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Cigarette Smoke-Induced Placental Adrenomedullin Expression and Trophoblast Cell Invasion

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Abstract

Smoking in pregnancy reduces preeclampsia risk, but the mechanism of this effect is unknown. Prior studies have demonstrated that women with preeclampsia have lower placental adrenomedullin (AM) expression, and cigarette smoke extract (CSE) treatment of placental trophoblast cells in culture increases AM cellular production. We hypothesized that CSE alters trophoblast invasion through an AM-mediated mechanism, and that placental AM expression is greater among smokers. HTR-8/SVneo trophoblast cells were incubated for 24 hours in Matrigel-invasion chambers with 6 treatment groups: nonstimulated (NS), AM, AM inhibitor ($AM_{22\text{-}52}$), 1% CSE, AM + $AM_{22\text{-}52}$, and 1% CSE + $AM_{22\text{-}52}$. Cells that penetrated the lower surface of the chambers were quantified, invasion indices were calculated, and compared using a 1-way analysis of variance with Bonferroni corrections for multiple comparisons. Trophoblast cells treated with both AM and 1% CSE demonstrated increased cellular invasion compared to NS controls (1.5-fold [$P < .01$] and 1.45-fold [$P < .01$], respectively). Cotreatment with the AM inhibitor significantly attenuated the increased invasion seen with both AM and CSE alone. Next, the placental tissue was obtained from 11 smokers and 11 nonsmokers at term and processed for immunohistochemistry (IHC) and real-time quantitative polymerase chain reaction (PCR) for AM. Placentas from smokers demonstrated more intense AM staining and increased AM gene (*ADM*) expression compared to placentas from nonsmokers ($P = .004$ for IHC, $P = .022$ for PCR). The CSE increases trophoblast cell invasion through an AM-mediated process, and placental AM expression is increased among term smokers compared to nonsmokers. These findings provide evidence that the AM pathway may play a role in the protection from preeclampsia seen in smokers.

Keywords

adrenomedullin, cigarette smoke extract, preeclampsia, pregnancy, smoking, trophoblast

Introduction

Preeclampsia, characterized by the onset of hypertension and proteinuria after 20 weeks of gestation, affects approximately 5% to 8% of pregnancies.[1] The condition is progressive and can affect multiple organ systems. Since delivery of the fetus and placenta is the only treatment option, preeclampsia is a common etiology of iatrogenic preterm delivery.[2] Preeclampsia has a unique pathophysiology, because it is thought to be due to a placental factor or abnormality leading to alterations in maternal physiology.[3] The process of normal placentation is the result of trophoblast cell invasion of the maternal decidua and the establishment of complex vascular networks requiring extensive angiogenesis.[3] It is theorized that inadequate trophoblast invasion is the initial event among a complex process leading to the maternal manifestations of preeclampsia.[4]

Although the exact cause of preeclampsia remains elusive, epidemiologic data indicate that smoking during pregnancy significantly reduces ones risk of developing the disease.[5] Frustratingly, how smoking protects the mother from developing preeclampsia is unknown. Wikstrom et al demonstrated that the protective effects of smoking on preeclampsia risk are likely to be due to some combustible product of smoking tobacco rather than nicotine.[6] There are roughly 5000 individual compounds present in cigarette smoke, making pinpointing 1 causative agent difficult, although current research has focused on carbon monoxide and soluble nitric oxide as possible candidates.[7-9]

Prior work in our laboratory demonstrated that cigarette smoke extract (CSE) treatment of HTR-8/SVneo trophoblast

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cells causes these treated cells to produce and secrete the hormone adrenomedullin (AM).[10] AM is a 52-amino acid peptide that binds a transmembrane receptor. The AM receptor is a heterodimer composed of the G protein-coupled receptor (GPCR) calcitonin-like receptor and GPCR activity-modifying protein 2.[11] AM has multiple functions including angiogenic, anti-inflammatory, and antimicrobial activities and has recently emerged as a strong clinical biomarker for a variety of cardiovascular disease conditions, including myocardial infarction.[12,13] AM is highly expressed in reproductive tissues, but its role in the pathogenesis of preeclampsia is not fully understood.[14,15] Zhang et al have demonstrated that AM increases the invasiveness of trophoblast cells in an in vitro model of invasion, and that this effect is reversible with the use of the competitive inhibitor $AM_{22\text{-}52}$, a truncated AM molecule that binds, but does not activate, the AM receptor, but significantly attenuates signaling through the AM receptor.[16] Furthermore, our work has demonstrated that CSE treatment of HTR-8/SVneo trophoblast cells leads to enhanced cellular viability and migration that are dependent on CSE-mediated cellular AM production.[10]

AM expression has been shown to be reduced in the placentas of women with preeclampsia compared to normal controls.[17] This finding supports the theory that altered AM levels in the placenta may mediate the maternal physiologic changes of preeclampsia. To our knowledge, there have been no reports comparing AM expression within the placentas of smokers versus nonsmokers. We hypothesized that CSE increases the invasiveness of trophoblast cells in vitro via a process mediated, at least in part, by AM, and that AM expression is higher in the placental tissue of smokers compared to nonsmokers at term.

Materials and Methods

Cigarette Smoke Extract

CSE was prepared with a modification of the method described by Mehendale et al.[18] Air was drawn through 3 lit cigarettes (Marlboro Red; Philip Morris, Richmond, Virginia) with a vacuum apparatus that bubbled the mainstream smoke through 50 mL of RPMI 1640 cell medium (Mediatech, Inc, Manassas, Virginia). The medium was then filtered with a 22-μm filter, and aliquots were stored at −80°C and considered 100% CSE. To normalize CSE concentrations between collections, total nicotine content was measured by liquid chromatography by the Center for Nicotine and Smoking Cessation Research, Duke University Medical Center. The amount of nicotine in each sample was used to standardize treatments between CSE collections, although all experiments described here utilized CSE from a single collection.

