Stretch and inflammation-induced Pre-B cell colony-enhancing factor (PBEF/Visfatin) and Interleukin-8 in amniotic epithelial cells[☆]

C.E. Kendal-Wright [a,*], D. Hubbard [a], J. Gowin-Brown [a], G.D. Bryant-Greenwood [b]

[a] Department of Obstetrics, Gynecology and Women's Health, John A. Burns School of Medicine, University of Hawai'i, Honolulu, HI 96813, USA
[b] Department of Cell and Molecular Biology, John A Burns School of Medicine, University of Hawai'i, Honolulu, HI 96813, USA

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ABSTRACT

Preterm birth continues to be a growing problem in the USA. Although approximately half of preterm births are caused by intrauterine infection, uterine over-distension is also a cause. In this study we have compared the effects of static stretch, cyclic stretch/release and an inflammatory stimulus alone and in combination on the expression of Pre-B cell colony-enhancing factor (PBEF) and IL-8 in primary amniotic epithelial cells (AEC). We then sought to identify some of the mechanism(s) by which these cells respond to stretching stimuli. We show that cyclic stretch/release is a more robust stimulus for both *PBEF* and *IL-8* than static stretch. Cyclic stretch/release increased both intracellular and secreted PBEF and a combination of both types of stretch was a more robust stimulus to *PBEF* that *IL-8*. However, when an inflammatory stimulus (IL-1β) was added to either kind of stretch, the effect on *IL-8* was much greater than that on *PBEF*. Thus, different kinds of stretch affect the expression of these two cytokines from AEC, but inflammation is a much stronger stimulus of *IL-8* than *PBEF*, agreeing with its primary role as a chemokine. Although the AEC showed morphological signs of increased cellular stress during stretching, blocking reactive oxygen species (ROS) had little effect. However, blocking integrin binding to fibronectin significantly reduced the responses of both *PBEF* and *IL-8* to cyclic stretch/release. The increased PBEF, both intracellularly and secreted, suggests that it functions both to increase the metabolism of the cells, at the same time as stimulating further the cytokine cascade leading to parturition.

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1. Introduction

Preterm birth continues be a growing problem as its rate has risen by 20% in the USA in the last ten years [1]. Approximately 50% of premature births are associated with intrauterine infection/inflammation [2], which triggers an immune response involving the proinflammatory cytokines, which then cause the synthesis and release of uterotonic prostaglandins [3]. However, normal term parturition also involves local cytokine signaling [4]. During normal pregnancy, the fetal membranes are subjected to a combination of chronic distension, allowing their accommodation to the growing fetus [5] and acute cyclical stretch/release both during Braxton–Hicks contractions, and during the major uterine contractions of labor. The stretching of the fetal membranes is known to increase a number of cytokines [6] which are associated with preterm birth [7]. In addition, inflammation caused by local infection or sterile inflammatory mediators such as thrombin also induces localized cytokine production. Therefore, in this study we attempted to mimic the *in vivo* situation where different stretch stimuli can occur simultaneously with inflammatory stimuli.

Pre-B-cell colony-enhancing factor (PBEF/visfatin) is a cytokine involved in the events of parturition [8,9]. It is expressed in all cellular layers of the fetal membranes [10] and both labor and sterile distension cause its increased expression [8,9]. Indeed, a number of stimuli associated with inflammation and infection; LPS, TNFα, IL-1β and IL-6 all up-regulate its expression [10] while in severe chorioamnionitis, it is produced by both the endogenous cells of the fetal membranes as well as the infiltrating neutrophils [9]. Although originally identified as a cytokine [12], more recently it was shown to be an adipokine and re-named Visfatin [13]. Although PBEF lacks a classical secretion sequence, its secretion has been demonstrated [12–15], although the mechanism is currently unknown [16]. The treatment of several different cell types with

PBEF increases production of proinflammatory cytokines and matrix metalloproteinases (MMPs) [17–20], showing it to be an important modulator of the immune response [15,21]. However, PBEF also functions intracellularly as the enzyme nicotinamide phosphoribosyltranferase (Nampt) and increases the amount of NAD$^+$ available for metabolism [22]. Thus, the static stretch-induced increased production of PBEF [8,23] may be a mechanism for providing the extra energy needed for the cell to successfully alter its cytoskeleton and gene expression profile required for adaptation to the stimulus and accommodation for the extra work required during labor. At the same time, its action to induce some key cytokines, such as TNFα [24] clearly involved in the parturition process [2], would further assist in the progression of labor.

