Pre-B-cell colony-enhancing factor, a novel cytokine of human fetal membranes

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OBJECTIVE: Our purpose was to determine whether pre-B-cell colony-enhancing factor (PBEF) is expressed in the human fetal membranes during normal gestation and parturition in the absence of infection and to show its effects on the expression of interleukin (IL)-6 and IL-8.

STUDY DESIGN: PBEF was immunolocalized in the fetal membranes from early pregnancy, at preterm, and at term. Its expression was quantitated by Northern analysis in separated uninfected amnion, chorion, decidua, and placenta of patients at term before labor and in full-thickness membranes before and after spontaneous labor at preterm and at term. Amnion-like epithelial (WISH) cells and fetal membrane explants were treated with recombinant PBEF (rhPBEF), and the expression of IL-6 and IL-8 was quantitated.

RESULTS: PBEF was immunolocalized throughout gestation in the amniotic epithelium and mesenchymal cells as well as the chorionic cytotrophoblast and parietal decidua. Northern analysis showed significantly more ($P < .01$) PBEF expressed in the amnion than in either chorion or placenta. Its expression increased after labor at both preterm and term and correlated with that of IL-8 ($r = 0.87$). rhPBEF treatment of WISH cells significantly increased IL-6 ($P < .05$) and IL-8 ($P < .01$) gene expression after 4 hours and of IL-8 protein after 24 hours ($P < .01$); similar 4-hour treatment of fetal membrane explants significantly increased IL-6 ($P < .01$) and IL-8 ($P < .05$) gene expression.

CONCLUSION: PBEF is a novel cytokine constitutively expressed by the fetal membranes during pregnancy. It increased the expression of IL-6 and IL-8 and may be important in both normal spontaneous labor and infection-induced preterm labor. (Am J Obstet Gynecol 2002;187:1051-8.)

Key words: Pre-B-cell colony-enhancing factor, fetal membranes, cytokines, labor

Preterm birth is a multifactorial disease involving activation of uterine contractions or decreased cervical competence, which can be the result of an inflammatory, infectious, or ischemic insult to the uteroplacental barrier.[1] Preterm labor distinguished by uterine contractions or preterm premature rupture of the fetal membranes before 37 weeks' gestation accounts for some 80% of all preterm deliveries.[2] There is little doubt that cytokine secretion by inflammatory cells plays an important role in the initiation of infection-induced parturition.[3] It seems likely that interleukin (IL)-6[4] and IL-8[5] are also involved in the normal localized growth required for successful pregnancy. However, their involvement in normal parturition appears to be facilitative rather than initiative. Thus, in the absence of chorioamnionitis, as the cervix dilates there is increased contact between the decidua/fetal membranes and the upper genital tract. This contact results in the locally increased production of several inflammatory cytokines, albeit at lower concentrations than in infection-induced parturition, and this augments and drives both uterine contraction and further cervical dilatation by the induction of prostaglandins[6] and matrix metalloproteinases.[7] In this manner, both an inflammatory response and decidual activation are initiated or augmented, accelerating the subsequent events leading to birth.

Pre-B-cell colony-enhancing factor (PBEF) was first identified from activated peripheral blood lymphocytes and shown to be involved in the maturation of B-cell precursors.[8] In our study of the effects of acute distension on the genes expressed by human amniotic epithelial cells (WISH), one of these genes was identified as PBEF.[9] When preterm and term full-thickness fetal membranes were similarly distended in vitro, we showed that the expression of the PBEF gene was greater in the preterm than in the term membranes.[10] This suggested that by term the tissue had attained its maximum distension and sensitivity to further distension was then limited. We subsequently analyzed a genomic clone of PBEF and showed it to be a highly regulated gene. An important finding in

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this study was the identification a nuclear factor-κB (NF-κB) binding element in the third intron, likely to be responsible for the responsiveness to distension.[11] We also localized PBEF with a specific antiserum and showed that the expression of the PBEF gene was significantly upregulated in severely infected membranes and that the neutrophils present in these tissues stained darkly for PBEF, suggesting that they are an additional source of PBEF in the setting of infection.[11] However, although lipopolysaccharide (LPS), tumor necrosis factor (TNF)-α, IL-1β, and IL-6 all induced PBEF gene expression, we showed that IL-8 treatment had no such effect.[11] This was particularly interesting because IL-8 and PBEF expression both increased when WISH cells and the fetal membranes were distended short term.[9,10] Therefore, like PBEF, IL-8 appears to be responsive to distension in a sterile situation, as well as being induced by infection, but its relationship with PBEF under these conditions is unknown. The aim of this study was therefore to determine whether PBEF is present in the fetal membranes during normal gestation and parturition in the absence of infection. In addition, we have produced a recombinant human PBEF (rhPBEF) to study its ability to stimulate the transcription of the classic cytokines IL-6 and IL-8 with use of the amnion-like epithelial (WISH) cell line and fetal membrane explants.

