Adrenomedullin Is Decreased in Preeclampsia Because of Failed Response to Epidermal Growth Factor and Impaired Syncytialization

Hongshi Li, Jamal Dakour, Susan Kaufman, Larry J. Guilbert, Bonnie Winkler-Lowen, Donald W. Morrish

Abstract—To explore the mechanisms of adrenomedullin (ADM) regulation in normal and preeclamptic (PE) states, we determined placentental production of ADM and ADM regulation by cytokines. Isolated, purified cytotrophoblast cultures from normal (n=8) and PE (n=10) placentas were cultured for 3 days in the absence or presence of 10 ng/mL epidermal growth factor (EGF), 1 ng/mL transforming growth factor (TGF)-β1, 10 ng/mL tumor necrosis factor (TNF)-α, or 100 U/mL interferon (IFN)-γ. Cells were also cultured for 3 days in 10% fetal bovine serum for determination of syncytial formation by desmoplakin staining. Pieces of normal and PE placentas were snap-frozen for ADM mRNA measurement. Results showed that basal ADM production into culture medium by radioimmunoassay was significantly lower in PE placentals. EGF significantly stimulated ADM production in normal trophoblasts but did not in PE placentas. None of the factors TNF-α, TGF-β1, or IFN-γ altered ADM secretion in either normal or PE placentas. ADM expression by Northern blot analysis demonstrated a 34.3±8.3% reduction in mRNA expression in PE placentas. Syncytialization, as assessed by desmoplakin-outlined syncytial units, was decreased in PE placentas (day 3: normal, 16.7±1.3%; PE, 5.5±2.0%; P<0.01, ANOVA). However, there was a normal increment in syncytialization in response to EGF in normal and PE trophoblast preparations (EGF day 3: normal, 43.8±5.6%; PE, 46.1±12.3%). We conclude that spontaneous placentental syncytialization is impaired in PE and that ADM production is markedly reduced in PE, possibly owing to an impaired EGF response. These abnormalities indicate poor placentental production of ADM as the likely cause of a failed compensatory increase in maternal serum ADM levels in PE. (Hypertension. 2003;42:895-900.)

Key Words: adrenomedullin ■ preeclampsia ■ interferon ■ epidermal growth factor ■ tumor necrosis factor ■ pregnancy

Adrenomedullin (ADM) is a novel, potent autacoid expressed in many tissues that induces vasodilatation, natriuresis, and diuresis.1 ADM levels are increased in hypertension and congestive heart failure, indicating that ADM is acting as a counterregulatory hormone to the hypertensive and volume-overloaded state. Infusion of ADM into humans and rats with congestive heart failure has demonstrated beneficial effects of increased cardiac output, natriuresis, and diuresis; decreased blood pressure and aldosterone; and increased renal blood flow.2,3 Preeclampsia (PE) is a unique state occurring in pregnancy that is characterized by hypertension, proteinuria, abnormalities of endothelial function, reduced fetoplacental blood flow, and hypoxia and that is cured by delivery of the placenta.4–6 Despite many abnormalities being described,5,7,8 there is as yet no clear cause of PE. Because of its vasodilatory properties, ADM has been intensively studied in pregnancy and PE as a potential pathogenic factor in this disease. ADM is widely expressed in maternal and fetal tissues, including villous and extravillous trophoblasts, chorion, decidua, and fetal membranes.9,10 We and others have shown a progressive increase in maternal plasma ADM levels throughout pregnancy.11–16 In PE, early reports suggested that maternal ADM levels were decreased, but subsequent larger series found no difference from controls.14,17,18 However, we believe that this indicates a lack of compensatory increase in response to the hypertensive state.16 Immunostaining data have shown either normal or decreased placental ADM expression.19,20 ADM also has demonstrable effects as a cytokine growth factor, being able to inhibit or stimulate cancer cell proliferation, depending on the cell type and culture conditions, and hence, might influence placental function by paracrine mechanisms.

Cytokine regulation of ADM has been extensively studied in various animal cells, but no data are available regarding its regulation in the placenta. It is known that multiple cytokines regulate placental function21 and that many cytokine abnor-
malities exist in PE, including elevated serum tumor necrosis factor-α (TNF-α).22 Other studies have demonstrated that TNF-α and interferon-γ (IFN-γ) induce trophoblast apoptosis and that this effect is blocked by epidermal growth factor (EGF).23 Because apoptosis is increased in PE,24 TNF-α and IFN-γ might therefore play a role in this process. We have also shown that EGF stimulates and TGF-β1 inhibits cytotrophoblast differentiation into syncytiotrophoblast.25-27 Abnormalities in EGF action have been linked as well to intrauterine growth restriction (IUGR), which often accompanies severe PE.28,29 Because of the possible roles of these cytokines in placental biology and pathophysiology, we wished to determine their effects on ADM secretion and whether such effects were different between normal and PE trophoblasts.

