The effects of pre–B-cell colony–enhancing factor on the human fetal membranes by microarray analysis

Simona Ognjanovic, PhD, Lily S. Tashima, PhD, and Gillian D. Bryant-Greenwood, PhD
Honolulu, Hawaii

OBJECTIVE: Our purpose was to show the effects of pre–B-cell colony–enhancing factor on the genes that are expressed by the human fetal membranes.
STUDY DESIGN: Explants of fetal membranes (amnion, chorion, and decidua) from three term patients were treated with 100 ng/mL recombinant human pre–B-cell colony–enhancing factor for 4 hours. RNAs were hybridized to gene chips that contained >18,000 known genes. One experiment was done in triplicate to assess replication. Data were analyzed to quantitate the signal intensities of each complementary DNA on the array. Confirmation of the results was carried out on tissues from nine other patients by the measurement of the proteins or quantitative real-time reverse transcriptase–polymerase chain reaction.
RESULTS: Replication gave <92.6% identical results, which showed high method reproducibility. Pre–B-cell colony–enhancing factor treatment caused a significant increase in 103 genes and decrease in 139 genes. Only 8 genes were up-regulated consistently and significantly in all three patients (three key inflammatory cytokines [tumor necrosis factor-α, interleukin-6, and interleukin-1β], four important chemokines [macrophage inflammatory protein-1α, macrophage inflammatory protein-1β, macrophage inflammatory protein-3α, and growth-related oncogene-γ], and prostaglandin-endoperoxide synthase 2). These data were confirmed by the measurement in the media with the use of specific enzyme-linked immunosorbent assays for tumor necrosis factor-α, interleukin-6, and interleukin-1β, macrophage inflammatory protein-1α, macrophage inflammatory protein-1β, and macrophage inflammatory protein-3α and by quantitative real-time reverse transcriptase–polymerase chain reaction for growth-related oncogene-γ and prostaglandin-endoperoxide synthase 2.
CONCLUSION: Pre–B-cell colony–enhancing factor appears to be at the proximal end of the pathway to labor initiation and may link sterile distention-induced labor with that of infection-induced labor. (Am J Obstet Gynecol 2003;189:1187-95.)

Key words: Pre–B-cell colony–enhancing factor, cytokine, chemokine, microarray, labor induction

Parturition in the human is particularly hazardous.[1] It is a complex physiologic process that requires synchronization of uterine contractions, cervical dilatation, and fetal membrane rupture, which are achieved in our species through autocrine/paracrine events at the maternal-fetal interface within the uterus.[2] Preterm birth is a major clinical problem and is the leading cause of perinatal and neonatal death, often resulting in significant long-term health problems for the survivors.[3] Bacterial infections within the uterus result in early preterm birth (<32 weeks of gestation) and the most serious outcomes.[3] Such infections cause an increased local production of cytokines, which mediate the inflammatory process and the common terminal pathway to labor.[4]

The importance of cytokines in the regulation of inflammatory and immune responses is now recognized clearly. They have pleiotropic regulatory effects on hematopoietic cells and many other cell types that participate in host defense and repair processes. Parturition, even in the absence of infection, appears to be accompanied by a localized inflammatory response within the uterus,[5] but in the setting of intrauterine infection, a precocious activation of immunocompetent cells occurs, which leads to labor.[4] This response removes the fetus from an environment that is hostile to its further growth and development. The proinflammatory cytokines that are produced by macrophages and decidual cells appear to play an important role in infection-induced preterm labor. This was shown by the intra-amniotic infusion of interleukin-1β (IL-1β) into preterm pregnant rhesus monkeys that rapidly induced the production of intra-amniotic tumor necrosis factor-α (TNF-α), prostaglandin E_2, and prostaglandin $F_{2\alpha}$, and uterine contractility.[6] However, the cytokines may be merely the mediators of

From the Department of Cell and Molecular Biology, University of Hawaii, Honolulu, Hawaii, USA.
Supported by National Institutes of Health grant No. HD-24314 and by grants to the University of Hawaii under the Research Centers in Minority Institutions Program of the National Center for Research Resources P20RR 11091 and P20RR 16467.
Received for publication January 9, 2003; revised April 15, 2003; accepted April 29, 2003.
Reprint requests: Simona Ognjanovic, PhD, University of Hawaii, 1960 East-West Rd, Biomed T-709, Honolulu, HI 96822.
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0002-9378/2003 $30.00 + 0
doi:10.1067/S0002-9378(03)00591-X

labor rather than the initiators. In any event, they stimulate the production of a subclass of cytokines, the chemokines, or chemoattractant cytokines (such as IL-8 and macrophage inflammatory proteins [MIPs]), establish a concentration gradient by which macrophages and neutrophils are attracted into a site of inflammation, and then become activated.[4] Thus, after the inflammatory response is initiated, the chemokines are produced, which recruit and activate circulating leukocytes and cause the release of proteolytic enzymes. This step represents a possible link from cytokines to preterm labor. Hence, the cytokines themselves do not cause preterm labor but mediate the maternal response to an inflammatory stimulus, it is this maternal immune response that involves the cytokines that mediates preterm labor.[4]