Material and methods

Tissues for immunolocalization. Early pregnancy fetal membranes (87, 94, and 101 days' gestation) were obtained after elective pregnancy termination and were kindly supplied by the Department of Pediatrics, University of Washington, Seattle. These tissues were dated by the day of the last menstrual period and collected within an hour of termination. They were collected with approval by the human subjects Institutional Review Board of the University of Washington and shipped to Hawaii for use with no patient identification. Placentas and fetal membranes were also collected as soon as possible after delivery at Kapiolani Medical Center for Women and Children (Honolulu, Hawaii) with informed consent and approval from the University Committee on Human Experimentation and the Hospital Institutional Review Board. These tissues were from patients after spontaneous preterm labor at 28 to 37 weeks' gestation (n = 4) and after normal spontaneous labor at term, 40 to 42 weeks' gestation (n = 4). Small pieces (3 × 3 cm) were rolled from each membrane and placed in Bouin's fixative for 18 hours at room temperature.

Tissues for Northern analysis. Placentas and fetal membranes were obtained from patients after elective cesarean section at term before labor (38-40 weeks' gestation, n = 5) and placed on ice, and the layers were separated by scraping the decidua from the chorionic surface with a blunt glass slide and by stripping the amnion from other surfaces of the chorion. The samples of placenta were cut from the villous trophoblast in the center of the tissue, avoiding the chorionic and basal plates. Samples were frozen separately in liquid nitrogen; however, three of the decidual samples were of inadequate weight and were therefore pooled (n = 3). For Northern analysis of PBEF gene expression in the fetal membranes obtained before and after the onset of labor at preterm (25-35 weeks' gestation) and term (39-41 weeks' gestation), tissues (n = 24) were collected and divided into four groups: preterm labor (n = 5); preterm cesarean section without labor (n = 6) performed because of intrauterine growth restriction, preeclampsia, or abdominal trauma; term labor (n = 6); and elective term cesarean section without labor (n = 7). For the Northern analysis of IL-6 and IL-8 gene expression after rhPBEF treatment, fetal membranes from elective term cesarean sections before labor (37-40 weeks' gestation, n = 6) were collected and cut into 3 × 3 cm explants. None of the preterm or term patients or tissues had any clinical or histologic signs of chorioamnionitis. The placentas and membranes were examined by a pathologist for histologic evidence of infection by use of the criteria of Naeye,[12] and all tissues with any sign of infection were excluded from this study.

Immunolocalization. Tissue sections (7 μm) were cut and mounted on Vectabond-treated slides (Vector Laboratories, Burlingame, Calif), deparaffinized, and hydrated in deionized water. They were treated with 0.3% hydrogen peroxide in methanol for 30 minutes to block endogenous peroxidase activity and washed in phosphate-buffered saline solution (PBS) for 20 minutes. Sections were treated with normal goat serum (1.5%) for 20 minutes to block all nonspecific binding sites and then incubated with the immunoglobulin G (IgG) fraction derived from a rabbit polyclonal antibody to human PBEF[8] at 5 μg/mL in 0.5% normal goat serum at room temperature for 30 minutes. The negative controls were adjacent sections processed with IgG fraction derived from normal rabbit serum at the same concentration as the primary antibody. The sections were rinsed three times in 0.015 mol/L PBS, 6 minutes total, and then incubated with a biotinylated secondary antibody for 30 minutes, rinsed three times in 0.015 mol/L PBS, 6 minutes total, and treated with the avidin-biotin-peroxidase complex (ABC) reagent (Vector Laboratories) for 30 minutes and diaminobenzidine (0.5 mg/mL) at room temperature for 7 minutes. The sections were washed in distilled water, counterstained with hematoxylin, dehydrated, and mounted in Pro-Texx (Baxter Scientific, Honolulu, Hawaii) and viewed under brightfield microscopy.

