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Pre-B cell colony enhancing factor (PBEF/NAMPT/Visfatin) and vascular endothelial growth factor (VEGF) cooperate to increase the permeability of the human placental amnion

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Fluid efflux across the region of the amnion overlying the placenta is an essential component of the intramembranous absorption pathway that maintains amniotic fluid volume homeostasis. Dysregulation of this pathway may result in adverse pregnancy outcomes, however the factors controlling amnion permeability are unknown. Here, we report a novel mechanism that increases placental amnion permeability. Pre-B Cell Colony Enhancing Factor (PBEF) is a stress-responsive cytokine expressed by the human amnion, and is known to induce Vascular Endothelial Growth Factor (VEGF) production by other cell types. Interestingly, VEGF is up-regulated in the ovine amnion when intramembranous absorption is augmented. In this study, we show that PBEF induced VEGF secretion by primary human amniotic epithelial cells (AEC) derived from the placental amnion, as well as from the reflected amnion that lines the remainder of the gestational sac. Further, PBEF treatment led to the increased expression of VEGFR2 in placental AEC, but not reflected AEC. To test the hypothesis that PBEF and VEGF increase placental amnion permeability, we monitored the transfer of 2′,7′-dichlorofluorescein (DCF) from the fetal to the maternal side of human amnion explants. A treatment regimen including both PBEF and VEGF increased the rate of DCF transfer across the placental amnion, but not the reflected amnion. In summary, our results suggest that by augmenting VEGFR2 expression in the placental amnion, PBEF primes the tissue for a VEGF-mediated increase in permeability. This mechanism may have important implications in amniotic fluid volume control throughout gestation.

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1. Introduction

To ensure proper fetal development, amniotic fluid turns over constantly and is regulated by the intramembranous absorption pathway of fluid efflux to maintain an ideal volume [1]. Intramembranous absorption (IA) is defined as the transfer of AF from the gestational sac into the blood vessels lining the fetal surface of the placenta, thus allowing the AF to re-enter the fetal circulation [2,3]. Dysregulated AF volume results in the pathologies polyhydramnios (too much AF) or oligohydramnios (too little AF), both associated with preterm birth and perinatal morbidity or mortality. Although there are fetal abnormalities or maternal conditions recognized to cause poly- or oligohydramnios, most incidents of these AF volume disorders are of an unknown etiology [4,5]. These idiopathic cases may result from dysregulated IA, however, the mechanisms governing this important pathway are unknown.

The amnion is the innermost layer of the fetal membranes, and is thought to be the primary site that limits the efflux of amniotic fluid [6]. Composed of a single layer of amniotic epithelial cells (AEC) and its underlying collagen matrix embedded with mesenchymal cells, the amnion is divided into multiple discrete, yet continuous regions: the "placental" amnion lies at the interface between the amniotic fluid (AF) and placenta, whereas the "reflected" amnion lines the remainder of the fetal sac [7]. Since the placental amnion is situated such that AF and solutes must transfer through it to access the fetal placental vessels, factors influencing the permeability of the placental amnion may ultimately regulate AF volume by controlling IA.

Pre-B cell colony enhancing factor (PBEF) is an ubiquitous, evolutionarily conserved protein that is constitutively expressed in

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the fetal membranes where its greatest expression is in the amnion [8]. PBEF was originally identified as a cytokine that potentiated the clonal expansion and differentiation of pre-B cells [9], but it is also acknowledged to be the ubiquitous intracellular enzyme nicotinamide phosphoribosyltranferase (NAMPT) [10] and the adipokine "visfatin" [11]. A stress-responsive gene, PBEF is up-regulated in hypoxic [12] and inflammatory conditions [13]. In such conditions, PBEF can prevent apoptosis in several cell types [14,15] and mediate an increase in lung epithelial permeability [16]. Interestingly, PBEF/visfatin also stimulates angiogenesis in endothelial cells by up-regulating their production of vascular endothelial growth factor (VEGF) [17].