We recently showed that a cytokine termed *pre–B-cell colony–enhancing factor (PBEF)* was up-regulated by the distension of human amniotic epithelial cells.[7] This cytokine was first identified from activated peripheral blood lymphocytes and shown to be involved in the maturation of B-cell precursors.[8] We have shown it to be expressed constitutively by the human fetal membranes during pregnancy but up-regulated by both normal and preterm labor in the absence of infection[9] and by severe chorioamnionitis.[10] This was confirmed recently in a macroarray study that showed increased expression of PBEF by both term labor and infection.[11]

PBEF has no structural homology to other known cytokines, and little is known about its actions. It was immunolocalized most prominently to both amniotic epithelial and mesenchymal cells of normal fetal membranes from early gestation, although the cells of the cytotrophoblast and decidua also immunostained later in gestation.[9] By Northern analysis, the amnion and decidua at term contained the highest levels of PBEF messenger RNA.[9] We analyzed its genomic clone[10] and produced a recombinant human PBEF (rhPBEF) to study its ability to stimulate the transcription of the more classic cytokines.[9] We have been attempting therefore to place PBEF into the cytokine cascade with a view to understanding its role in the inflammatory process. In this study, we have broadened this approach and used DNA microarrays to analyze the effects of this rhPBEF when it is added to explants of normal, uninfected, nonlabored, term fetal membranes. Confirmation of the results has been performed at the protein level for the secreted products in the media or by quantitative real-time reverse transcriptase–polymerase chain reaction (RT-PCR) if an enzyme-linked immunosorbent assay (ELISA) was inappropriate or not available.

Material and methods

Patients and tissues. For use on microarrays, full-thickness fetal membranes with adhering decidua were collected from three patients at elective cesarean delivery at term (38-40 weeks of gestation), before labor and within 30 minutes of expulsion. Institutional review board approval was obtained, but consent was not required, because these tissues were collected anonymously. None of the patients had any clinical signs of infection, and the placentas and membranes were examined histologically by a pathologist to eliminate localized infection, as previously reported.[9] Explants (3 × 3 cm) were cut and treated with rhPBEF (100 ng/mL); adjacent squares of tissues were incubated in media alone (controls) for 4 hours as previously described.[9] The dose of PBEF was based on our previous work with an amnion cell line.[9] We used the highest dose (100 ng/mL) here because the full-thickness fetal membrane was treated, as opposed to a single layer of confluent cells.[9] The rhPBEF was produced in a bacterial system as described and assayed for lipopolysaccharide content, which was shown to be well below the acceptable limit.[9] The explants were incubated in 50 mL of Ham's F-12 and Dulbecco's modified Eagle's medium that was supplemented with 0.5% fetal calf serum at 37°C in 95% air/5% carbon dioxide. Explants were harvested and stored at −80°C until RNA extraction. For confirmation of the Affymetrix array data (Affymetrix, Santa Clara, Calif), nine similar tissues were collected, and the explants were treated with rhPBEF for 4 hours and then stored at −80° until used. Media from all explant experiments were collected and stored at −80° until used for ELISAs.

RNA extraction. The explants were homogenized, and RNA was extracted with the RNeasy Total RNA Kit (Qiagen, Chatsworth, Calif). The yields and quality were confirmed by spectrophotometry and formaldehyde gel electrophoresis.

Experimental design and microarray hybridization. rhPBEF-treated and control explants from three patients were used for the microarray hybridizations. One of these explants was carried out in triplicate (fetal membrane No. 1), and the RNA from the rhPBEF-treated and control explants were labeled and hybridized on three different dates to determine interassay variability and replication (Fig 1). Labeling, hybridization, washes, and staining were performed according to the Affymetrix protocols . Briefly, the total RNA was used as a template for double-stranded complementary DNA synthesis, which in turn became a template for the in vitro transcription of biotin-labeled complementary RNA. The biotinylated complementary RNA was fragmented and hybridized to an Affymetrix Human Genome U133A chip. This array chip contains >22,000 probe set identifications, including >18,000 known genes. Affymetrix protocols were also followed for posthybridization washes, staining, and scanning. All the Affymetrix microarray protocols were performed at the University of Hawaii Biotechnology Core Facility.