Northern analysis. Total RNA was extracted[13] and messenger RNA (mRNA) prepared.[14] Samples of mRNA (10-20 μg) were electrophoresed, and the RNA transferred to a nylon membrane (Magna Graph, MSI, Westborough, Mass) as previously described.[11] The complementary

DNA (cDNA) probes were labeled with the Random Primed DNA labeling kit according to the manufacturer's instructions (Invitrogen, Carlsbad, Calif). The filters were hybridized at 68°C for 1 hour with Express Hyb solution (Clontech, Palo Alto, Calif). A final wash stringency of 0.1× saline–sodium citrate (SSC) buffer, 0.1% sodium dodecylsulfate (SDS) was performed at 50°C. Filters were deprobed in 0.5% SDS for 10 minutes at 100°C and rehybridized sequentially with cDNA probes to PBEF, IL-6, and IL-8 and finally with a human glyceraldehyde-3 phosphate dehydrogenase (G3PDH) cDNA probe (Clontech), as an internal standard. The Northern blot was quantitated with a PhosphoImager (Amersham Pharmacia Biotech, Piscataway, NY), using the G3PDH value for each sample to standardize sample loading. Results were expressed as a ratio to G3PDH. Statistical analysis was performed with the Tukey-Kramer multiple comparison test.

The cDNA probes for PBEF (600 bp) and for IL-6 (639 bp) were both prepared as previously described.[9,15] The IL-8 cDNA probe was a generous gift from Dr Kouji Matsushima, Cancer Center, Kanazawa, Japan.

Recombinant human PBEF protein production. rhPBEF was produced in a bacterial system with pTrcHis2 vector (Invitrogen). PBEF was amplified by polymerase chain reaction (PCR) from a library prepared from fresh human amnion, chorion, and decidua with 5′ and 3′ primers CAACAAGAATTCATGAATCCTCGCGCAGAAG and CTTAAGCGCCGGCGATGATGTGCTGCTTCCAGTTC, respectively. The PCR product was subcloned in the pTrcHis2 vector, and positive colonies were identified by colony lift. One clone was sent for sequencing at the University of Hawaii Biotechnology Facility, and the rhPBEF sequence was confirmed. The production of the rhPBEF protein was carried out according to the Invitrogen pcDNA 3.1/V5-His TOPO TA Cloning Kit protocol. Briefly, a single colony was used to inoculate Luria-Bertani (LB) media, and the culture was incubated until the optical density at OD_{600} reached 0.6, when rhPBEF production was induced with 50 mmol/L isopropyl β-D-thiogalactopyranoside (IPTG). It was stopped after 8 hours, and the cells were then collected by centrifugation and lysed by freeze-thawing (alternating methanol–dry ice and 37°C waterbath). The lysates were spun, filtered through 0.8 μm syringe filters, and stored at –20°C. The rhPBEF protein was purified with use of ProBond resin from Xpress System Protein Purification (Invitrogen,) according to the manufacturer's manual. Briefly, the resin was equilibrated with native binding buffer (20 mmol/L phosphate buffer, 500 mmol/L sodium chloride [NaCl], pH 7.8) and the lysates loaded. The washes were carried out in native wash buffer (20 mmol/L phosphate buffer, 500 mmol/L NaCl, pH 6.0), and rhPBEF was eluted with 350 mmol/L imidazole. All eluates were collected, imidazole was replaced with 50 mmol/L sodium phosphate buffer, pH 7.2, and concentrated with Centricon Plus-20 filtration units (Millipore Corporation, Bedford, Mass). The concentration of rhPBEF was determined with the BioRad Protein Assay (BioRad Laboratories, Hercules, Calif). An aliquot (50 μg) was kindly sequenced by Dr E. Petricoin, Food and Drug Administration/National Institutes of Health, Washington, DC, and six separate peptides confirmed its identity. In addition, an aliquot was assayed for LPS content by Biowhittaker Inc (Walkersville, Md). This showed a concentration of 0.03 EU/ng, well below the acceptable limit of 10 EU/ng.

Amnion-like epithelial (WISH) cell culture and treatment. The amnion-like epithelial (WISH) cells were obtained from American Type Culture Collection (ATCC No. CCL-25) (ATCC, Manassas, Va). WISH cells were cultured in Dulbecco's modified Eagle's medium:Ham F-12 (DMEM:F12) (1:1) supplemented with 10% fetal bovine serum (FBS) (Life Technologies, Grand Island, NY), penicillin (50 U/mL)-streptomycin (50 μg/mL) and incubated at 37°C in 5% carbon dioxide/95% air. After reaching confluency, the cells were trypsinized and plated into 6-well culture dishes at a density of 1×10^5 cells in 4 mL of media. After 48 hours, or when the cells reached 80% confluency, the growth medium was replaced with minimal media (0.5% FBS and 0.2% lactalbumin in DMEM:F12) and incubated for 12 hours. Treatment of the cells began with addition of rhPBEF prepared in minimal media. The concentration of rhPBEF was determined with use of the BioRad protein assay, and 1, 10, and 100 ng/mL was added to the cells for 4 and 24 hours. Treatment was carried out in triplicate and each experiment was performed at least three times on different occasions. Controls received minimal media containing 0.5% bovine serum albumin (15 μL) in PBS. After treatment of the cells, the media were collected and stored frozen at –20°C and the cells lysed in 1 mL of Trizol reagent (Life Technologies, Grand Island, NY). Total RNA was isolated from the lysates according to the manufacturer's protocol.