Cell Culture

An immortalized first-trimester human cytotrophoblast cell line (HTR-8/SVneo; a gift from Dr C.H. Graham, Queen's University, Kingston, Ontario, Canada)[19] was cultured in RPMI 1640 supplemented with 5% fetal bovine serum and maintained in a humidified 5% CO_2 incubator at 37°C. The cells were trypsinized with 0.05% trypsin-EDTA (Gibco, Life Technologies, Carlsbad, California).

Matrigel Invasion Assay

Invasion of HTR-8/SVneo trophoblast cells was measured using 24-well Matrigel-invasion chamber plates (35-4480; Becton Dickinson Labware, Bedford, Massachusetts) in accordance with the published methods.[20,21] The cells (8×10^4 cells/well) were plated in the upper chamber of the Matrigel-coated insert in a total volume of 500 μL of serum-free RPMI 1640 media supplemented with 0.1% bovine serum albumin. To measure and control for migration, the cells were also plated at the same density in non-Matrigel-coated control 8.0 μm pore polyethylene terephthalate membranes cell culture inserts (35-4578; Becton Dickinson Labware). Two Matrigel-coated and 2 non-Matrigel-coated inserts were utilized for each run of the experiment, and the experiment was performed in quadruplicate. Six treatment groups were created for each run of the experiment: nonstimulated (NS), AM, AM inhibitor ($AM_{22\text{-}52}$), 1% CSE, AM + $AM_{22\text{-}52}$, and 1% CSE + $AM_{22\text{-}52}$. In the total volume of 500 μL media in the upper chamber was added nothing for NS, AM 10^{-7} mol/L ($AM_{1\text{-}52}$; American Peptide Company, Sunnyvale, California), $AM_{22\text{-}52}$ 10^{-6} mol/L ($AM_{22\text{-}52}$; American Peptide Company), 1% CSE, AM + $AM_{22\text{-}52}$, and 1% CSE + $AM_{22\text{-}52}$. The dosing for AM (10^{-7} mol/L) and $AM_{22\text{-}52}$ (10^{-6} mol/L) were selected based on our previous work.[10] The $AM_{22\text{-}52}$ is a competitive inhibitor of the AM receptor and so was utilized in 10-fold higher concentration compared to AM. To the lower chamber was added 750 μL of RPMI 1640 supplemented with 5% serum that acted as a chemoattractant. The cells were then incubated at 37°C with 5% CO_2 for 24 hours. Noninvasive cells on the upper surface of the chambers were then wiped away with a sterile cotton-tipped applicator, and any remaining cells were removed from the rim of the chamber with a sterile 200 μL pipette tip. Cells on the lower surface of the chamber were then quantified using the CellTiter 96 AQ_{ueous} Non-Radioactive Cell Proliferation Assay (G5421; Promega, Madison, Wisconsin) by placing each individual chamber into the inverted cap of a cryovial to which 200 μL of phenol-red-free RMPI 1640 and 40 μL of the CellTiter reagent had been added. After incubation for 3 hours, 2 separate 100 μL aliquots of media from each sample were transferred to a 96-well plate, and the optical absorbance at 490 nm was read, providing 2 readings per chamber. The readings were averaged for each chamber. For descriptive and morphologic analysis, the invasion chamber membranes were stained with the Hema-3-stain kit (22-122911; Thermo Fisher Scientific, Waltham, Massachusetts) and later viewed with and imaged with a Olympus Vanox microscope at 20× magnification. The percent invasion and invasion indices (InIs) were calculated for each group in the following manner:

$$\text{Percent invasion} = \frac{\text{Absorbance of cells penetrating Matrigel insert}}{\text{Absorbance of cells penetrating control insert}}$$

$$\text{Invasion index, InI} = \frac{\text{Percent invasion of treatment group}}{\text{Percent invasion of non} - \text{stimulated group}}$$

The InIs for the different treatment arms were then compared using a 1-way analysis of variance with Bonferroni corrections for multiple comparisons. $P < .05$ was considered significant.

Placental Tissue Collection

Placental tissue was identified from patients who had previously given informed consent to participate in the Duke Pregnancy and Tissue Repository (Duke IRB Number, Pro000011659). Placentas were collected at the time of delivery, and 1-in^2 tissue samples from the placenta were obtained using sterile scissors. These samples were then placed in optimal cutting temperature (OCT) media and immediately frozen and stored at -80°C.

Placental Immunohistochemistry for AM

Placental tissue samples from 11 smokers and 11 nonsmokers (self-reported smoking status) who delivered at term were prepared for immunohistochemistry (IHC). Tissue samples were thawed, fixed in formalin, sectioned, mounted, and paraffin embedded on slides with deidentified labels to blind both preparers and reviewers of the tissue sections. The samples were then deparaffinized and prepared for IHC using the UltraVision LP Detection System's (TL-125-HD; Thermo Scientific, Fremont, California) standard protocol. The primary antibody used was a mouse monoclonal anti-AM antibody (AB-18092, Abcam, Cambridge, Massachusetts) at a 1:100 dilution. This was incubated overnight at 4°C. Sections of amnion were used as a positive control for the staining process. Negative controls were sections of placental tissue incubated with the IHC reagents in the absence of the primary antibody as well as sections of placental tissue incubated with AM primary antibody that had been preabsorbed with AM peptide. After completion of the IHC staining protocol, the sections were counterstained with hematoxylin.

