Maternal Autoantibodies From Preeclamptic Patients Activate Angiotensin Receptors on Human Mesangial Cells and Induce Interleukin-6 and Plasminogen Activator Inhibitor–1 Secretion

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Background: Preeclampsia affects 3–5% of all pregnancies. It is a major cause of maternal and fetal morbidity and mortality. Recent studies demonstrate that autoantibodies against the angiotensin II type 1 (AT₁) receptor are present in the serum of preeclamptic patients. In this study, we investigated the role of AT₁ receptor–agonistic autoantibody (AT₁-AA) regarding interleukin-6 (IL-6) and plasminogen activator inhibitor–1 (Pai-1) secretion in human mesangial cells.

Methods: The study included ten patients: five severely preeclamptic and five normotensive pregnant women. Immunoglobulin-G (IgG) was purified from each individual. The presence of AT₁-AA was determined based on its ability to stimulate an increase in the contraction rate of rat neonatal cardiomyocytes. Primary human mesangial cells were chosen to study IgG-induced secretion of IL-6 and Pai-1. Losartan and epitope peptides were used to determine whether AT₁-AA interaction with AT₁ receptor was associated with stimulation of IL-6 and Pai-1 secretion and was mediated through AT₁ receptor activation.

Results: The IgG from preeclamptic patients stimulated an increased contraction rate in rat neonatal cardiomyocytes. The IgG from preeclamptic patients induced the AT₁ receptor–specific secretion of IL-6 and Pai-1 from human mesangial cells at a significantly higher level than that achieved with IgG from normotensive patients. Competition with an epitope peptide suggested that the AT₁ receptor was stimulated by AT₁-AA.

Conclusions: Our findings suggest that a maternal autoantibody with the ability to activate AT₁ receptors may account for the development of renal damage seen in preeclamptic patients. Am J Hypertens 2005;18:330–336 © 2005 American Journal of Hypertension, Ltd.

Key Words: Preeclampsia, angiotensin receptor, mesangial cells, plasminogen activator inhibitor–1, interleukin-6, renal damage, hypertension, disseminated intravascular coagulation, inflammation.

Preeclampsia is a disease specific to pregnancy. It is clinically defined by gestational hypertension and proteinuria, associated with edema and vascular abnormalities. It is a leading cause of morbidity and mortality for the mother and developing fetus, affecting 3% to 5% of all pregnancies.1 Although this problem is widely recognized, the etiology of the disease remains unknown.2 It has been reported that disseminated intravascular coagulation plays a role in the development of acute renal failure.3 The pathology of this system includes the disappearance of pro-coagulatory agents, intravascular reduction in fibrin degradation, and organ damage resulting from microthrombi. Mesangial cells in the glomerulus can be induced by angiotensin II (Ang II) to secrete plasminogen activator inhibitor–1 (Pai-1).4 High circulating levels of Pai-1 in preeclamptic patients are likely to be associated with the pathologic renal condition of disseminated intravascular coagulation.5,6 There is also evidence that a localized inflammation occurs in the kidney in renal disease.7 Angiotensin II–induced pro-inflammatory re-
response in the kidney is likely to play a role in the overall symptoms of edema and glomerular damage in preeclampsia. Interleukin-6 (IL-6) is an Ang II–induced cytokine secreted by mesangial cells. The release of IL-6 from mesangial cells may cause localized inflammation and the release of oxygen radicals, which may contribute to renal and glomerular damage. This factor has a higher concentration in the circulation of preeclamptic patients compared with normotensive individuals.

Agonistic autoantibodies that activate the Ang II type 1 (AT$_1$) angiotensin receptor are associated with preeclampsia and have been suggested as a possible cause for the development of the preeclamptic pathologic condition. Investigation into a possible association between AT$_1$ receptor activation in preeclamptic patients and the release of IL-6 and Pai-1 by mesangial cells is warranted, given these findings. Here we report our finding that AT$_1$ receptor–activating autoantibodies (AT1-AA) in the serum of preeclampsia patients cause the induction of Pai-1 and IL-6 secretion by mesangial cells.