Microarray data analysis. Data from all 10 arrays (one study was done in triplicate, and there were two additional studies; Fig 1) were analyzed by both Microarray Suite 5.0 (Affymetrix) and Data Mining Tool (Affymetrix). Each array was analyzed individually and in pairs (PBEF-treated vs control), then all sets were analyzed together (Fig 1). All the data sets were scaled to 150 target intensity (TGT) to compensate for differences in overall hybridization signals. For each probe set identification (approximately 22,000) in an array, a probe set signal (detection signal) was computed after background correction was made. Then, each pair of rhPBEF-treated versus control probe set was compared against each other to generate a difference value for each probe set. The Microarray Suite 5.0 uses a number of quantitative algorithms. One set of algorithms generates a "change probability value" and an associated "change" call (I = increased; D = decreased; NC = no change; MI = marginally increased; MD = marginally decreased,). The Wilcoxon signed rank test was used for this comparison analysis. A second set of algorithms generated a quantitative change in gene expression in the form of a "signal log ratio," which was based on the mean difference in probe pair hybridization intensities (PBEF vs control). The data were further analyzed with the Data Mining Tool, which is based on confidence statistical algorithms that use rank-based (nonparametric) methods. This software performs more complex analyses and has the ability to combine such sample replicates (as conducted for fetal membrane no. 1; Fig 1) with the other experimental data (fetal membranes No. 2 and No. 3, Fig 1). This analysis could then be used for the Mann-Whitney and the Student *t* tests.

ELISA. The ELISAs for TNF-α, IL-1β, IL-6, MIP-1α, MIP-1β, and MIP-3α were purchased from R&D Systems (Minneapolis, Minn) and were used according to the manufacturer's protocols to determine the concentrations of each protein in the culture media. These ELISAs were carried out in the media from the three tissues used for the Affymetrix array analysis and the nine additional tissues. The sensitivities for each of the ELISA assays were IL-6 <0.7 pg/mL, TNF-α <4.4 pg/mL, IL-lβ <1 pg/mL, MIP-1α <10 pg/mL, MIP-1β <4 pg/mL, and MIP-3α <0.5 pg/mL.

Quantitative real-time RT-PCR. Total RNA was isolated from each explant with the RNeasy Maxi Kit (Qiagen Inc, Valencia Calif), DNase treated (Ambion, Austin, Tex), and reverse transcribed with random hexamers; and the resulting complementary DNA was used as a template for quantitative real-time RT-PCR in the DNA Engine Opticon System (MJ Research, Boston, Mass). The contents of the PCR mix were primers (200 nmol/L): fluorescence resonance energy transfer (FRET) probe (100 nmol/L), magnesium (5.5 mmol/L), and Amplitaq Gold (0.025 U/μL). The PCR conditions were 1 cycle of 95°C (10 minutes) and 40 cycles of 95°C (15 seconds) and 60°C (1 minute). FRET probes and primers for prostaglandin-endoperoxide synthase 2 (PGHS-2) and 18 S recombinant RNA were purchased from Biosource International (Camarillo, Calif). The sequences for growth-related oncogene gamma (GRO-γ) were CAAACCGAAGTCATAGCCACACT, GGTGCTCCCCTTGTTCAGTATC, and FAM-AAGCTTGTCTCAACCCCGCATCC-BHQ1 (FRET probe). Data normalization was performed with the use of 18S recombinant RNA.

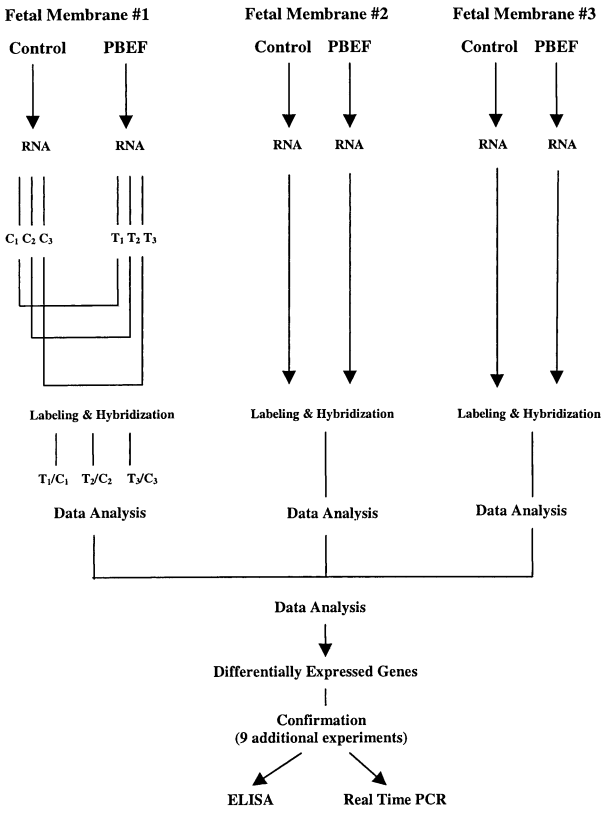

Fig 1. The experimental design for the microarray experiments with fetal membrane explants that were treated with rhPBEF (100 ng/mL) for 4 hours.

Results

Microarrays. The replication experiment with fetal membrane No. 1 (Fig 1), which was labeled and hybridized on three separate occasions, showed >92.6% identical change calls (ie, increase, decrease, or no change). Thus, there was little chip-to-chip variability and high method reproducibility with this technique.