For each patient, we imaged 10 random 20× fields with a Zeiss Axio Imager microscope. Three independent blinded reviewers scored all digital images using a standardized semiquantitative 4-point scale for intensity of AM immunoreactivity in the syncytiotrophoblasts of the tertiary villi (1, nominimal staining; 2, low-moderate staining; 3, high-moderate staining; 4, strong staining; Supplemental Figure 1). Once scoring was complete, the results were unblinded and tabulated into a cumulative score for each patient sample. For statistical analysis, the mean score for each group was calculated and compared using a Student t test after confirming normalcy of the distribution of scores in each group with a Kolmogorov-Smirnov test for normalcy. Statistical analysis for demographic factors of the patient samples including maternal age, gestational age at delivery, and birthweight were also performed using Student t tests as well. $P < .05$ was considered significant.

Real-Time Polymerase Chain Reaction

To determine whether the placentas of smokers demonstrated greater *ADM* gene expression compared to the placentas of nonsmokers, quantitative real-time polymerase chain reaction (RT-PCR) was performed. Placental samples from 11 smokers and 11 nonsmokers (same samples utilized for IHC) were obtained, and total RNA was extracted from each sample using an RNeasy Mini kit (QIAGEN, Valencia, California). Following RNA extraction, RNA concentrations were calculated using the Nanodrop ND-1000 spectrophotometer. A reverse transcription reaction was performed to create complementary DNA using Superscript III (Invitrogen, Carlsbad, California) first strand synthesis system. Reverse transcription quantitative RT-PCR was performed with a Bio-Rad iCycler (Bio-Rad, Hercules, California) using the SYBR green detection method. Primer pairs (Eurofins MWG Operon, Huntsville, Alabama) utilized for *ADM* were forward (5′-3′) ATGAAGCTGGTTTCCGTCG and reverse (5′-3′) GCCCACTTATTCCACTTCTTTCG and for *GAPDH* forward (5′-3′) CATGAGAAGTATGACAACAGCCT and reverse (5′-3′) AGTCCTTCCACGATACCAAAGT. *ADM* cycle threshold values were normalized to *GAPDH* and analyzed using the Pfaffl model with mean fold change reported and compared using Student t test.[22]

Results

Nicotine Content of CSE

CSE has been used widely as a model to study the effects of cigarette smoking on biologic processes.[23] Only the respiratory tract is exposed to aerosolized cigarette smoke; the other in vivo tissues are not directly exposed to cigarette smoke but rather to its soluble components. CSE treatment of cells in culture mimics this by exposing cells to thousands of soluble components of cigarette smoke.[24] Similar to other groups, we measured the nicotine content in our 100% CSE as a method to standardize exposure to the various components in CSE. After measuring the nicotine content by high-performance liquid chromatography of our 100% CSE preparation, we chose to utilize a CSE dilution of 1%, which estimates the nicotine exposure seen in a heavy smoker (1-2 packs per day leads to nicotine blood levels of approximately 200 ng/mL).[18] Furthermore, prior work from our lab demonstrated that 1% CSE treatment of HTR-8/SVneo trophoblast cells lead to enhanced cellular growth that was dependent on AM.[10] The CSE allows for a good model of the effects of cigarette smoke on tissues, although identifying the individual component of CSE that leads to the changes in biologic behavior would be difficult.[24]

The concentration of nicotine in CSE preparations was determined in order to standardize CSE concentrations between