Fetal membrane explant treatment. Each fetal membrane was cut into several 3 × 3 cm explants. One explant was used to check for infection and another was treated with 100 ng/mL rhPBEF in minimal media for 4 hours; an adjacent explant was used as a control and incubated in minimal media only. Total RNA was isolated with the RNeasy Maxi kit (Qiagen Inc, Valencia, Calif) according to the manufacturer's protocol and used for Northern analysis.

Enzyme-linked immunosorbent assay. The IL-8 enzyme-linked immunosorbent assay (ELISA) kit was purchased from R&D Systems (Minneapolis, Minn) and used to determine the IL-8 protein concentration in the culture supernatants according to the manufacturer's protocol. The sensitivity of the assay was 15 pg/mL. The basal levels of IL-6 protein in conditioned media of WISH cells were reported to be at the limit of sensitivity of ELISA[16]

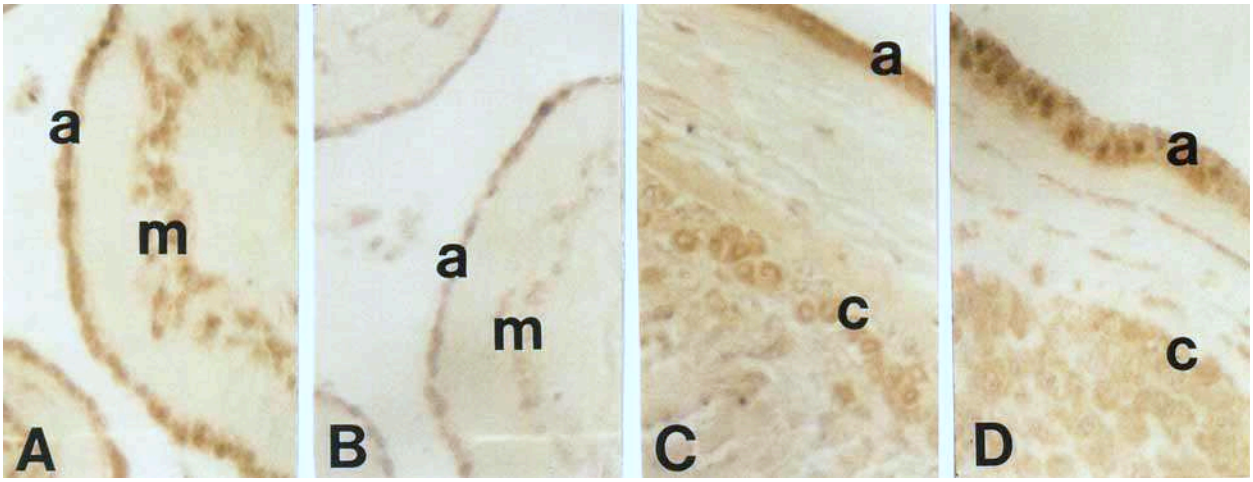

Fig 1. Examples of immunostaining for PBEF in the human fetal membranes. **A,** Early pregnancy (87 days' gestation) and a serial section **(B)** as control, with nonimmune IgG at the same concentration as anti-PBEF in **A.** (Original magnification ×318). Positive staining in amniotic epithelium *(a)* and mesenchyme *(m)* cells. **C,** Fetal membrane from a patient delivered at preterm after labor with no evidence of chorioamnionitis. (Original magnification ×300.) Positive staining in the amniotic epithelium, chorionic cytotrophoblast, and decidua (not shown). **D,** Fetal membrane from a patient after normal term vaginal delivery. (Original magnification ×260.) Positive perinuclear staining can be seen in the cells of the amniotic epithelium and the mesenchymal cells of the connective tissue directly beneath. The cells of the chorionic cytotrophoblast have positively stained cytoplasm and the decidual cells stained similarly but are not visible in this section.

and could not be considered reliable; therefore, the levels of IL-6 protein were not measured.