Studies in pregnant sheep have shown that amniotic VEGF expression is up-regulated *in vivo* simultaneous with increases in IA [18,19], qualifying VEGF as a candidate factor affecting amnion permeability. Although VEGF is classically recognized to mediate the proliferation and permeability of the endothelium, it can also increase the permeability of epithelial tissues [20,21]. VEGF increases caveolin-1 activity in ovine AECs, implicating a transcytotic caveolae-mediated mechanism of amniotic fluid transport across the amnion [22]. However, VEGF treatment alone does not alter the permeability of ovine AEC monolayers *in vitro* [23], suggesting other factors may be involved. Given that PBEF induces VEGF production in endothelial cells, and the concomitant up-regulation of VEGF and its receptors has been noted in several tissue types [24,25] including the amnion in pre-term labor [26], we sought to determine if these same phenomena occur in human AEC. In addition, as VEGF is likely involved in the regulation of IA, we tested the hypothesis that PBEF could augment a VEGF-mediated increase in human placental amnion permeability.

2. Materials and methods

2.1. Tissue collection and amniotic epithelial cell culture

Fetal membranes were collected anonymously, immediately following singleton, 38–40 week gestation, elective Cesarean sections prior to labor at Kapi'olani Medical Center for Women and Children (Honolulu, HI, USA) with approval from the Hospital Institutional Review Board.

Leaving a 1–2 cm margin around the placenta, the reflected fetal membranes were removed and the placental amnion was peeled from the chorionic plate. If the membranes were utilized for explant permeability studies, two sutures were inserted in the placental amnion during removal for orientation purposes.

Primary AEC were isolated as previously described [14,27]. Laboratory observations revealed that reflected AEC attached to cultureware and proliferated more efficiently than placental AEC, so the reflected were plated on tissue culture-treated dishes (BD Falcon) at 70, 000 cell/cm^2 and the placental at 80,000 cells/cm^2. AEC were cultured in complete DMEM:F12 with 10% FBS (Invitrogen, Carlsbad, CA), penicillin (10 U/ml)-streptomycin (50 μg/ml) and incubated at 37 °C in 95% air/5% CO_2. Media was changed every 2–3 days and the cells were utilized without passage upon reaching confluence (6–10 days).

2.2. Recombinant PBEF production

Hexahistidine-tagged PBEF was prepared as previously reported [8].

2.3. PBEF treatment of isolated AEC

Upon reaching confluence, primary AEC were serum-starved for 16 h in 0.5% FBS-containing DMEM:F12 medium then treated with 0, 10, 100, and 250 ng/ml PBEF for 24 h. The media was collected, centrifuged 16,000 g for 10 min at 4 °C, and the supernatant was stored at −80 °C. Whole cell lysates were collected for western blotting (below) and stored at −80 °C.

2.4. VEGF ELISA

Sandwich ELISA assays were conducted using the Quantikine VEGF Immunoassay (#DVE00, R&D Systems) according to the manufacturer instructions. Each sample was tested in duplicate, and the optical density was measured on a VersaMax plate reader (Molecular Devices, Sunnyvale, CA).

2.5. Immunohistochemistry

Placenta and fetal membranes were washed in PBS and placed in formalin within 1 h following collection. Overnight fixation was followed by daily changes to 70% ethanol for 3 days and subsequent paraffin embedding. Prior to staining, the slides were deparaffinized and hydrated through xylenes and a graded alcohol series, antigen unmasked for 30 min in steam using 10 mM sodium citrate pH 6.0 for VEGFR1 labeling or 1 mM EDTA pH 8.0 for VEGFR2, and peroxidase-quenched with 0.3% hydrogen peroxide for 30 min. Sections were incubated with VEGFR1 (1:250) antibody (#ab32152, Abcam, Cambridge, MA) and the VEGFR2 (1:300) antibody (#2479, Cell Signaling Technology, Danvers, MA). All slides were stained using Vectastain ABC Elite kits (Vector Laboratories, Burlingame, CA) according to the provided protocol, and developed using the DAB Peroxidase Substrate Kit (Vector Laboratories) and mounted using Pro-Texx (Lerner Laboratories, Pittsburgh, PA).