Materials and Methods

Patients

Ten patients were selected for this study. Patients were identified by the obstetrics faculty of the University of Texas Medical School attending to patients admitted to Memorial Herman Hospital. Five patients were diagnosed with severe preeclampsia based on the definition set by the National Blood Pressure Education Program Working Group Report. The criteria were based on the presence of persistent hypertension of $\geq 160/110$ mm Hg and significant proteinuria appearing after 20 weeks of pregnancy. Significant proteinuria was defined as a positive reading on urinanalysis or $\geq 300$ mg of protein in a 24-h urine collection. These women had no medical history of chronic hypertension before pregnancy. Other criteria included the presence of a persistent headache, visual symptoms, epigastric pain, or the hemolysis, elevated liver enzymes, and low platelets (HELLP) syndrome in association with hypertension $\geq 140/90$ mm Hg. These samples were coded to prevent patient identification. The research protocol was approved by the Institutional Committee for the Protection of Human Subjects.

Quantification of Cardiomyocyte Contraction Rate

Rat neonatal cardiomyocytes were prepared as previously described. Contraction rates were also determined as previously reported.

Preparation of Serum From Patient Blood Samples

For samples of $\leq 10$ mL of whole blood, red-capped (no additive) tubes were spun at 1800 rpm for 10 min at room temperature to separate serum from the cells. The serum was aliquoted and stored at $-80^\circ$C.

Preparation of Immunoglobulin-G Fractions From Serum

For this procedure, Bio-Rad Laboratories polyprep columns (Bio-Rad, Hercules, CA) (75 mm long, 2 mL volume) were used. To prepare a column, 200 $\mu$L of GammaBind G (Amersham, Piscataway, NJ) Sepharose gel was added on the top of the column filter. Immunglobulin-G (IgG) was collected according to the manufacturer’s suggested protocol. To measure the total concentration of the immunoglobulin, $A_{280}$ readings were recorded from a spectrophotometer. The conversion ratio for mg/mL of IgG is 1 OD = 0.75 mg/mL IgG.

Human Mesangial Cells

Primary human mesangial cells were obtained from a commercially available source, Biowhitaker/Clonetics. The human mesangial cells were grown in 24-well plates at a cell density of roughly 50% confluence. The cells were allowed to grow overnight before being switched to serum-free medium overnight. The next day the medium was replaced again. This was done to remove any potential stimulating agents that would cause the secretion of Pai-1 and IL-6. The cells were incubated with the doses of angiotensin II, IgG, losartan (Merck, Whitehouse Station, NJ), or the competitive AFHYEQ epitope peptide fragment. Preliminary experiments determined that a 20 $\mu$M dose of losartan and a concentration of 125 $\mu$M for epitope peptide was required for experiments with the human mesangial cells (data not shown). These experiments also included the use of a noncompetitive control peptide, VFFIENE. After 24 h, serum was collected and prepared for treatment on the Immubind Pai-1 plate (American Diagnostica, Stamford, CT) and IL-6 EIA kit (Diaclone, Besancon, France). In some cases samples were frozen at $-20^\circ$C overnight ($-70^\circ$C long term) before testing.

Cell Lysate Normalization

After the tissue culture supernatant was collected, wells containing human mesangial cells were washed with PBS and then aspirated. A 50-$\mu$L quantity of RIPA buffer prepared as 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM PMSF, 1 mM EDTA, 1% Triton X-100, and 0.1% SDS, with protease inhibitor cocktail (Complete, Roche, Nutley, NV) was added to each well in the 24-well plate. The plates were scraped to remove the cell lysate and stored in microcentrifuge tubes at $-70^\circ$C. The protein concentration for each sample was calculated by Bradford assay. The Pai-1 (in nanograms per milliliter) or IL-6 (in picograms per milliliter) secreted in the medium was normalized to protein content from cell lysate (in micrograms of protein per well), and untreated samples were referenced for fold induction measurements.
Statistical Analysis

All values are expressed as mean ± SEM. Data were graphed and analyzed for statistical significance using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA). Statistical significance was determined by analysis of variance. A P value of <.05 was interpreted to mean that observed experimental differences were statistically significant.