Results

Immunolocalization. The PBEF protein was immunolocalized in a consistent and uniform manner in the fetal membranes obtained in early pregnancy; an example from an 87-day gestation is shown in Fig 1, *A*. Good staining was obtained in the amniotic epithelium and mesenchymal cells of the amniotic connective tissue in each tissue studied, as seen in Fig 1, *A*. A consecutive section was used as a control with IgG derived from nonimmune rabbit serum at the same concentration, Fig 1, *B*. The fetal membranes from patients delivered preterm showed positive staining for PBEF, which was also uniform over each section. The staining was primarily in the amniotic epithelium, with good staining also in the cells of the chorionic cytotrophoblast and the parietal decidua; an example is shown in Fig 1, *C*. The decidual cells are not easily seen in this section but also stained quite well. An example of a normal fetal membrane from a term spontaneous labor and delivery is shown in Fig 1, *D*. There was good staining in the amniotic epithelial cells, which appeared to be perinuclear in many cells. The mesenchymal cells embedded in the connective tissue of the amnion and chorion were quite prominently stained in this section (Fig 1, *D*). There was also uniform staining of the cytoplasm of the cells of the chorionic cytotrophoblast and the decidua; the latter is not seen in this section.

Northern analysis of PBEF in the fetal membranes, decidua, and placenta. The Northern blot with separated amnion, chorion, decidua, and placental trophoblast obtained at term before labor and delivery is shown in Fig 2. All three PBEF transcripts of 2.0, 2.4, and 4.0 kb when quantitated separately were expressed in each of the different tissues in similar proportions (data not shown) and all transcripts were therefore included in the quantitation. The actual blot showing these transcripts is shown in Fig 2. Some of the samples showed stronger hybridization of all transcripts than did others, in spite of equal loading of the RNA. The quantitation of this blot is shown beneath and is expressed as counts per microgram RNA as a result of lack of a suitable housekeeping gene, which can be used with both membranes and placenta (Fig 2). There was significantly more ($P < .01$) PBEF expressed in the amnion than in either the chorion or the placenta.

Northern analysis of PBEF, IL-6, and IL-8 before and after the onset of labor at preterm and term. In the absence of labor, PBEF was constitutively expressed at similar levels in both preterm and term fetal membranes. The process of labor caused up-regulation of PBEF expression to the same extent both at preterm and term compared with the respective nonlabored group, but this up-regulation was not significant because of the large patient-to-patient variability in the nonlabored group. The basal levels of IL-6 and IL-8 expression, in the absence of labor, regardless of gestational age, were lower than those of PBEF, and labor acted as a stronger inducer of their expression (Fig 3). This resulted in a significant increase of

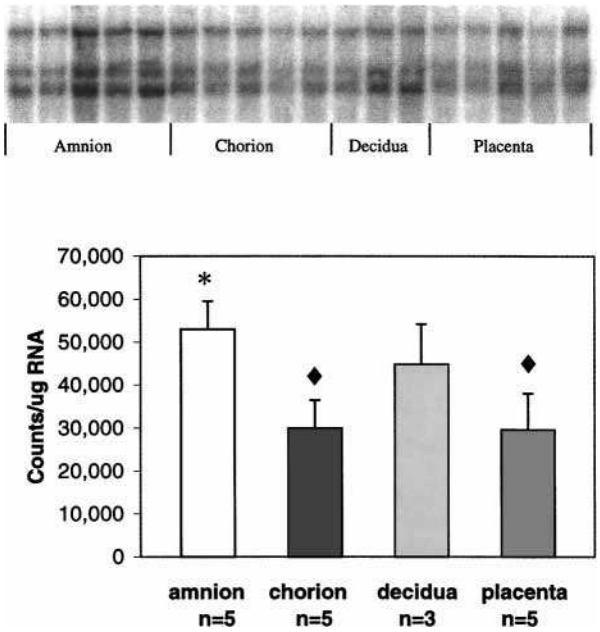

Fig 2. Quantitative Northern analysis of PBEF gene expression in the fetal membranes and placenta. The three PBEF transcripts are shown above their quantitation, expressed as counts per microgram. *Asterisk,* There was significantly more ($P < .01$) PBEF expressed in the amnion compared with the chorion or placenta.