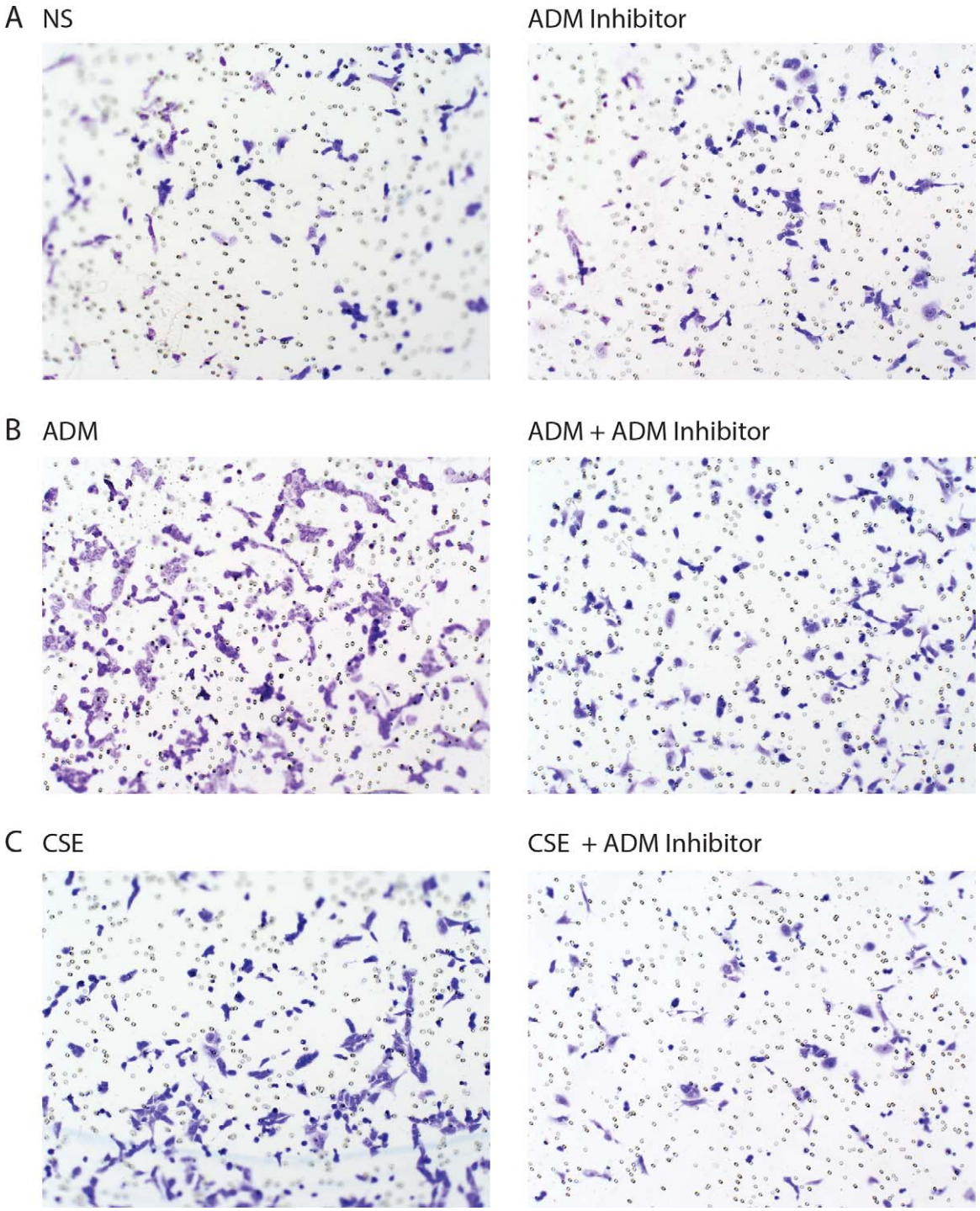

Figure 1. Effect of AM and CSE on trophoblast invasion. Matrigel invasion assay was performed to evaluate HTR-8/SVneo cell invasion in the presence of varying treatments. Images represent ×20 fields of Matrigel-coated 8 μm pore PET membranes after removing all noninvasive cells. A, Representative example of HTR-8/SVneo cells (stained purple) that invaded through the Matrigel and migrated through the PET membrane in nonstimulated (left) and AM inhibitor-treated conditions (right). B, Representative images demonstrating HTR-8/SVneo cellular invasion in AM treated (left) and AM + AM inhibitor cotreatment (right). AM enhanced cellular invasion compared to nonstimulated cells (panel B compared to panel A), and this was reversed with AM-inhibitor cotreatment. C, Representative example of HTR-8/SVneo cells treated with 1% CSE (left) and cells cotreated with 1% CSE and AM inhibitor (right). CSE treatment of cells enhanced invasion compared to nonstimulated cells (panel C compared to panel A), and this was reversed with AM-inhibitor cotreatment. AM indicates adrenomedullin; CSE, cigarette smoke extract; PET, polyethylene terephthalate.

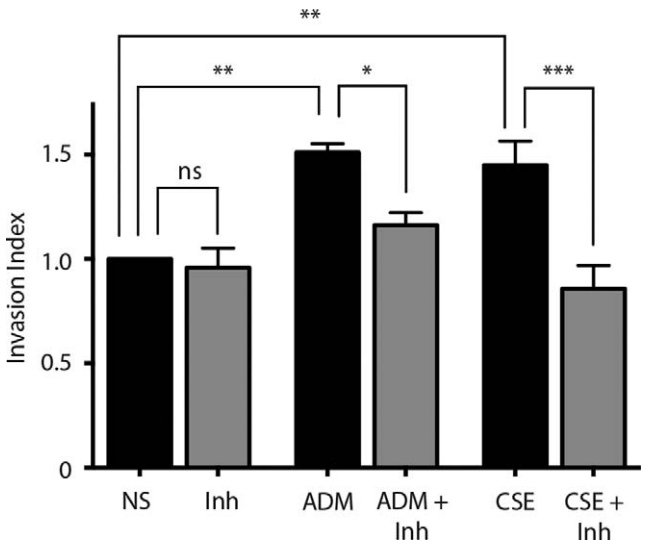

Figure 2. Summary data comparing invasion indices of HTR-8/SVneo cells by treatment type. Invasion indices account for the number of cells that invade through the Matrigel insert among different treatment groups, as well as the number of cells that migrate through a control insert in different treatment groups (see Methods section), were calculated and compared by treatment group. The invasion index of NS cells was set at 1. Treatment with the AM inhibitor (Inh) alone had no significant (ns) effect on the invasion index. Both AM and CSE treatments increased cellular invasion compared to NS controls by 1.5- and 1.4-fold, respectively (**$P < .01$). Cotreatment of cells with AM and AM inhibitor (AM + Inh) significantly reduced invasion compared to AM alone (*$P < .05$). Cotreatment of cells with CSE and AM inhibitor (CSE + Inh) significantly reduced the increased invasion seen with CSE treatment alone (***$P < .001$). Summary data represents 4 independent experiments. Comparisons made using analysis of variance with Bonferroni corrections for multiple comparisons. AM indicates adrenomedullin; CSE, cigarette smoke extract; NS, nonstimulated.

individual CSE collections and to determine the appropriate dose of CSE to use in experiments. High-performance liquid chromatography analysis of 100% smoke extract showed a mean nicotine concentration of 13 363 ng/mL. The nicotine content from 1% CSE dilution was 130 ng/mL and is equivalent to that found in the serum of a heavy smoker (100-200 ng/mL).[18]

CSE Increases Trophoblast Invasion via an AM-Mediated Pathway

A Matrigel-based invasion assay was performed to test the effects of CSE on HTR-8/SV neo trophoblast cell invasion. Increased cellular invasion through Matrigel-coated membranes was seen in cells each treated with AM (Figure 1B) or CSE (Figure 1C) when compared to NS cells or cells treated with the AM inhibitor alone (Figure 1A). The addition of the AM inhibitor to either AM- or CSE-treated cells attenuated the increased invasion seen with AM or CSE alone (Figure 1B and C).

The InIs were calculated for all the treatment groups, setting the NS cells as the reference group with an InI of 1 and are summarized in Figure 2. Calculation of an InI accounts for the potential effects that the treatments may have on cellular migration as well as cellular invasion. Cells treated with either AM or CSE demonstrated increased invasion compared to NS cells (InI 1.51-fold or 1.45-fold, respectively, $P < .01$ when each compared to NS). Cotreatment of cells with AM and the AM inhibitor significantly attenuated the increased invasion seen with AM treatment alone (1.51- vs 0.96-fold, $P < .05$). Moreover, a similar effect was found when CSE-treated cells were cotreated with the AM inhibitor. Comparison of the InIs of cells treated with CSE alone compared to cells cotreated with CSE and the AM inhibitor was significant (1.45 vs 0.86, $P < .001$), suggesting that inhibition of the AM pathway can reverse the increased invasion seen with CSE treatment of trophoblast cells.