Results

We found that the cardiomyocyte contraction assay serves to identify AT1 receptor activating antibody in the serum of preeclamptic individuals. Cardiomyocyte contraction rates can be stimulated by AT1 receptor activation. A dose–response curve relating the concentration of angiotensin II and the contraction rate of the cardiomyocytes is shown in Fig. 1A. Losartan, an AT1 receptor antagonist, blocked the angiotensin II–induced increase in contraction rate. Wallukat et al capitalized on the highly conserved nature of AT1 receptors between rodents and humans to develop an in vitro bioassay to detect the presence of AT1 receptor activating autoantibody (AT1-AA) by measuring an increase in cardiomyocyte contraction rate. 15 We used this assay to identify AT1-AA present in preeclamptic patients. Initially, three IgG dilutions (1/400, 1/100, and 1/10) were compared for one preeclamptic patient and one normotensive pregnant individual. The results (Fig. 1B) demonstrate that IgG from the preeclamptic patient stimulated a dose-dependent increase in contraction rate, whereas IgG from the normotensive subject did not. To determine whether IgG from the preeclamptic patient stimulated an increase in contraction rate through AT1 receptor activation the IgG was tested in the presence of losartan, an AT1 receptor–agonist autoantibody (AT1-AA) epitope from the second extracellular loop of the AT1 receptor. The VFFIENE epitope peptide did not compete for receptor-ligand-type interaction. *

Wallukat et al13 reported that the ability of AT1-AA to activate AT1 receptors was blocked by adding a 7–amino acid peptide, AFHYESQ, corresponding to a sequence present in the second extracellular loop of the AT1 receptor. To determine whether IgG in the preeclamptic patients enrolled in our study is directed against the same epitope, we incubated IgG with increasing concentrations of the epitope peptide before the addition to the cultured cardiomyocytes. Figure 1D illustrates that IgG-mediated stimulation of cardiomyocyte contraction rates was reduced by the addition of increasing doses of AFHYESQ, indicating that this peptide competes with the interaction of AT1-AA with the AT1 receptor. Overall, these results are in good agreement with those of Wallukat et al13 and show that the rat neonatal cardiomyocyte contraction assay can be used as a bioassay to identify AT1-AA.

We also observed that the AT1 receptor agonistic autoantibody stimulates IL-6 production in human mesangial cells. Ogino found monocyte invasion in the mesangium.
of preeclamptic patients. Secreted by mesangial cells, IL-6 is a likely facilitator in this process. To establish whether AT1 receptor activation in human mesangial cells results in increased IL-6 secretion, we set up an angiotensin II dose–response curve. For standardization, the fold induction was normalized to the protein content of the human mesangial cell lysate. A dose-dependent, losartan-sensitive induction of IL-6 secretion was observed with increasing concentrations of angiotensin II (Fig. 2A). The conclusion from this experiment is that angiotensin II stimulated the induction and secretion of IL-6 through the AT1 receptor activation in a dose-dependent manner.

To make an initial determination whether preeclamptic patients show an AT1 receptor–specific increase in stimulation of IL-6 secretion, we compared five preeclamptic patients with five normotensive pregnant individuals using 1/40 dilutions of IgG fractions. The IgG from preeclamptic patients consistently showed higher IL-6 secretion compared with IgG from normotensive pregnant subjects. This separation of the patient groups into two different clusters was highly significant (P < .001). In conclusion, these data show that preeclamptic patients show a greater induction of IL-6 secretion than do normotensive pregnant individuals.

To determine whether the induced secretion of IL-6 after IgG treatment met the agonistic autoantibody criteria, three patients were evaluated for the following: 1) induction of IL-6 secretion by 1/40 dilution IgG fractions from preeclamptic patients; 2) test for AT1 receptor–specific activation with the addition of the AT1 receptor antagonist losartan; 3) successful competition and reduced induction after incubation of the immunoglobulin fraction with the competitive peptide AFHYESQ; and 4) failure to block the induction by co-incubation of the IgG fraction with the nonepitope peptide VFFIENE. The results (Fig. 2D) demonstrated the following: 1) IgG from preeclamptic patients had a higher induction than IgG from normotensive pregnant subjects; 2) the addition of losartan blocked the AT1 receptor–specific activity present in the IgG fraction from the preeclamptic patients; 3) the epitope peptide AFHYESQ blocked the IgG-mediated activation of the
AT1 receptor; and 4) the noncompetitive peptide had no effect in blocking AT1-AA mediated activation of the AT1 receptor. In conclusion, these data demonstrate that an AT1 receptor–specific autoantibody caused the induction of IL-6 secretion.