Methods

These studies received institutional ethics approval. PE was defined according to the Report of the National High Blood Pressure Education Program Working Group criteria as hypertension starting during pregnancy after 20 weeks’ duration with blood pressure >140 mm Hg systolic and >90 mm Hg diastolic and proteinuria ≥1.30 Presence or absence of IUGR was assessed by established criteria.24 No subjects were smokers, had diabetes or other disease, or were taking medication other than antihypertensives.

Term normal (n=8) or PE (n=10) human placental tissues were obtained by vaginal delivery and/or cesarean section and were immediately transported on ice to the laboratory. Isolated, purified cytotrophoblast cultures were prepared by trypsin–DNAse I digestion, as previously described.25 These cells are >95% pure cytotrophoblasts, and in vitro, they rapidly and spontaneously differentiate toward syncytiotrophoblast.31 Cells (2×10⁶ per well) were plated in 12-well dishes (Linbro) in Dulbecco’s modified Eagle’s medium—10% fetal bovine serum–penicillin-streptomycin. After 2 hours, the medium was changed to serum-free Dulbecco’s modified Eagle’s medium, and cytokines were added (10 ng/mL recombinant human EGF, 1 ng/mL TGF-β1, 10 ng/mL TNF-α, or 100 U/mL IFN-γ). Duplicate cultures of each cytokine were done. These doses have been shown to have biologic effects.23,25,28 The medium was changed daily and frozen for subsequent ADM radioimmunoassay (RIA). Cytokines were added after each change of medium. For desmoplakin studies, cells were further purified on CD9/HLA class I and class II immunoelimination columns.33 These cells show slow, spontaneous differentiatination in culture even in the presence of 10% fetal bovine serum,27 which is required for desmplakin expression.23,32 Immunopurified cells otherwise have biologic and biochemical functions identical to less purified cells.27,34,35 Cells from 4 normal and 4 PE placentas were prepared. Pools of each group were cultured in triplicate for 3 and 5 days with and without 10 ng/mL EGF present and stained on days 3 and 5 for desmoplakin, as previous described.23 Syncytial unit formation was quantified by counting nuclei in cytotrophoblasts (mononuclear) and syncytiotrophoblasts (>2 nuclei per unit) units from 9 separate, randomly chosen microscope fields from each replicate. Syncytial formation was expressed as the ratio between the number of nuclei in syncytiotrophoblast divided by the total number of nuclei ×100%.

Eight normal and 10 PE placental pieces were collected and snap-frozen. RNA was extracted first by homogenization in LNE followed by treatment with Trizol. Northern blot analysis was performed as described.36 mRNA expression in both immunopurified and nonpurified cultured cells was also performed at 3 days of culture. cDNA probing was performed as previously described with a specific human ADM cDNA probe.36 Blots were reprobed with an 18S rRNA to assess RNA loading, and the results were compared by laser densitometry.36 Ethidium bromide staining was also performed.

ADM RIA was performed as previously described.16 In brief, medium was collected into tubes containing EDTA plus 500 KIU aprotinin per 5-mL tube and frozen for subsequent RIA. Samples were extracted as described by Lewis et al.37 Samples were mixed with an equal volume of phosphate alkaline–treated casein buffer. Phosphate alkaline–treated casein buffer contains 0.05 mol/L phosphate buffer, pH 7.4, 0.1% alkali-treated casein, 0.1% Triton X-100, 0.1% sodium EDTA, and 0.2% NaNO₃. Alkali-treated casein was prepared as described.38 Sep-Pak C-18 columns (Waters Corp) were preequilibrated with 5 mL methanol and 10 mL 0.9% saline. The medium-buffer mixture was added and the columns washed with 5 mL 0.9% saline. ADM was eluted with 2 mL 80% isopropanol/0.013 mol/L HCl into a tube containing 10 μL 1% Triton. The eluate was dried under N₂ and the extract stored at −70°C until ready for RIA. Extraction efficiency was 85%. Each sample was assayed in duplicate with a RIA kit from Phoenix Pharmaceuticals. The intra-assay and interassay coefficients of variation were 7.5% and 10.0%, respectively, as previously reported.16

Results were expressed as the mean±SEM. Statistical analysis was performed with Sigma Stat software by applying a 2-way, repeated-measures ANOVA followed by a Student-Newman-Keuls test for each cytokine versus control; comparison of normal versus PE data for each cytokine was performed separately.