All probe sets (22,000) were analyzed by the Data Mining Tool software, which took into consideration both the absolute differences between the groups and the variance. With use of the Student *t* test, 103 genes were up-regulated significantly ($P < .05$, Table I), and 139 genes were down-regulated significantly (Table II) by the

Table I. Genes up-regulated with probability values of < .05

*GenBank accession no. and name**	*P value*
NM_014944 KIAA0911 protein	.004
NM_004233 CD83 antigen (activated B lymphocytes, immunoglobulin superfamily)	.004
NM_013259 Neuronal protein	.004
NM_000963 Prostaglandin-endoperoxide synthase 2 precursor	.004
NM_002983 Small inducible cytokine A3	.004
NM_000600 Interleukin 6 (interferon, beta 2)	.004
NM_001200 Bone morphogenetic protein 2 precursor	.004
NM_000594 Tumor necrosis factor (cachectin)	.004
NM_002090 GRO3 oncogene	.004
NM_005723 Tetraspan 5	.004
NM_022567 Nyctalopin	.004
NM_003074 SWI/SNF-related matrix-associated actin-dependent regulator of chromatin c1	.012
NM_018356 Hypothetical protein FLJ11193	.012
NM_004049 BCL2-related protein A1	.012
NM_006065 Signal-regulatory protein beta 1 precursor	.012
NM_023070 Hypothetical protein LOC65243	.012
NM_000231 Gamma sarcoglycan	.012
NM_012122 Carboxylesterase 3	.012
NM_000575 Interleukin 1, alpha	.012
NM_016577 RAB6B, member RAS oncogene family	.012
NM_006139 CD28 antigen (Tp44)	.012
NM_003403 YY1 transcription factor	.012
NM_017634 Hypothetical protein FLJ20038	.012
NM_003790 Tumor necrosis factor receptor superfamily, member 12	.012
NM_013275 Nasopharyngeal carcinoma susceptibility protein	.012
NM_022144 Tenomodulin protein	.012
NM_014111 PRO2086 protein	.012
NM_017716 Membrane-spanning 4-domains	.012
NM_014095 PRO1600 protein	.012
NM_030911 Protein kinase NYD-SP15	.012
NM_000739 Cholinergic receptor, muscarinic 2	.012
NM_006474 Lung type-I cell membrane-associated glycoprotein precursor NM_013317	.012
NM_001834 Clathrin, light polypeptide B (Lcb) isoform	.012
NM_002421 Matrix metalloproteinase 1 preproprotein	.020
NM_004992 Methyl CpG binding protein 2	.024
NM_001952 E2F transcription factor 6	.024
NM_015032 Androgen-induced prostate proliferative shutoff associated protein	.024
NM_002553 Origin recognition complex, subunit 5-like	.024
NM_000576 Interleukin 1, beta	.024
NM_003401 X-ray repair cross complementing protein 4	.024
NM_000399 Early growth response 2 protein	.024
NM_020980 Aquaporin 9	.024
NM_005658 TNF receptor-associated factor 1	.024
NM_002422 Matrix metalloproteinase 3 preproprotein	.024
NM_000450 Selectin E precursor	.024
NM_005069 Single-minded (*Drosophila*) homolog 2 long isoform	.024
NM_000899 Mast cell growth factor, isoform b	.024
NM_006058 Nef-associated factor 1	.024
NM_000208 Insulin receptor	.024
NM_012117 Chromobox homolog 5 (HP1 alpha homolog, *Drosophila*)	.024
NM_001352 D site of albumin promoter (albumin D-box) binding protein	.024
NM_000065 Complement component 6 precursor	.024
NM_005746 Pre–B-cell colony–enhancing factor	.024
NM_018093 Hypothetical protein FLJ10439	.024
NM_024683 Hypothetical protein FLJ22729	.024
NM_024688 Hypothetical protein FLJ13031	.024
NM_012152 Endothelial cell differentiation gene 7	.024
NM_000576 Interleukin 1, beta	.024
NM_003488 A kinase (PRKA) anchor protein 1	.044
NM_001927 Desmin	.044
NM_004148 Ninjurin 1	.044
NM_001792 Cadherin 2, type 1 preproprotein	.044
NM_002984 Small inducible cytokine A4	.044
NM_016315 CED-6 protein	.044
NM_014810 Centrosome-associated protein 350	.044
NM_004591 Small inducible cytokine subfamily A (Cys-Cys)	.044
NM_000905 Neuropeptide Y	.044
NM_001478 UDP-N-acetyl-alpha-D-galactosamine	.044
NM_024318 Immunoglobulin-like transcript 8	.044
NM_003821 Receptor-interacting serine-threonine kinase 2	.044
NM_001285 Calcium activated chloride channel 1 precursor	.044
NM_014216 Inositol 1,3,4-triphosphate 5/6 kinase	.044
NM_006439 Mab-21-like protein 2	.044
NM_001372 Dynein, axonemal, heavy polypeptide 9	.044
NM_000723 Calcium channel, voltage-dependent	.044
NM_006750 Basic beta 2 syntrophin, isoform a	.044
NM_004244 CD163 antigen	.044
NM_019839 Seven transmembrane receptor BLTR2; leukotriene B4 receptor BLT2	.044
NM_022136 SAM domain	.044
NM_018297 Peptide:N-glycanase similar to yeast PNG1	.044
NM_017415 Kelch-like 3	.044
NM_021196 Sodium bicarbonate transporter 4	.044