Table 1. Clinical Characteristics of Patients From Whom Placentas Were Utilized for ADM Immunohistochemistry.

Characteristic	Nonsmokers (n = 11)	Smokers (n = 11)	*P* Value[a]
Age, years[b]	30.9 ± 5.6	24.7 ± 6.0	.02
Cesarean delivery, n (%)	10 (90.9)	7 (63.6)	.31
Gestational age delivery, weeks[b]	39.2 ± 0.9	39.2 ± 1.5	1.0
Birthweight, g[b]	3312 ± 406	3141 ± 561	.4

Abbreviation: SD, standard deviation.
[a] Student *t* test for continuous variables; chi-square for categorical variables.
[b] Values are mean ± SD.

Placental AM Protein and Gene Expression Levels Are Increased Among Smokers

To determine whether AM protein expression varies within the placentas of smokers compared to nonsmokers, we performed IHC for AM on sections obtained from term placentas. There were no significant differences in gestational age at delivery or newborn birthweight between smokers and nonsmokers from whom placental samples were obtained. The smokers were younger compared to nonsmokers (mean age 24.7 vs 30.9 years, $P = .02$ for difference in means), and there was no difference in mode of delivery between the 2 groups (Table 1).

Placentas from both smokers and nonsmokers demonstrated positive immunostaining for AM in the villous syncytiotrophoblasts (Figure 3A). The intensity of AM staining, when measured using a 4-point semiquantitative intensity scale (Supplemental Figure 1) was significantly higher in smokers compared to nonsmokers (Figure 3B, $P = .004$). When the results were analyzed individually from each of the 3 observers, the results were similar. Staining was noted in the amnion layer as a positive control (Supplemental Figure 1). Negative controls incubated both without the primary antibody (Supplemental Figure 1) or with primary AM antibody preabsorbed with AM peptide (not shown) failed to demonstrate staining.

Using real-time quantitative PCR, mean *ADM* gene expression was 3.3-fold greater in the placentas of smokers compared