### TABLE 1. Characteristics of Controls and Patients With PE

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Normal Control</th>
<th>PE</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of subjects</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>Blood pressure, mm Hg, mean±SEM</td>
<td>99±13</td>
<td>162±29</td>
</tr>
<tr>
<td>Systolic</td>
<td>99±3</td>
<td>99±0</td>
</tr>
<tr>
<td>Diastolic</td>
<td>39.2±0.5</td>
<td>35.5±1.1</td>
</tr>
<tr>
<td>Gestation at delivery, wk, mean±SEM</td>
<td>700±65</td>
<td>562±116</td>
</tr>
<tr>
<td>Placental weight, g, mean±SEM</td>
<td>3526±165</td>
<td>2443±387</td>
</tr>
<tr>
<td>Fetal weight, g, mean±SEM</td>
<td>140 mm Hg systolic and proteinuria ≥1</td>
<td></td>
</tr>
<tr>
<td>Delivery type</td>
<td>4 Spontaneous vaginal, Cesarean section</td>
<td></td>
</tr>
<tr>
<td>Proteinuria</td>
<td>None</td>
<td>1+ (n=1), ≥2+ (n=9), HELLP (n=2)</td>
</tr>
</tbody>
</table>

All controls and PE patients were nonsmokers and had no illness other than PE. One PE patient but no control patients had IUGR. HELLP indicates HELLP syndrome (hemolysis, elevated liver function test, low platelet count).
Results

Table 1 shows the characteristics of the normal and PE patients from whom the placentas were obtained. All PE patients were hypertensive and had >1+ proteinuria. PE patients demonstrated significantly reduced fetal and placental weights, but only 1 patient fulfilled the criteria for IUGR. No control patients had IUGR. All normal controls were screened to ensure that they had normal blood pressure (<140/90), had no other disease, were nonsmokers, were taking no medications, and did not have any other diseases. Figure 1 shows the mRNA expression of ADM in 8 normal and 10 PE placentas. There was a significant 34.3±8.3% reduction in ADM mRNA expression (P<0.05, ANOVA). Figure 2 shows the ADM secretion into culture medium from 9 normal and 9 PE placental cultures. Unstimulated normal placentas showed a significantly greater secretion of ADM than did PE placentas (P<0.05). EGF induced a significant increase in ADM secretion in normal placentas (P<0.001) but no significant increase in PE placenta (P>0.05, NS). EGF induction of ADM was greater than any effect of TNF-α, TGF-β1, or IFN-γ. Neither normal nor PE placentas showed a significant effect of TNF-α, IFN-γ, or TGF-β1 on ADM secretion (P>0.05). However, for all cytokines, PE placental cells had lower ADM secretion than did correspondingly treated normal cells (P<0.05). ADM secretion on days 1 and 2 of culture was similar to that on day 3 for all cytokines and controls (data not shown).

Figure 3 shows the effects of EGF on syncytialization in normal (Figures 3a and 3b) and PE (Figures 3c and 3d) cytotrophoblast cells. Table 2 lists the syncytialization rates at days 3 and 5 of culture. After 3 days, there was a significantly lower spontaneous syncytialization rate of 5.5±2.0% in PE cells (P<0.01). Normal control cells showed no significant difference between day 3 and day 5 (P=NS). However, EGF stimulation produced a similar increment in syncytialization rate in both normal and PE cells. In both cell types, there was a significant and large increase in syncytialization after EGF exposure, consistent with prior data.25 Similar responses were noted at 5 days of culture. Both immunopurified and nonpurified cells demonstrated similar expression of ADM with and without EGF stimulation at day 3 of culture (Figure 4). ADM expression was greater in EGF-stimulated cells, because EGF induces more rapid differentiation of cytotrophoblasts into syncytiotrophoblasts.27

Discussion

These data demonstrate for the first time that PE placentas have decreased mRNA expression and decreased secretion of ADM into culture medium. Previously, there have been
conflicting data as to whether qualitative estimates of immunoreactive ADM have been normal or decreased in PE. Our results agree with those of Kanenishi et al that ADM mRNA is decreased in PE. The reason for the differences with the results of Makino et al is unclear but might relate to sample selection or to the fact that PE placentas are heterogeneous, with areas of histologically normal and abnormal tissue. In our studies, cell cultures used extracts from most of the placenta in multiple areas, and hence, heterogeneity would not affect the results. mRNA expression in pieces of placenta was in agreement with that in the cell cultures, suggesting that there was not a selection artifact in these results.

In PE, there is cytotrophoblast proliferation and increased syncytial sprouts, both of which might be a response to hypoxia and increased apoptosis, characteristics of the disease. There also might be reduced syncytial mass with a reduced proportion of syncytium, indicating a failure of cytotrophoblasts to differentiate into syncytium. Recent data suggest that these morphological abnormalities might reflect a component of IUGR rather than PE without IUGR. However, only 1 of our patients had IUGR, and hence, the results cannot be explained by this process.