*Plus 21 probe sets with unidentified names/function.

rhPBEF treatment. Among the most significantly up-regulated genes were prostaglandin-endoperoxide synthase 2 (PGHS-2), TNF-α, IL-6, and MIP-1α (Table I). In addition, two members of the TNF receptor superfamily (member 12 [$P < .012$] and the TNF receptor–associated factor 1 [$P < .024$]) were up-regulated significantly. IL-lα ($P < .012$) and IL-1β ($P < .024$) were also up-regulated significantly, as were two important prepromatrix metalloproteinases (MMP-1 [$P < .02$] and MMP-3 [$P < .024$], Table I). PBEF itself was also up-regulated significantly ([$P < .024$).

The down-regulated genes with probability values of <.05 that are shown in Table II included a disintegrin ($P < .004$), phospholipase A2 ($P < .024$), and the estrogen-related receptor β ($P < .024$).

When the data were analyzed with the Microarray Suite 5.0, there were hundreds of increased genes in any

Table II. Genes down-regulated with a probability values of <.05

*GenBank accession no. and name**	*P value*
NM_005582 Lymphocyte antigen 64 homolog, radioprotective 105 kd	.004
NM_000810 Gamma-aminobutyric acid (GABA) A receptor, alpha 5 precursor	.004
NM_006344 Macrophage lectin 2 (calcium dependent)	.004
NM_018287 Hypothetical protein FLJ10971	.004
NM_001524 Orexin precursor	.004
NM_004770 Potassium voltage-gated channel, Shab-related subfamily	.004
NM_004194 A disintegrin and metalloproteinase domain 22	.004
NM_003296 Testis specific protein 1 (probe H4-1 p3-1)	.004
NM_004555 Nuclear factor of activated T cells, cytoplasmic	.004
NM_025207 Hypothetical protein PP591	.004
NM_032924 Hypothetical protein MGC16040	.004
NM_005671 Reproduction 8	.004
NM_002877 RAD51-like 1, isoform 1 NM_133509 RAD51-like 1, isoform 1	.004
NM_018231 Hypothetical protein FLJ10815	.004
NM_013363 Procollagen C-endopeptidase enhancer 2	.004
NM_019066 MAGE-like protein 2	.004
NM_018488 T-box 4	.004
NM_015985 Angiopoietin 4	.004
NM_000312 Protein C (inactivator of coagulation factors Va and VIIIa)	.008
NM_004156 Protein phosphatase 2 (formerly 2A), catalytic subunit, beta isoform	.012
NM_002470 Myosin, heavy polypeptide 3, skeletal muscle, embryonic	.012
NM_005096 Zinc finger protein 261	.012
NM_001143 Amelogenin (Y chromosome)	.012
NM_002586 Pre–B-cell leukemia transcription factor 2	.012
NM_013354 CCR4-NOT transcription complex, subunit 7, isoform 1	.012
NM_002793 Proteasome (prosome, macropain) subunit 7, beta type, 1	.012
NM_001700 Azurocidin 1 (cationic antimicrobial protein 37)	.012
NM_024947 Hypothetical protein FLJ12729	.012
NM_018040 Hypothetical protein FLJ10252	.012
NM_025268 Hypothetical protein MGC4659	.012
NM_017741 Hypothetical protein FLJ20280	.012
NM_024084 Hypothetical protein MGC3196	.012
NM_019039 DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide	.016
NM_003514 H2A histone family, member N	.016
NM_015853 Unknown protein LOC51035	.024
NM_012143 Tuftelin-interacting protein	.024
NM_024666 Hypothetical protein FLJ11506	.024
NM_000137 Fumarylacetoacetase	.024
NM_014763 Mitochondrial ribosomal protein L19	.024
NM_000300 Phospholipase A2, group IIA (platelets, synovial fluid)	.024
NM_014844 KIAA0329 gene product	.024
NM_001666 Rho GTPase activating protein 4	.024
NM_015993 Plasmolipin	.024
NM_015071 GTPase regulator associated with the focal adhesion kinase pp125	.024
NM_016948 Tax interaction protein 40	.024
NM_000102 Cytochrome P450, subfamily XVII polypeptide	.024
NM_004452 Estrogen-related receptor beta	.024
NM_004437 Erythrocyte membrane protein band 4.1	.024
NM_002738 Protein kinase C, beta 1	.024
NM_000766 Cytochrome P450, subfamily IIA (phenobarbital-inducible)	.024
NM_015880 RIG-like 14-1	.024
NM_019093 UDP glycosyltransferase 1 family, polypeptide A3	.024
NM_005673 Solute carrier family 25, member 16	.024
NM_002970 Spermidine/spermine N1-acetyltransferase	.024
NM_000192 T-box 5, isoform 1 NM_080717 T-box 5, isoform 3	.024
NM_004207 Solute carrier family 16 (monocarboxylic acid transporters), member 3	.024
NM_016372 Seven transmembrane domain orphan receptor	.024
NM_021020 Leucine zipper, putative tumor suppressor 1	.024
NM_024320 Hypothetical protein MGC11242	.024
NM_013333 EH domain-binding mitotic phosphoprotein	.024
NM_001805 CCAAT/enhancer binding protein (C/EBP), epsilon	.032
NM_000339 Solute carrier family 12 (sodium/chloride transporters)	.032
NM_003194 TATA box binding protein	.044
NM_000435 Notch homolog 3	.044
NM_003983 Solute carrier family 7	.044
NM_021098 Calcium channel, voltage-dependent, alpha 1H subunit	.044
NM_005849 Immunoglobulin superfamily, member 6	.044
NM_005210 Crystallin, gamma B	.044
NM_000764 Cytochrome P450, subfamily IIA (Phenobarbital-inducible)	.044
NM_014946 Spastin	.044
NM_005377 V-myc myelocytomatosis viral oncogene homolog 2	.044
NM_002572 Platelet-activating factor acetylhydrolase	.044
NM_014458 Kelch motif containing protein	.044
NM_002722 Pancreatic polypeptide	.044
NM_000162 Glucokinase, isoform 1 NM_033507 glucokinase	.044
NM_003742 ATP-binding cassette, sub-family B (MDR/TAP)	.044
NM_006258 Protein kinase, cGMP-dependent, type I	.044
NM_003568 Annexin 31	.044
NM_002105 H2A histone family, member X	.044
NM_003032 Sialyltransferase 1	.044
NM_018254 Hypothetical protein FLJ10876	.044
NM_021213 Phosphatidylcholine transfer protein	.044
NM_018696 elaC homolog 1	.044
NM_024774 Hypothetical protein FLJ21924	.044
NM_024557 Hypothetical protein FLJ11608	.044
NM_024720 Hypothetical protein FLJ23510	.044
NM_002551 Olfactory receptor, family 3, subfamily A, member 2	.044
NM_022911 Solute carrier family 26, member 6, isoform a	.044
NM_019052 HCR (a-helix coiled-coil rod homologue)	.044

