**References**


S1. Gene expression levels of LIGHT in the placental tissues of normal pregnancy individuals (NT) and preeclampsia patients (PE). Placental mRNA of LIGHT expression was quantified by RT-PCR. Data are expressed as means ± SEM. *P < 0.05 compared to NT group. N=5-7.
S2. Circulating LIGHT levels in the pregnant mice with saline or LIGHT-injected pregnant mice. LIGHT and saline were injected to pregnant mice on gestation E13.5 and E14.5 twice. Then Blood was withdrawn and serum was collected on gestation E18.5. Circulating LIGHT levels were measured by ELISA. Data are expressed as means ± SEM. *P < 0.05 compared to saline-injected group. N=8-10.
S3. LIGHT-induced increased blood pressure and proteinuria were significantly reduced and associated with decreased circulating LIGHT and sFlt-1 levels in pregnant mice postpartum. Pregnant mice were injected with saline or LIGHT. LIGHT injection resulted in hypertension (A) and proteinuria (B) in pregnant mice. Both hypertension and proteinuria significantly reduced in the mice postpartum day 5. Additionally, circulating LIGHT and sFlt-1 also were significantly reduced in the pregnant mice postpartum day 5. Data are expressed as means ± SEM. * \( P < 0.001 \) versus saline injected pregnant mice; ** \( P < 0.01 \) versus LIGHT injected pregnant mice).
S4. Intra-carotid mean arterial blood pressure (MAP) was measured in non-pregnant mice with injection of saline or LIGHT on day 5. Data are expressed as means ± SEM. *$p < 0.05$ compared to saline-injected group. n=-5-8/group.