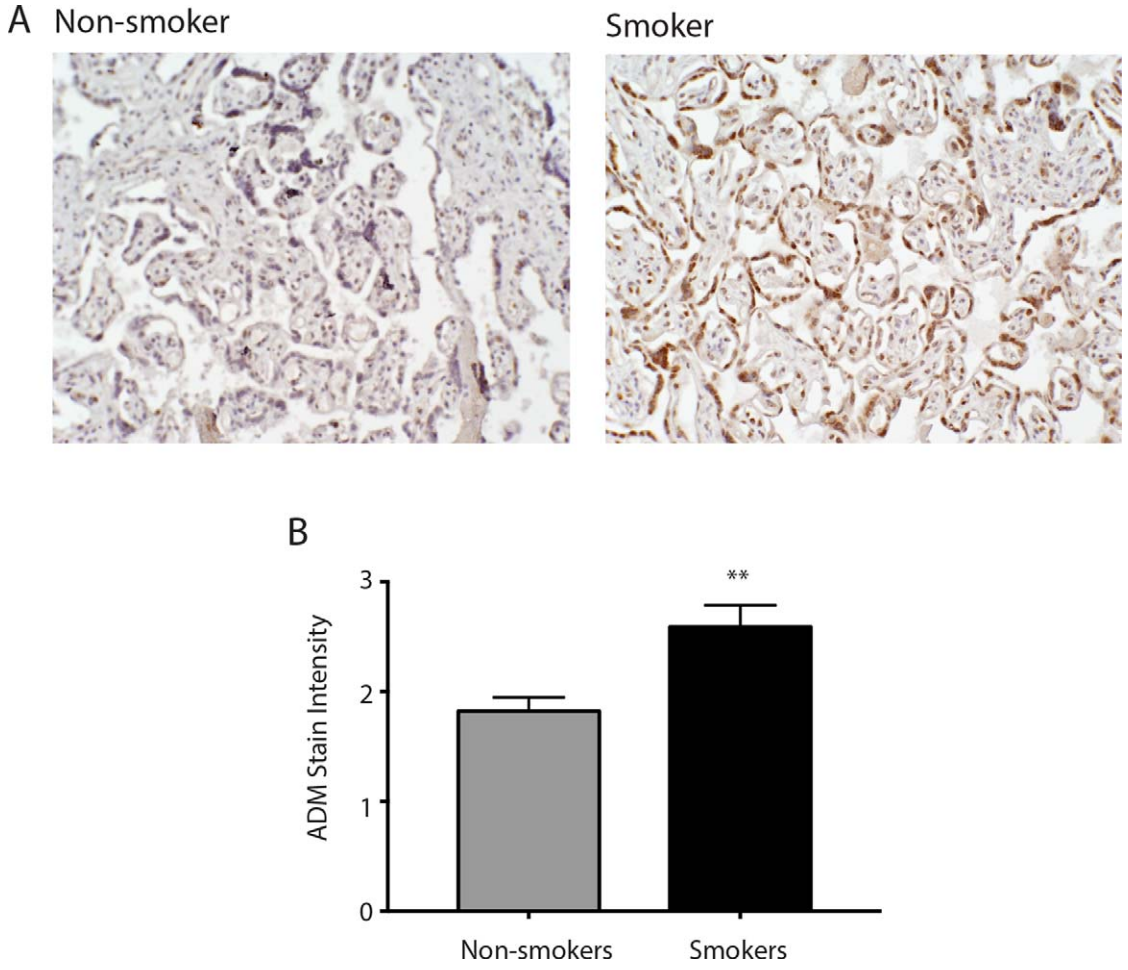

Figure 3. Immunohistochemistry for AM expression in term placentas from smokers and nonsmokers. A, Representative examples of ×20 images from term placentas stained with antibodies directed against AM from a nonsmoker (top) and smoker (bottom). The placenta from the smoker demonstrates enhanced staining for AM compared to the nonsmoker. B, Three independent, blinded observers evaluated 10 images from each patient (n = 22) and scored the intensity of AM staining using a 4-point scale (Supplemental Figure 1). A mean score intensity was obtained for each patient and then after unblinding, a mean score was calculated for the smokers and nonsmokers groups and compared using a Student *t* test. Placentas from smokers demonstrated enhanced AM staining compared to nonsmokers ($P = .004$). AM indicates adrenomedullin.

to the placentas of nonsmokers ($P = .022$ for difference in mean fold change).

Discussion

In this study, we demonstrate that the treatment of a first-trimester cytotrophoblast cell line with 1% CSE increases cell invasion through a Matrigel layer compared to NS controls, and that this increased invasion can be reversed by cotreatment with the competitive inhibitor to the AM receptor. Moreover, treatment of the same cells with AM alone also caused increased invasion in a similar manner to CSE treatment, and this increased invasion with AM was also reversed by cotreatment with the competitive AM inhibitor. Prior work from our laboratory has demonstrated that treatment of HTR-8/SVneo cells with 1% CSE increases cellular AM production, while treatment with nicotine alone had no effect.[10] Taken together, these results suggest that CSE leads to increased invasiveness of HTR-8/SVneo cells by causing the cells to increase endogenous production of AM.

Our study did not address potential mechanisms by which CSE enhances cellular invasion that is mediated by AM, though recent work in a lung cancer model implicates aryl hydrocarbon receptor (AHR) activation.[25] Portal-Nuñez et al demonstrated that CSE treatment of lung cancer cells in vitro lead to a dose- and time-dependent increase in AM production that was mediated by AHR activation. Furthermore, cigarette smoke enhanced lung cancer growth by activating the AHR, which increased cellular production of AM. Cigarette smoke-dependent tumor growth was then reversed with AM receptor antagonist cotreatment.[25] Their work provides further evidence that cigarette smoke enhances cellular production of AM, which then affects cellular growth.

The placentas of murine fetuses lacking the AM gene (*ADM*) exhibit typical pathological features of preeclampsia.[26] The fetal labyrinth vessels within the placenta of these mice demonstrate reduced branching. Furthermore, the number of

decidual natural killer (NK) cells is reduced with a concomitant decrease in the remodeling of maternal spiral arterioles.[26] The findings from mice lacking *ADM* demonstrate the importance of AM for normal placental development.

The most commonly accepted theory for the development of preeclampsia is that poor placental development and inadequate trophoblast-mediated remodeling of the spiral arterioles results in reduced placenta perfusion, which increases oxidative stress, resulting in the release of antiangiogenic factors causing maternal systemic endothelial dysfunction.[3] We propose that cigarette smoking enhances placental trophoblast invasion (by increasing cellular production of AM), which may improve placental function and improve spiral arteriole remodeling, thereby decreasing placental release of antiangiogenic factors, which have been implicated in preeclampsia.[3,26,27]

Multiple studies have demonstrated that smoking during pregnancy reduces the risk of developing preeclampsia by approximately 50%.[5,6,28,29] Wikstrom et al evaluated preeclampsia risk in smokers and found that the risk reduction did not apply to women who smoked in the first trimester but quit by the onset of the third trimester, rather smoking throughout pregnancy provided greatest protection.[6] Moreover, the same group found that the use of smokeless tobacco (ie, snuff) conferred no protection from preeclampsia.[6] Finally, our group has shown that nicotine treatment of trophoblast cells did not affect cell viability, migration, or AM production, while CSE treatment enhanced cellular viability and migration, both of which were dependent on AM.[10] These findings suggest that smoking throughout pregnancy is required to maintain protection, and that nicotine products do not provide similar protection.

To determine whether AM expression was enhanced in term placental tissue of smokers, we performed IHC staining directed at AM and real-time PCR for *ADM* gene expression in the placentas of smokers and nonsmokers. Our results demonstrate that the trophoblast cells in the tertiary villi of smokers express more AM at term than the same tissue from nonsmokers. Another group demonstrated decreased placental AM production in placentas obtained from preeclamptic women compared to nonpreeclamptics.[17] Our findings, together with the findings of Kanenishi et al, suggest that smoking's protection against the development of preeclampsia may be partially mediated by increased placental production of AM. As placental AM expression is higher in term placentas from smokers compared to nonsmokers, this offers 1 possible mechanism as to how a third-trimester alteration in placental physiology may be an associated factor in the pathogenesis of preeclampsia.

The AM is highly expressed throughout the reproductive tract including the endometrium, fetal membranes, and placenta. Originally described for its vasodilatory properties, AM has been shown to have angiogenic, growth inducing, immunomodulatory, and antimicrobial properties. With regard to AM's role in reproduction, Caron et al have shown that AM knockout mouse pups die in midgestation with a hydropic phenotype, and that the placentas of these mice show reduced fetal vessel branching and reduced decidual NK cells resulting in decreased remodeling of the maternal spiral arterioles, both of which are quintessential pathologic findings of preeclampsia.[14,26,30,31] The AM plasma levels rise over the course of gestation in normal pregnancies, but there are conflicting data about plasma levels of AM in preeclamptic pregnancies.[32] Possible explanations for the conflicting data include different patient phenotypes and differences in sample processing or assays used to measure AM levels. Researchers who have found increased AM levels in pregnancy theorize that the rise in AM is a compensatory vasodilatory response attempting to counteract the vasospasm at the root of preeclamptic hypertension.[33] Conversely, theories as to how lower circulating AM levels fit with the pathogenesis of the disease focus on the decreased paracrine vasodilation as one of the root causes of the preeclamptic phenotype.[34] However, the predominant rationale for these explanations is based on the function of AM as a vasodilator, without consideration for the local effects of AM peptide on trophoblast cells of the placenta.