We also found that AT1 receptor agonistic autoantibody stimulates Pai-1 production in human mesangial cells. Disseminated intravascular coagulation contributes to renal damage in preeclamptic patients, possibly in association with increased Pai-1 levels.17–19 Mesangial cells can be a source of secreted Pai-1, influenced by AT1 receptor activation.4 The IgG preparations from preeclamptic patients were evaluated for the ability to activate AT1 receptors and to induce Pai-1. Angiotensin II–mediated induction of Pai-1 (Fig. 3A) was observed in a manner similar to that of IL-6 (Fig. 2B). We found AT1 receptor–specific activity in the IgG of a preeclamptic patient but was absent in IgG from a normotensive pregnant subject (Fig. 3B). When IgG treatments from five preeclamptic patients and five normotensive patients were compared for Pai-1 secretion, a highly significant separation of the patient groups (P < .001) was revealed (Fig. 3C). Three patients were evaluated for AT1 receptor–specific induction of Pai-1 secretion by the suspected agonistic autoantibody. Losartan sensitivity and inhibition by the epitope peptide (Fig. 3D) indicated that the stimulation of Pai-1 secretion was mediated through activation of the AT1 receptor. In conclusion, these data demonstrate the presence of an AT1 receptor agonistic autoantibody causing the induction of Pai-1 secretion.

**Discussion**

Renal damage is one of the hallmark symptoms of preeclampsia. Recent work published by Luft et al10 demonstrated renal damage based on high angiotensin II stimulation in a rat model. In studies that allowed renal biopsies, evidence for lymphocyte infiltration has been shown.16 In these rodent and human studies, the pathologic condition of the glomerulus has drawn connections to angiotensin II signaling and disease-state pathology. The role of the mesangial cell in the glomerular structure is to maintain the proper interaction of the blood flowing through the arteriole and the porous space into the Bowman’s capsule that begins the excretory system into the urinary tract. The literature surrounding this topic reveals the following: 1) angiotensin II signaling can influence the secretion of Pai-1 and IL-6 in mesangial cells4,9; 2) high levels of Pai-1 are likely to be associated with the disseminated intravascular coagulation present in the glomerular structure of preeclamptic patients17; and 3) release of IL-6 by mesangial cells may result in the activation the pro-inflammatory response of circulating macrophages and lymphocytes in the kidney tissue.7 Both Pai-1 and IL-6 have been reported to occur at higher levels in the circulation of preeclamptic patients compared with normotensive pregnant women.6,12 The experiments presented here propose a case for AT1-AA as a potential culprit for causing mesangial cells in the kidney tissue to secrete factors that cause renal damage.

Preeclampsia is characterized by a sustained inflammatory
response during the second and third trimesters of pregnancy. The process of lymphocytes adhering to vascular endothelial cells and migrating through cell layers disrupts the normal integrity of the tissue, resulting in vascular injury and disruption of normal physiologic function. Lymphocytes can be found to infiltrate into tissues, resulting in edema. Links have been established with the renin–angiotensin system and secretion of proinflammatory cytokines by endothelial, smooth-muscle, and mesangial cells. Novel data reported here show the impact of AT1 receptor activation and secretion of IL-6 in human mesangial cells. Preeclamptic patients have higher levels of IL-6, a proinflammatory cytokine, in the circulation. It has been observed that IL-6 has properties that activate macrophage and lymphocyte responses. This raises the possibility of an association between the presence of an agonistic autoimmune response (that is, AT1 receptor activation) and the release of the proinflammatory cytokines. The proinflammatory response in the kidney is likely to play a role in the overall symptoms of edema and glomerular damage in preeclampsia. Interleukin-6 is an example of a cytokine that participates in the local inflammatory response. The release of IL-6 from mesangial cells may also cause the release of oxygen radicals, which may contribute to renal and glomerular damage. Investigation into a possible association between AT1 receptor activation in preeclamptic patients and the release of IL-6 by mesangial cells is suggested by our findings.