*Plus 50 probe sets with unidentified names/function.

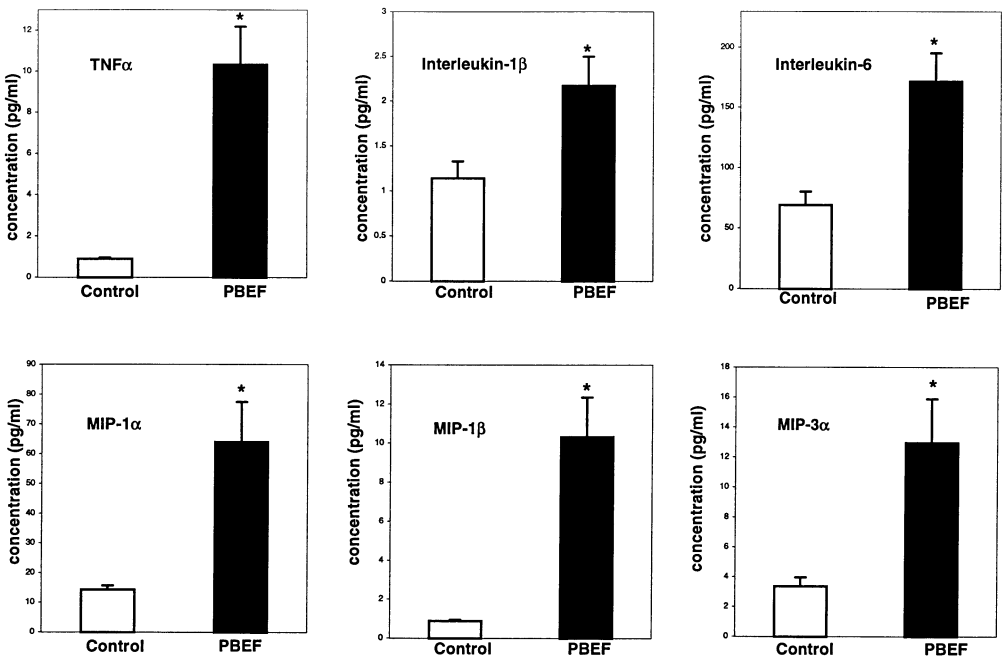

Fig 2. Confirmation of the Affymetrix data was carried out by the measurement of the proteins in the media of nine additional experiments with the use of tissue from different patients, together with the three primary experiments (n = 12). rhPBEF significantly up-regulated TNF-α (*asterisk, P* < .0002), IL-1β (*asterisk, P* < .0001), and IL-6 (*asterisk, P* < .0001). In addition, the macrophage inflammatory proteins were all significantly up-regulated: MIP-1α, MIP-1β, and MIP-3α (all *P* < .0001).