2.6. Western blotting

Whole cell lysates were prepared by washing the cells with ice-cold PBS and scraping into a modified RIPA buffer containing EDTA-Free Protease Cocktail Inhibitor and PhosSTOP Phosphatase Inhibitor Cocktail (Roche, Indianapolis, IN). Protein concentrations were determined using the BCA Protein Assay Kit (Thermo Pierce Scientific, Rockford, IL).

Cell lysates (25 μg) were heated at 95 °C for 7 min in 1× SDS buffer with 5% beta-mercaptoethanol. Electrophoretically separated proteins were transferred to nitrocellulose membranes. Immunoblotting was performed by blocking with either 5% non-fat milk in PBS with 0.01% Tween-20 or Odyssey Blocking Buffer (Li-Cor Biosciences, Lincoln, NE) containing 0.01% Tween-20. The membranes were incubated with primary antibodies to VEGFR1 (1:10,000), VEGFR2 (1:1000), or Beta-actin (1:5000) (Abcam #ab8227, Cambridge, MA) for 1 h at room temperature in blocking buffer. The membranes were incubated with IRDye 800CW goat anti-rabbit IgG secondary antibody (1:5000, Licor) for 45 min at room temperature in blocking buffer. The membranes were scanned and the signals were quantitated (Odyssey Infrared Imaging System, LiCor Biosciences). The relative levels of receptor expression were normalized to the actin signal for each sample.

2.7. Permeability assays

The manufacturer's synthetic membranes were removed from 12-well transwell inserts (Corning, Lowell, MA), the plastic scored and re-sterilized in 70% ethanol and dried under UV overnight. 2.5 cm^2 pieces of placental ($n = 12$) and reflected amnion ($n = 7$) were cut and attached to the transwell inserts using sterile 5/16 inch dental bands to ensure a complete seal. The tissues were oriented so the fetal side of the membrane faced up in the 12-well dishes. Explants were allowed to equilibrate in a 37 °C, 95% air/5% CO_2 incubator for a minimum of 2 h before experimentation. Explants were separated into 4 treatment groups: 1) untreated controls; 2) PBEF-pretreatment (100 ng/ml) for 18 h; 3) acute VEGF treatment (20 ng/ml); 4) PBEF-pretreatment (100 ng/ml) for 18 h plus acute VEGF treatment (20 ng/ml). All treatments were performed in triplicate. PBEF-pretreated explants were exposed to PBEF in both the top and bottom wells for 18 h. Subsequently, the media in the top and bottom wells were exchanged for fresh media with additives according to the treatment group. Culture medium added to the top well of all treatment groups contained 25 μM 2,7-dichlorosfluorescein (DCF) (Sigma), and contained VEGF for treatment groups 3 and 4. Medium in the bottom wells of treatment groups 3 and 4 also contained VEGF. Ten microliter aliquots were transferred from the bottom well into black walled 96-well plates (Corning) at 2.5, 5, 10, 15, 20, 30, 40, 60, 80, 100, 120, 140, and 160 min following DCF ± VEGF addition. At the end of the time course, 40 μl of PBS was added to each well and the fluorescent values were measured (SpectraMAX Gemini XS, Molecular Devices) at 484/535 excitation/emission. DCF μM values were calculated using a DCF standard curve ($n = 52$, average $r^2 = 0.999$). Damaged or improperly sealed tissue explants were identified after the experiment; if the fluorescent readings within the first 10 min of the experiment were greater than 10-fold the average of all the values at any given time-point, these explants were considered compromised and excluded from analysis.

2.8. Statistics and data analyses

2.8.1. ELISAs and westerns

The ELISA data were normalized to the untreated control to reflect the fold change in VEGF secretion following PBEF treatment. Normalization was due to the wide variation in baseline VEGF production, which is normal for primary cells derived from different patients. The data were analyzed by repeated-measures ANOVA with a Dunnett post test using Prism 5.0 (GraphPad Software, La Jolla, CA). Western blot densitometry values also varied among experiments, therefore the values were adjusted to fold change compared to untreated controls and analyzed by Mann–Whitney tests.

2.8.2. Analysis of the explant permeability data

The average μM DCF value was calculated at each time point for each treatment group and correlating averages from all experiments were pooled for statistical analysis. Prism 5.0 was used for all statistical and regression analyses.

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