Distension of the fetal membranes also increases expression of the IL-8 gene [8,23,25]. IL-8 has potent chemotactic and neutrophil activation properties [26]. It is constitutively expressed by the endogenous cells of the fetal membranes [27] and its expression is increased during normal gestation, resulting in accumulation in amniotic fluid during the third trimester [7]. However, its expression is increased in acute infection [28] and IL-8 is therefore involved in both the initiation of normal term parturition and in infection-induced preterm birth.

The aims of this study were (1) to compare the effects of static stretch, cyclic stretch/release, and inflammation (alone and in combination) on the expression of PBEF and IL-8 in primary amniotic epithelial cells (AEC). (2) To show which pathways are activated and cause their up-regulation, focusing on the roles of reactive oxygen species (ROS) and integrins.

2. Methods and materials

2.1. Tissue collection and amniotic epithelial cell culture

Fetal membranes ($n = 33$) were collected from patients having elective Cesarean sections before labor (38–40 weeks gestation) at Kapiolani Medical Center for Women and Children (Honolulu, HI, USA) with approval from the University Committee on Human Experimentation and the Hospital Institutional Review Board. All tissues were examined by a pathologist for histological evidence of infection, and if positive were excluded.

Primary AEC were isolated as previously described [29] and as used in our prior studies [30]. In brief, the amnion was stripped from adjacent choriodecidua and the epithelial cells isolated by consecutive trypsin (0.2%) digestion (Sigma, St. Louis, MO). The purity of epithelial cells obtained from each patient was similar to that previously reported [30]. The cells were utilized without passage and were seeded at a density of 2 million per well in a 6 cm culture plate in DMEM:F12 supplemented with heat inactivated 10% FBS (Invitrogen), penicillin (50 U/ml)–streptomycin (50 μg/ml) and incubated at 37 °C in 95% air/5% C0$_2$ for 4 days. Media was changed every 3 days until the cells were 70–80% confluent (7–10 days).

2.2. Culture of amniotic epithelial-like cells (WISH)

Human amnion-derived WISH cell (ATCC CCL25) were obtained from the American type Culture Collection (ATCC, Manassas, VA) and grown in Dulbecco's Modified Eagle Medium: Ham F-12 (DMEM:F12) (1:1) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Life technologies, Carlsbad, CA), penicillin (50 U/ml)–streptomycin (50 μg/ml) and incubated at 37 °C in 95% air/5% CO$_2$. When confluent, cells were plated onto Flexcell collagen IV (Flexcell International Corp, Hillsborough, NC) coated plates, at a density of 500,000 per well in DMEM:F12 medium containing 10% FBS and always used between passages 5 and 9.

2.3. Cell stretching experiments

When primary AEC (or WISH cells) reached sub-confluency, the medium was replaced with 0.5% FBS DMEM/F12 for 12–16 h prior to their use. In addition, fresh DMEM:F12 containing 0.5% FBS was added before exposure to any stimulation. Stretching was performed using a Flexcell instrument (Flexcell tension PlusT, FX-4000T) that simulates biological strain conditions using a vacuum to deform the cells cultured on a silastic flexible growth surface. This was set up and used based on the parameters designed by the manufacturer. The 20% static stretching of these cells was performed as-described in our previous studies [23] and represents the maximum amount of stretch achievable with this instrument. Cyclic stretch/release was performed using repetition of 23 s of 20% stretch followed by 7 s of release to 0% stretch. In order to block integrin binding, the RGD peptide GRGDNP, 50 μM (Biomol

International, Plymouth Meeting, PA) was pre-incubated with the cells for 2 h before stretching. Integrins bind to ligands with an exposed arginine-glycine-aspartate (RGD) sequence. Therefore, RGD peptides mimic this adhesion and can be used to block this interaction of the integrin with its usual extracellular matrix substrate. In order to inhibit reactive oxygen species, N-acetyl-L-cysteine (NAC, 30 μM) (Sigma) was added to the cells 2 h before stretching.