Table III. Genes up-regulated in fetal membranes explants incubated with PBEF

*Probe set identification**	*GenBank accession no.*	*Gene name*	*Signal log ratio change*†	*Fold change*‡	*P value*§
Inflammatory cytokines					
207113	NM_000594	Tumor necrosis factor (TNT superfamily)	1.3 ± 0.3	2.4 ± 0.5	<.004
39402	M15330	Interleukin 1-beta mRNA	0.5 ± 0.3	1.5 ± 0.4	<.024
205207	NM_000600	Interleukin 6 (interferon, beta 2)	0.9 ± 0.1	2.0 ± 0.1	<.004
Chemokines					
205114	NM_002983	Small inducible cytokine A3 (homologous to mouse MIP-1α)	0.9 ± 0.2	2.0 ± 0.3	<.004
204103	NM_002984	Small inducible cytokine A4 (homologous to mouse MIP-1β)	1.1 ± 0.6	2.4 ± 0.9	<.004
205476	NM_004591	Small inducible cytokine subfamily A, MIP-3α	1.1 ± 0.4	2.3 ± 0.7	<.044
207850	NM_002090	Growth-related oncogene gamma GROγ	1.1 ± 0.5	2.3 ± 0.8	<.004
Prostaglandin synthase					
204748	NM_000963	Prostaglandin-endoperoxide synthase 2, (COX2)	1.0 ± 0.3	2.1 ± 0.4	<.004

*An identifier number from the Affymetrix U133A microarray.
†Derived from quantitative algorithms that are based on the mean difference in probe pair hybridization intensities (PBEF versus control)
‡Calculated from signal log ratios: Fold change = $2^{\text{Signal log ratio}}$.
§Based on confidence algorithms that use rank-based (non-parametric) methods.

experimental pair (PBEF-treated and control), but only 8 genes were up-regulated consistently (increased = I) in all experimental pairs (Table III)., There were no genes that were down-regulated (D) in all pairs. These eight up-regulated genes included three key inflammatory cytokines that were involved in infection-driven labor induction: TNF-α (*P* < .004), IL-6 (*P* < .04), and IL-1β (*P* < .024). The PBEF treatment also caused significant

increases in four important chemokines: MIP-1α ($P < .004$), MIP-1β ($P < .004$), MIP-3α ($P < .044$), and GRO-γ ($P < .004$). In addition, PGHS-2 was also up-regulated significantly ($P < .004$; Table III).

ELISA and real-time RT-PCR. Confirmation of these results was carried out with the use of media or tissue from the three primary experiments and nine additional experiments on similar tissues from nine different patients by the measurement of the proteins in the media (n = 12) or by real-time quantitative RT-PCR, if an ELISA was inappropriate or unavailable. The results from the ELISA for TNF-α ($P < .0002$), IL-1β ($P < .0001$) and IL-6 ($P < .0001$) showed that all these proteins were up-regulated highly significantly after the PBEF treatment (Fig 2). In addition, the macrophage inflammatory proteins were also up-regulated significantly: MIP-lα ($P < .0001$), MIP-1 β ($P < .0001$), and MIP-3α ($P < .0001$; Fig 2). The real-time quantitative RT-PCR confirmed the up-regulation of the genes for PGHS-2 ($P < .0004$) and GRO-γ ($P < .024$) in all experiments (Fig 3).

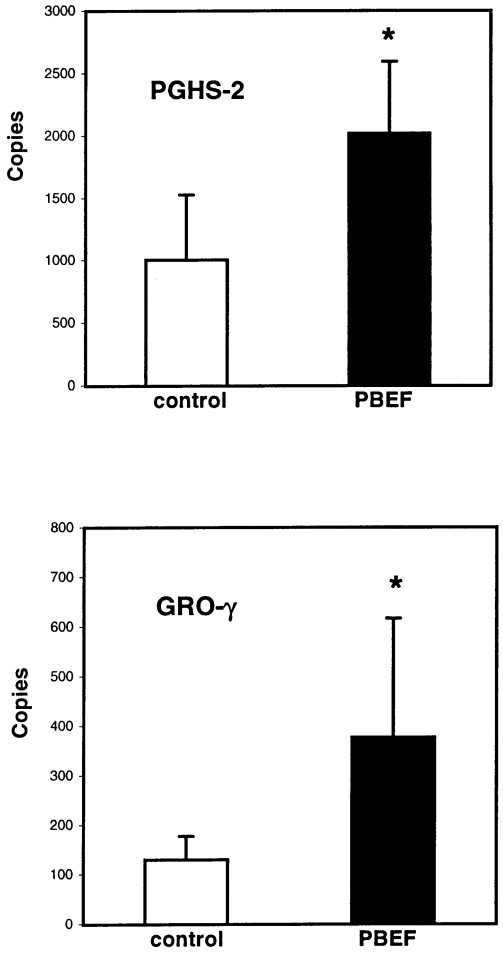

Fig 3. Confirmation by quantitative real-time RT-PCR with the use of tissues from the nine additional experiments (different patients) and from the three primary experiments (n = 12) for the expression of PGHS-2 (*asterisk*, $P < .0004$) and GRO-γ (*asterisk*, $P < .024$) in the fetal membranes after rhPBEF treatment. Quantitation of the data was normalized to ribosomal 18S standard.