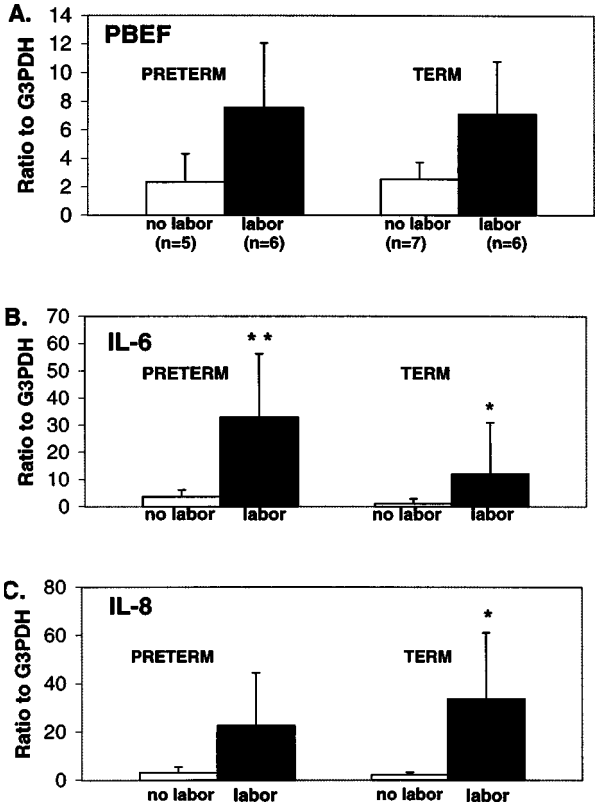

Fig 3. The expression of PBEF, IL-6, and IL-8 in the human fetal membranes: *open bar,* no labor; *solid bar,* labor. **A,** PBEF expression was up-regulated by labor, but this did not reach significance at either gestational age. **B,** IL-6 expression after labor and delivery was significantly up-regulated both at preterm and term compared with the nonlabored tissues of matched gestational age (*asterisk,* $P < .01$ in each case). IL-6 expression at preterm labor was (*two asterisks,* significantly higher than that at term labor, $P < .05$). The correlation between PBEF and IL-6 expression ($r = 0.64$) was significant ($P < .1$) and that between PBEF and IL-8 expression ($r = 0.86$) was also extremely significant ($P < .0001$). **C,** IL-8 expression was increased after labor both at preterm and term, where the latter reached statistical significance (*asterisk,* $P < .05$) compared with the nonlabored tissues of matched gestational age.

IL-6 expression after labor and delivery both at preterm and term compared with the nonlabored tissues of matched gestational age ($P < .01$ in each case). IL-6 expression increased significantly more with preterm than with term labor ($P < .05$). IL-8 expression was significantly increased only after term labor ($P < .05$) compared with nonlabored term tissues. Comparison of IL-8 expression in preterm tissues with and without labor showed an increase, which did not reach significance. In spite of the greater patient variability of PBEF expression, there was a good correlation between PBEF and IL-8 ($r = 0.87$).

The effects of PBEF on the expression of IL-6 and IL-8 in WISH cells and fetal membrane explants. The amniotic epithelial cell line, WISH cells, were used to study the effects of PBEF on the expression of IL-6 and IL-8. The expression of the IL-6 and IL-8 genes were quantitated by Northern blotting by use of total RNA isolated from the cells and calculated as the degree of up-regulation (fold) compared with the respective control at each time (Fig 4). The treatment of WISH cells with rhPBEF caused a dose-dependent increase (15%, 29%, and 44%, respectively) in IL-6 gene expression after 4 hours with both 10 and 100 ng/mL, which were both significant ($P < .05$) (Fig 4, *A*). The treatment with 10 ng/mL rhPBEF caused a 35% increase in IL-8 gene expression after 4 hours of treatment, which was significant ($P < .01$); this effect was absent with 100 ng/mL (Fig 4, *B*). The IL-8 protein levels in the media showed a basal expression level of 34 pg/mL, which remained the same at 4 and 24 hours (data not shown). The 10 ng/mL dose of rhPBEF caused a small (15%) increase in secreted IL-8 after 4 hours ($P < .05$) (not shown) and a greater increase (30%) after 24 hours treatment ($P < .01$), which reached significance (Fig 4, *C*). Thus, the same dose of rhPBEF (10 ng/mL) increased expression of both the IL-8 gene and protein. Fetal membrane explants were also treated with rhPBEF because WISH cells are an amnion-derived cell line. Explants were treated with the highest dose (100 ng/mL) of rhPBEF because we anticipated that more would be required with tissue to show an effect. The expression of the IL-6 and IL-8 genes were quantitated by Northern