Adrenomedullin 2 (ADM2), which is also known as intermedin, is a 47-amnio acid peptide that contains some structural homology to AM.[35] It is also expressed widely in the reproductive tract and has also been shown to increase trophoblast cellular invasion.[20] Furthermore, infusion of an ADM2 antagonist to pregnant mice leads to fetal growth restriction.[36] It is unknown whether CSE treatment of trophoblast cells affects ADM2 production and cellular invasion but is certainly an important area of future research.

Cigarette smoking has also been shown to affect other angiogenic markers that are implicated in preeclampsia.[10,18] Soluble fms-like tyrosine kinase 1 (s-Flt1) is the soluble form of the vascular endothelial growth factor (VEGF) receptor. The s-Flt1 binds free serum VEGF, preventing VEGF signaling, thereby acting as a VEGF antagonist.[37] Women with preeclampsia have been found to have elevated levels of s-Flt1, whereas pregnant smokers have lower levels of s-Flt1.[38] Furthermore, there is a reduction in the secretion of s-Flt1 from term placental explants treated with CSE in culture.[18] Together, this suggests that in addition to the effects of AM on trophoblast function, cigarette smoking also likely decreases the risk of preeclampsia by its affect on serum angiogenic factors.[10,18,38]

This study, in addition to prior work in our lab, is one of the first to address the role of AM in explaining the protective effect of smoking on the development of preeclampsia.[10] We used an immortalized trophoblast cell line in a Matrigel-based assay in order to model early placental function, mainly trophoblast invasion through the maternal decidua. This cell line, which arose spontaneously from primary cultures of normal first-trimester chorionic villous samples, has been shown to retain its phenotypic function.[19] Despite some of the limitation of CSE, CSE treatment of cell culture is recognized as a robust model for studying the effects of smoking.[24] This study lends further evidence to the idea that the AM pathway is activated by CSE due to the finding that increased cell invasion with CSE was attenuated by cotreatment with the AM inhibitor. Prior work has demonstrated that the AM inhibitor works directly on the AM receptor, although additional off target

effects cannot be completely excluded here.[39] However, in our study the fact that AM inhibitor treatment of trophoblast cells alone did not alter invasion compared to NS controls is reassuring that off target effects, if they exist, are minimal. Loss-of-function experiments where AM is competitively blocked or the receptor is knocked down will be conducted to determine specificity of the observed relationship.

In summary, we have demonstrated that treatment of a first-trimester cell line with CSE increases cell invasion through an AM-mediated process, and that placental AM levels are increased among smokers at term compared to nonsmokers. Smoking throughout pregnancy is required for protection from preeclampsia. Here, we model early placental development with the Matrigel assay and then demonstrate in third-trimester placentas that AM placenta expression is increased among smokers. Together, the increased invasiveness of trophoblast cells treated with CSE concentrations typical of heavy smoking may lead to improved placentation, and continued placental production of AM throughout pregnant may, in part, explain how smoking protects against the development of preeclampsia.

Authors' Note

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Declaration of Conflicting Interests