After 3 days of culture, normal cytotrophoblasts demonstrated a modest, spontaneous differentiation rate into syncytiun (Table 2), as previously described. However, PE cytotrophoblasts had a significantly lower spontaneous differentiation rate (Table 2) at day 3 of culture. Although there was a normal syncytialization response to EGF, it is known that syncytialization, which includes cell fusion to form giant cells (syncytiun) as well as numerous biochemical changes, is regulated by a large variety of factors that might be abnormal in PE. The data also show that despite a normal syncytialization response to EGF, the syncytiun thus formed were abnormal and unable to secrete normal amounts of ADM, as would be expected of normal control cells (Figure 2). By day 5 in culture, the PE trophoblasts were similar to normal trophoblasts in terms of spontaneous syncytialization. The cause of this is unclear and might represent an artifact of recovery during in vitro culture. Nonetheless, the PE cytotrophoblasts displayed a reduced syncytialization ability in early culture. The data are thus consistent with a defect in early, spontaneous differentiation in vitro but a normal morphological formation of syncytiun in response to EGF that overrode these defects.

Hypoxia is considered to be a likely etiologic factor in PE. It is also known that ADM is likely a hypoxia-induced gene. Thus, the low levels of ADM in PE indicate either a failure of the hypoxic transduction mechanism in PE or that nonhypoxic factors are responsible for the poor ADM production. No studies are available to address this question.

Prior studies have shown that EGF receptors are important in early mammalian development, including, for example, the trophectoderm, and that targeted deletion of the EGF receptor results in widespread abnormalities, including poor placental development. Early in pregnancy, at 4 to 5 weeks’ gestation, EGF might act also to induce proliferation, whereas, at 6 to 12 weeks and later, it induces differentiation. In IUGR, in which placentas and fetuses are small, EGF receptors show decreased phosphorylation and numbers of receptors, with some abnormal receptor sizes. It is unknown whether such abnormalities are present in PE, but in severe PE the placentas are often also small, consistent with a defect in syncytial formation. Recent data suggest that the morphological abnormalities described are attributable to the IUGR component as a separate but concurrent disease.
process rather than PE without IUGR. However, although the patients whose placentas were used in this study had quite severe PE, only 1 had associated IUGR (Table 1). Thus, the current data support a separate non–IUGR-related defect in PE to account for the reduced placental differentiation. Our data thus suggest that there is a functional abnormality in the EGF signal transduction pathway that results in lack of induction of ADM production but that preserves postreceptor pathways that lead to cell fusion. The nature of these transduction defects is unknown.

These experiments are important in understanding the potential role of ADM in the pathogenesis of PE. Previous work has established that ADM levels are elevated in hypertensive and in sodium- and volume-overloaded states, such as congestive heart failure. This indicates that ADM does not cause hypertension per se but rather acts as a counterregulatory hormone to decrease blood pressure and sodium and volume overload induced by other mechanisms. Work by ourselves and others has indicated that ADM increases progressively throughout pregnancy. Although early, small series demonstrated low ADM levels in PE, other larger series including our own have not confirmed this. It is unknown whether this represents patient selection, there is a spectrum of disease states, or concomitant IUGR was present in the patients studied. We did not find in our prior series that there was a correlation between degree of proteinuria or hypertension and ADM levels. Because ADM is a counterregulatory hormone in hypertensive states, it would be anticipated that serum ADM levels should be increased in PE, but this does not occur. We therefore postulate that ADM production by PE placentas is deficient, resulting in this lack of an expected increase in serum ADM. The current data support this hypothesis, demonstrating a marked reduction in placental expression in vitro. The major nonplacental source of serum ADM is thought to be secretion from endothelial cells. A reduced placental contribution might therefore not be sufficient to reduce overall serum levels if endothelial production represents a much larger proportion of ADM production. No data are available to verify this possible explanation. The failure of an adequate placental output in PE might nonetheless contribute to the increased vascular resistance and hypertension noted in PE due to a relative deficiency of ADM available for uteroplacental bed vasodilation on a paracrine basis.

Perspectives
ADM is a physiologic regulator of blood pressure, and its levels increase 3-fold during pregnancy. However, maternal serum levels are not decreased in PE. In contrast, we have found that PE results in a significant suppression of placental ADM production. We postulate that this lack of placental production might contribute to the pathophysiologic of PE through the lack of a paracrine vasodilatory effect on uteroplacental blood flow and that impaired responses to EGF and other as-yet unidentified factors might be involved in the process.

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References


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