In preeclampsia, the glomerulus becomes enlarged due to hypertrophy of the intercapillary cells, including endothelial and mesangial cells. The basement membrane bordering the epithelial cells becomes thicker. These changes can cause occlusion of the capillary lumen, defined pathologically as “glomerular capillary endotheliosis.” This is a common complication seen in patients with preeclampsia. A possible explanation for this is that the swelling of endothelial cells in the glomerulus causes occlusion in the glomerular capillaries, causing damage, lesion formation, and loss of normal glomerular function. Hypertrophy is noted in the endothelial cells lining both the arteriole capillary membrane and the mesangial cells in the glomerulus. Histopathologic findings of renal failure in preeclampsia show that the glomerular endothelial cells become enlarged. Fibrin thrombi can be found in the afferent arterioles and glomeruli. Many of the features of glomerular capillary endotheliosis could be explained by activation of AT1 receptors present on glomerular endothelial cells, resulting in increased Pai-1 production and cell proliferation. Two recent studies have shown that angiotensin II stimulates increased Pai-1 production by cultured endothelial cells, suggesting that AT1 receptor activation may mediate the inhibition of fibrinolysis in the vasculature. It has been found that AT1 receptor activation is also associated with endothelial cell hypertrophy. Thus, AT1-AAA may contribute to renal glomerular pathology by activating AT1 receptors on glomerular endothelial cells and on mesangial cells.

Antibodies capable of activating G-protein–coupled receptors are associated with a number of human diseases. One of the best studied examples is Graves’ disease, a condition characterized by the presence of antibodies capable of activating the thyroid-stimulating hormone receptor on thyroid cells, resulting in excessive production of thyroid hormones. Antibodies directed against the cardiac β1-adrenergic receptor are associated with patients with dilatative cardiomyopathy. These IgG antibodies function as agonists for the β1-adrenergic receptor, resulting in a positive chronotropic effect on cultured cardiomyocytes. Antibodies capable of activating the α1-adrenergic receptor are associated with various forms of hypertension. Finally, antibodies capable of activating the muscarinic M2 receptor are associated with idiopathic dilated cardiomyopathy. The AT1 receptor–activating antibodies have been associated with preeclampsia, as described here, but have also been associated with various forms of essential hypertension. Thus, the presence of agonistic antibodies directed to G-protein–coupled receptors has been observed in numerous disease states. It is likely that these antibodies mediate their agonistic effect by cross-linking and thereby stabilizing the active conformation of the receptor. This hypothesis is supported by recent data regarding the effect of monoclonal antibodies on the β1-adrenergic receptor or the muscarinic M2 receptor. These antibodies realize their agonistic properties via stabilization of the dimeric conformation of the receptors. A large volume of recent data indicating that many G-protein–coupled receptors are activated by dimerization also supports this view. So, the simplest view is that these agonistic antibodies activate their target receptors by promoting receptor dimerization.

Our findings add to the growing body of literature that supports a role for the AT1 receptor agonist autoantibody in the etiology of preeclampsia. The production and secretion of tissue factor has been associated with the presence of the AT1-AAA and increased activity of NADPH oxidase. These two factors may play a role in vascular injury and oxidative stress. Abnormal placentalation has also been implicated, with decreased invasiveness of trophoblasts and increased secretion of Pai-1, possibly associated with abnormal spiral arteriole remodeling. More recently, evidence has been presented that AT1-AA-mediated AT1 receptor activation results in calcium mobilization and the activation of genes under the control of calcineurin and nuclear factor of activated T cells signaling. All of these reports share the common association that AT1 receptor activation by an agonistic autoantibody is a potential major contributor to the pathophysiology of preeclampsia.

References