2.4. RNA isolation and quantitative real-time PCR

Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA) and DNase (Ambion, Austin, TX) treated, in accordance with the manufacturer's instructions. Reverse transcription was performed with random hexamers. The resulting complementary DNA was used as a template for quantitative real-time PCR. Fluorescence resonance energy transfer (FRET) probes and primers for PBEF, IL-8 and 18S, were purchased from Applied Biosystems Taq Man Assays on Demand (ABI, Foster City, CA) and the ABI protocol used; one cycle for 10 min at 95 °C and 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Each 96-well plate included a water blank and a reverse transcriptase blank. cDNA dilutions were used to generate standard curves for each gene. Real-time PCR was carried out on an MJ Research Opticon 2 Continuous Fluorescence Detector (MJ Research, Waltham, MA). The contents of the PCR mix were; primers (200 nmol/L), FRET probe (100 nmol/L), magnesium (5.5 mmol/L) and Amplitaq Gold (0.025 U/μl). Each reaction was performed in triplicate and the results normalized to the expression of 18S in each sample.

2.5. Immunofluorescence localization of PBEF and the integrin β1 subunit

After stretching, the cells were fixed in 3.7% formaldehyde in 1× PBS followed by three washes in 1× PBS for 10 min total. Non-specific binding was blocked with 1% BSA (Sigma) in 1× PBS for 45 min. The cells were again washed and PBEF antibody at 1/500, (Phoenix Pharmaceuticals) or Integrin β1 subunit antibody at 1/500 (Chemicon) added to each well. After incubation for 1h, the cells were washed and secondary antibody; anti-rabbit or anti-mouse alexafluor 546 at 1/10,000 (Molecular Probes, Eugene, OR), was added for 45 min. The cells were washed again and Flexcell membranes, with cells still attached, were cut around the edges with a scalpel to release them from the plastic tissue culture plate. These were then mounted in Mowiol/glycerol mounting media (Sigma) containing 1,4-diazabicyclo [2.2.2]octane (DABCO) (Sigma) and viewed under fluorescence microscopy.

2.6. Phalloidin-TRITC staining for actin cytoskeleton

After stretching, cells adherent to the Flexcell plates were fixed in 4% formaldehyde in 1× PBS containing calcium (0.901 mM) and magnesium (0.493) followed by three washes in 1× PBS for 10 min total. The cells were then permeabilized in 0.2% Triton X-100 in 1× PBS for 5 min and washed three times with 1× PBS followed by blocking in 10% FBS (Invitrogen) in 1× PBS for 40 min. The cells were washed again and then stained with Phalloidin-TRITC (Sigma) at 1/2500 in 1× PBS for 1 h. The cells were washed again before the Flexcell membranes were cut around the edges with a scalpel to release the membrane with the adherent cells. These were then mounted in Mowiol/glycerol mounting media (Sigma) containing 1,4-diazabicyclo[2.2.2]octane (DABCO) (Sigma) and viewed under fluorescence microscopy.

2.7. Western blotting for PBEF, phoshorylated FAK and paxillin

Cell lysates (20 μl) were boiled for 5 min in 4× SDS loading buffer with 5 mM DTT and loaded into a 10% SDS polyacrylamide gel. The electrophoretically separated proteins were then transferred onto nitrocellulose membranes. Immunoblotting was performed by overnight blocking with 5% non-fat milk (BioRad) 0.1% Tween-20 (Fisher) in 1× PBS. The membranes were then incubated with primary antibodies for 1 h in blocking solution. Antibodies were used at; 1/1000 PBEF (Phoenix Pharmaceuticals, Belmont, CA), 1/300 phosphorylated FAK (Try 576), 1/500 phosphorylated Paxillin (Try 118) (Upstate). After washing, the membranes were incubated with secondary anti-rabbit antibody conjugated to horseradish peroxidase at 1/3000 (BioRad) for 45 min. The membranes were again washed and developed using an enhanced chemiluminescence kit (Amersham Inc, Pitscataway, NJ). The blots were immediately exposed to hyperfilm-enhanced chemiluminescence (Amersham) and the signals quantitated with a densitometer (Kodak EDAS290 System: Eastman Kodak Co, Rochester, NY). The membranes were washed overnight with PBS 0.1% Tween and then re-probed with GAPDH antibody (Abcam, Cambridge, MA) conjugated to HRP at 1/1000 at room temperature for 1h as a protein loading control. The membranes were again washed and developed using an enhanced chemiluminescence kit, the blots exposed and the signals quantitated with a densitometer, the relative levels of each protein were normalized to the amount of GAPDH in each sample.

2.8. Statistical analysis

The Mann–Whitney U test was performed and only p values <0.05 were considered significant. All statistics were calculated with GraphPad software (GraphPad Software Inc, San Diego, CA).