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References

1. Report of the National High Blood Pressure Education Program Working Group on High Blood Pressure in Pregnancy. *Am J Obstet Gynecol*. 2000;183(1):S1-S22.
2. Behrman R, Butler A, eds. *Preterm Birth: Causes, Consequences, and Prevention. Institute of Medicine Committee on Understanding Premature Birth and Assuring Healthy Outcomes*. Washington, DC: National Academies Press; 2007.
3. Redman CW, Sargent IL. Latest advances in understanding preeclampsia. *Science*. 2005;308(5728):1592-1594.
4. Fisher SJ. The placental problem: linking abnormal cytotrophoblast differentiation to the maternal symptoms of preeclampsia. *Reprod Biol Endocrinol*. 2004;2:53.
5. Castles A, Adams EK, Melvin CL, Kelsch C, Boulton ML. Effects of smoking during pregnancy. Five meta-analyses. *Am J Prev Med*. 1999;16(3):208-215.
6. Wikstrom AK, Stephansson O, Cnattingius S. Tobacco use during pregnancy and preeclampsia risk: effects of cigarette smoking and snuff. *Hypertension*. 2010;55(5):1254-1259.
7. Schramm S, Carre V, Scheffler JL, Aubriet F. Analysis of mainstream and sidestream cigarette smoke particulate matter by laser desorption mass spectrometry. *Anal Chem*. 2011;83(1):133-142.
8. Zhai D, Guo Y, Smith G, Krewski D, Walker M, Wen SW. Maternal exposure to moderate ambient carbon monoxide is associated with decreased risk of preeclampsia. *Am J Obstet Gynecol*. 2012; 207(1):57.e1-57.e9.
9. Meher S, Duley L. Nitric oxide for preventing pre-eclampsia and its complications. *Cochrane Database Syst Rev*. 2007;(2): CD006490.
10. Beiswenger TR, Feng L, Brown HL, Heine RP, Murtha AP, Grotegut CA. The effect of cigarette smoke extract on trophoblast cell viability and migration: the role of adrenomedullin. *Reprod Sci*. 2012;19(5):526-533.
11. Gibbons C, Dackor R, Dunworth W, Fritz-Six K, Caron KM. Receptor activity-modifying proteins: RAMPing up adrenomedullin signaling. *Mol Endocrinol*. 2007;21(4):783-796.
12. Caron K, Hagaman J, Nishikimi T, Kim HS, Smithies O. Adrenomedullin gene expression differences in mice do not affect blood pressure but modulate hypertension-induced pathology in males. *Proc Natl Acad Sci USA*. 2007;104(9):3420-3425.
13. Caron KM, Smithies O. Multiple roles of adrenomedullin revealed by animal models. *Microsc Res Tech*. 2002;57(1):55-59.
14. Lenhart PM, Caron KM. Adrenomedullin and pregnancy: perspectives from animal models to humans. *Trends Endocrinol Metab*. 2012;23(10):524-532.
15. Di Iorio R, Marinoni E, Letizia C, Cosmi EV. Adrenomedullin in perinatal medicine. *Regul Pept*. 2003;112(1-3):103-113.
16. Zhang X, Green KE, Yallampalli C, Dong YL. Adrenomedullin enhances invasion by trophoblast cell lines. *Biol Reprod*. 2005; 73(4):619-626.
17. Kanenishi K, Kuwabara H, Ueno M, Sakamoto H, Hata T. Immunohistochemical adrenomedullin expression is decreased in the placenta from pregnancies with pre-eclampsia. *Pathol Int*. 2000; 50(7):536-540.
18. Mehendale R, Hibbard J, Fazleabas A, Leach R. Placental angiogenesis markers sFlt-1 and PlGF: response to cigarette smoke. *Am J Obstet Gynecol*. 2007;197(4):363. e1-e5.
19. Graham CH, Hawley TS, Hawley RG, et al. Establishment and characterization of first trimester human trophoblast cells with extended lifespan. *Exp Cell Res*. 1993;206(2):204-211.
20. Chauhan M, Yallampalli U, Dong YL, Hankins GD, Yallampalli C. Expression of adrenomedullin 2 (ADM2)/intermedin (IMD) in human placenta: role in trophoblast invasion and migration. *Biol Reprod*. 2009;81(4):777-783.
21. Imamura H, Takao S, Aikou T. A modified invasion-3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide assay for quantitating tumor cell invasion. *Cancer Res*. 1994;54(13): 3620-3624.
22. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res*. 2001;29(9):e45.
23. Kim H, Liu X, Kobayashi T, et al. Reversible cigarette smoke extract-induced DNA damage in human lung fibroblasts. *Am J Respir Cell Mol Biol*. 2004;31(5):483-490.
24. Shapiro SD. Smoke gets in your cells. *Am J Respir Cell Mol Biol*. 2004;31(5):481-482.

25. Portal-Nunez S, Shankavaram U, Rao M, et al. Aryl hydrocarbon receptor-induced adrenomedullin mediates cigarette smoke carcinogenicity in humans and mice. *Cancer Res*. 2012;72(22):5790-5800.
26. Li M, Schwerbrock NMJ, Lenhart PM, et al. Fetal-derived adrenomedullin mediates the innate immune milieu of the placenta. *J Clin Invest*. In press.
27. Moffett-King A. Natural killer cells and pregnancy. *Nat Rev Immunol*. 2002;2(9):656-663.
28. England L, Zhang J. Smoking and risk of preeclampsia: a systematic review. *Front Biosci*. 2007;12:2471-2483.
29. Conde-Agudelo A, Althabe F, Belizan JM, Kafury-Goeta AC. Cigarette smoking during pregnancy and risk of preeclampsia: a systematic review. *Am J Obstet Gynecol*. 1999;181(4): 1026-1035.
30. Caron KM, Smithies O. Extreme hydrops fetalis and cardiovascular abnormalities in mice lacking a functional Adrenomedullin gene. *Proc Natl Acad Sci USA*. 2001;98(2):615-619.
31. Li M, Yee D, Magnuson TR, Smithies O, Caron KM. Reduced maternal expression of adrenomedullin disrupts fertility, placentation, and fetal growth in mice. *J Clin Invest*. 2006;116(10):2653-2662.
32. Senna AA, Zedan M, El-Salam GE, El-Mashad AI. Study of plasma adrenomedullin level in normal pregnancy and preclampsia. *Medscape J Med*. 2008;10(2):29.
33. Gratton RJ, Gluszynski M, Mazzuca DM, Nygard K, Han VK. Adrenomedullin messenger ribonucleic acid expression in the placentae of normal and preeclamptic pregnancies. *J Clin Endocrinol Metab*. 2003;88(12):6048-6055.
34. Dikensoy E, Balat O, Pence S, Balat A, Cekmen M, Yurekli M. The changes of plasma malondialdehyde, nitric oxide, and adrenomedullin levels in patients with preeclampsia. *Hypertens Pregnancy*. 2009;28(4):383-389.
35. Roh J, Chang CL, Bhalla A, Klein C, Hsu SY. Intermedin is a calcitonin/calcitonin gene-related peptide family peptide acting through the calcitonin receptor-like receptor/receptor activity-modifying protein receptor complexes. *J Biol Chem*. 2004; 279(8):7264-7274.
36. Chauhan M, Yallampalli U, Reed L, Yallampalli C. Adrenomedullin 2 antagonist infusion to rats during midgestation causes fetoplacental growth restriction through apoptosis. *Biol Reprod*. 2006;75(6):940-947.
37. Levine RJ, Maynard SE, Qian C, et al. Circulating angiogenic factors and the risk of preeclampsia. *N Engl J Med*. 2004;350(7): 672-683.
38. Jeyabalan A, Powers RW, Durica AR, Harger GF, Roberts JM, Ness RB. Cigarette smoke exposure and angiogenic factors in pregnancy and preeclampsia. *Am J Hypertens*. 2008;21(8): 943-947.
39. Mazzocchi G, Albertin G, Andreis PG, et al. Distribution, functional role, and signaling mechanism of adrenomedullin receptors in the rat adrenal gland. *Peptides*. 1999;20(12):1479-1487.