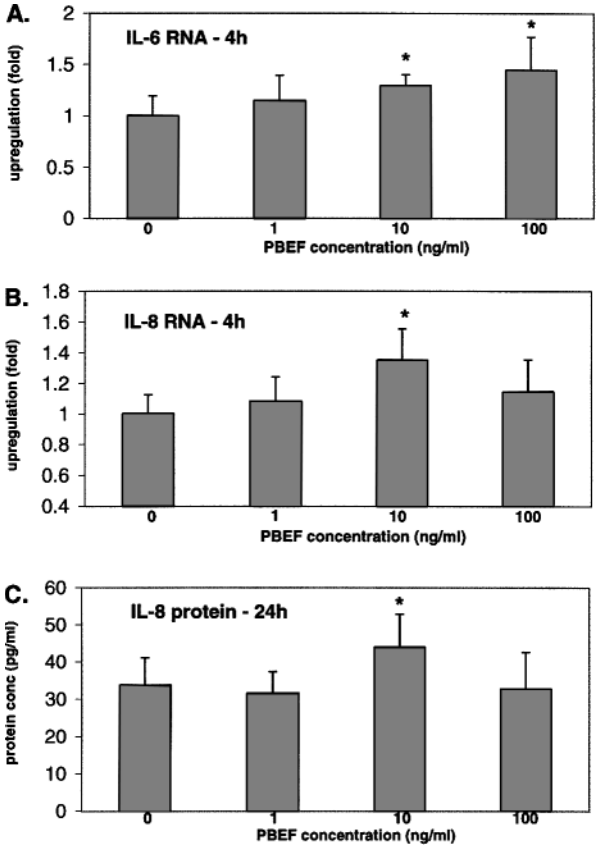

Fig 4. The effect of rhPBEF treatment on the expression of IL-6 and IL-8 in amniotic epithelial (WISH) cells. **A,** The dose-dependant increase in IL-6 gene expression after treatment with rhPBEF at 1, 10, and 100 ng/mL for 4 hours. *Asterisk,* Significantly ($P < .05$) increased expression compared with the control. **B,** The dose-dependent effect of rhPBEF on IL-8 gene expression: 10 ng/mL rhPBEF caused a significant ($P < .01$) increase in IL-8 gene expression after 4 hours. **C,** The effect of rhPBEF on IL-8 protein expression in the media after 24 hours of treatment measured by ELISA: 10 ng/mL rhPBEF caused a significant ($P < .01$) increase compared with the control.

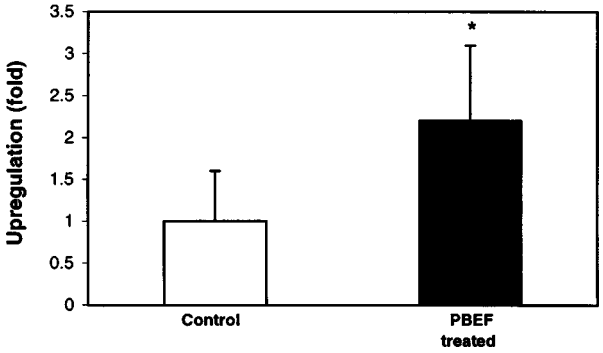

Fig 5. The effect of rhPBEF treatment on the expression of IL-8 in fetal membrane explants. RhPBEF (100 ng/mL) caused a significant (*asterisk,* $P < .05$) increase in IL-8 gene expression after 4 hours of treatment compared with the control.

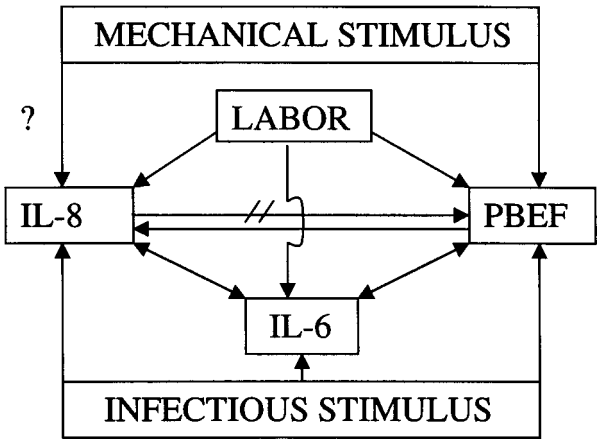

Fig 6. Diagrammatic summary of the possible relationships between PBEF, IL-6, and IL-8 and their controls by mechanical stimulation, by infectious stimulation, and by labor.

analysis and the results for the latter are shown in Fig 5. rhPBEF treatment caused a 40% up-regulation of IL-6 and 120% up-regulation of IL-8 gene expression compared with the control ($P < .01$ and $P < .05$, respectively). The statistical analysis was performed with use of the Wilcoxon matched pairs test.

Comment

In this study we show that PBEF is constitutively expressed in the fetal membranes and placenta during normal gestation with no marked increase with gestational age. In this respect, PBEF appears to be like IL-8, which is also constitutively expressed and has been suggested to have a physiologic role during normal gestation.[17] However, the expression of these two cytokines increases when the fetal membranes are distended short term.[10] As gestation advances, the membranes become increasingly distended[15]; thus PBEF and IL-8 may act as growth regulators to facilitate the accommodation of the tissue and prevent it from rupturing prematurely. It has recently been reported that PBEF is antiapoptotic for neutrophils,[18] similar to IL-8.[19] The distension of the fetal membranes in vivo may increase apoptosis in this tissue; thus PBEF and IL-8 may be protective against this effect of distension.

It has been reported that both IL-6[20] and IL-8[21] are more highly expressed in choriodecidua in comparison to amnion. In our study, we separated the fetal membranes into three layers (amnion, chorion, and decidua), showing the highest expression in the amnion. However, taking the sum of the expression of PBEF in chorion and decidua and comparing it with the expression of IL-6 and IL-8, the same pattern of expression for PBEF is then evident.[20,21] In these patients with no chorioamnionitis, IL-6 gene expression was significantly increased with labor at both preterm and term, with a greater increase at preterm than term. This agrees with previously published

data.[20,22] The expression of IL-8, on the other hand, significantly increased only with labor at term, also agreeing with published work.[23,24] However, PBEF expression showed increases to approximately the same levels with labor at both preterm and term, with neither reaching a level of significance compared with the nonlabored controls. This was due to the considerably greater variation in the expression of PBEF in the nonlabored patients, not the case for either IL-6 or IL-8. Greater sample numbers may have shown a significant effect of labor on PBEF gene expression.

We have previously shown that the in vitro distension of the fetal membranes increased the expression of both the genes for IL-8 and PBEF.[10] Most recently, we have shown that the expression of both these genes also increased in the fetal membranes of patients with severe chorioamnionitis.[11] The mechanical stimulation of the fetal membranes resulting from uterine contractions at the time of labor may be a cause of their increased expression. On the other hand, the cervical changes during labor allow bacteria from the vagina to stimulate cytokine expression from the fetal membranes.[23] This may explain the labor-related increases in the expression of IL-1,[24] TNF-α,[25] and IL-6.[20] Thus, the increased expression of IL-6, IL-8, and PBEF with labor at both preterm and term in this study may be a consequence of such subclinical infection. The increase in IL-6 in this study suggests this mechanism because IL-6 was not increased by a mechanical stimulus alone, whereas IL-8 and PBEF were.

The rhPBEF was produced to study the relationship of PBEF with IL-6 and IL-8. We have used WISH cells as a model system to exclude the possibility of infection. In this study we have shown that the addition of rhPBEF increased the expression of mRNA for IL-6 in dose-dependent manner. Because there may be a dissociation between the events of transcription and translation for the cytokines and increased transcription may not necessarily be followed by increased translation,[26] we measured the protein levels in the media, where possible. Because it has been reported that IL-6 protein secreted by WISH cells is below the level of detection of an ELISA,[16] we did not attempt to measure it here. However, the IL-8 protein in the media of these cells was detectable by ELISA, even under basal conditions. The addition of 10 ng/mL rhPBEF caused a significant increase in the expression of the IL-8 gene after 4 hours and of its protein in the media after 24 hours of treatment; these time frames suggest that PBEF may have a direct effect on the expression of IL-8. However, the extent of this up-regulation of the genes for IL-6 and IL-8 in WISH cells was moderate and might not reflect any biologic significance; therefore, we treated fetal membrane explants with rhPBEF to confirm these results. The higher dose (100 ng/mL) of rhPBEF caused a 120% increase in the expression of IL-8 in fetal membrane explants compared with the lower dose (10 ng/mL) of rhPBEF in WISH cells, which caused a maximal effect of 35% increase. On the other hand, IL-6 gene expression was only increased by 40% after rhPBEF (100 ng/mL) in the fetal membrane explants, which approximated to the maximal effect of rhPBEF (100 ng/mL), 44% in WISH cells.

The proinflammatory cytokines TNF-α and IL-1β have been shown to stimulate IL-6,[16] IL-8,[16,27] and PBEF[11] transcription. We show here that rhPBEF was able to stimulate the expression of both IL-6 and IL-8; however, we previously showed that IL-6, but not IL-8, could cause increased expression of PBEF from WISH cells.[11] Thus, we summarize these interactions diagrammatically in Fig 6 and attempt to place PBEF into the cytokine network stimulated by both mechanical and infection pathways in the fetal membranes. We propose that a mechanical stimulus may directly affect the expression of PBEF through the specialized NF-κB site in its third intron.[11] We do not know whether a mechanical stimulus can directly cause increased IL-8 transcription, or whether this only occurs through the up-regulation of PBEF and its subsequent stimulation of IL-8. Labor may represent a combined mechanical and infection stimulus because, in addition to PBEF and IL-8, IL-6 expression was also increased. Thus, further studies are now needed to clarify the interactions between IL-8 and PBEF in normal gestation and with preterm and term labor and to determine the mechanisms involved in its mechanical stimulation.

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