Comment

These data show that the treatment of term nonlabored fetal membranes with rhPBEF induced both the expression of the genes and the translation of the proteins for some of the potentially important regulators of labor. PBEF is a product of the human fetal membranes in normal pregnancy and is up-regulated by both sterile distension and intrauterine infection.[7,10] The present study was designed to elucidate some of its actions in the fetal membranes with the in vitro addition of rhPBEF to nonlabored, noninfected explants and to seek its effects on some 18,000 genes on the human Affymetrix gene chip. Our selection of a 4-hour treatment probably biased the results towards the identification of cytokines. A time-course experiment would have been ideal, but costly. The data that were obtained suggest that PBEF may play a key role in the early events of both normal and pathologic infection-driven preterm labor. The data also support the idea that normal parturition is an inflammatory response that may involve the same cytokine and hormone players as infection-induced parturition, albeit at a much lower level of expression. Thus, PBEF may be a critical link between a sterile distension pathway and the infection-induced pathway to labor. However, because of the redundancy in the cytokine system, we do not know if PBEF were knocked-out, whether this would have any effect. Such a knock-out mouse has not yet been produced, but an antisense system to specifically block PBEF translation has been developed and will be used to try to answer this question.

We were very careful to eliminate tissues with any signs of intrauterine infection; likewise, we avoided tissues from patients who had started labor, because both were known to affect the endogenous expression of PBEF and therefore could blunt its response. We also designed the experiment to assess the reproducibility of the Affymetrix gene chip system and found this to be excellent. As in all studies that use macroarrays or microarrays, the results must be confirmed, preferably by different methods. In this case, further experiments were conducted, and the medium used for the quantitation of the respective proteins that were derived from the genes was shown to be up-regulated. However, this was inappropriate for the membrane-associated prostaglandin synthase enzyme (PGHS-2). There was no commercially available ELISA for the measurement of GRO-γ; therefore, to be consistent, quantitative RT-PCR was used for these two genes to confirm the microarray results.

The increased expression of TNF-α, IL-1, and IL-6 by PBEF potentially place this novel cytokine at the proximal end of the cytokine cascade.[12] The increased expression of TNF-α, IL-1, and IL-6 in the onset of normal labor or as

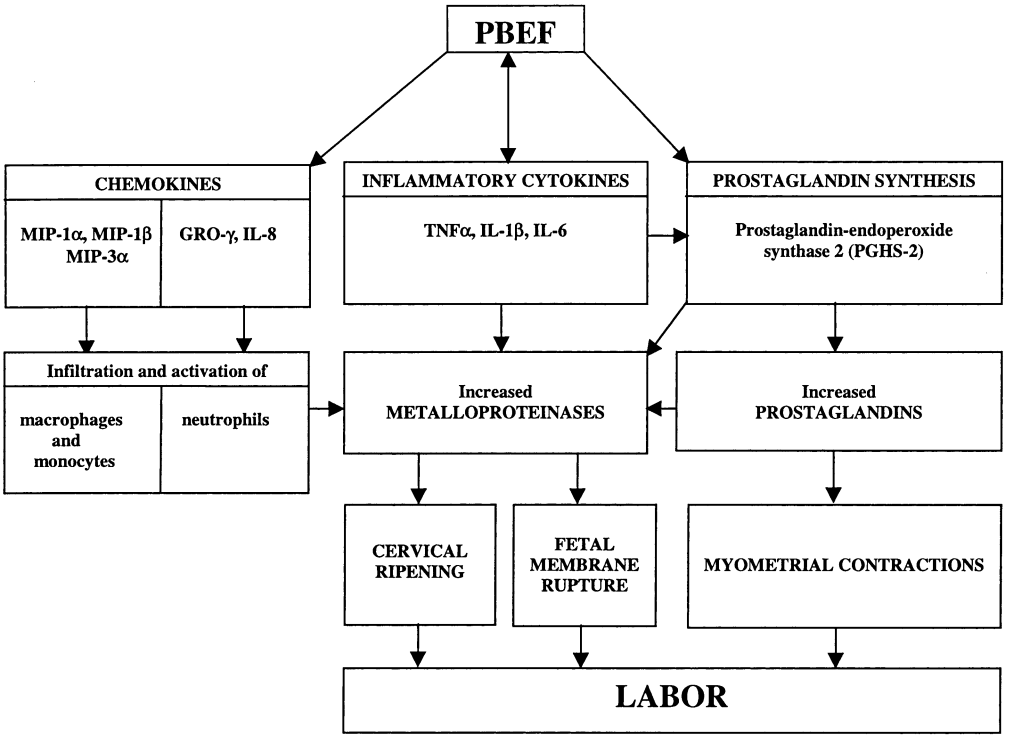

Fig 4. Diagrammatic summary of the possible relationships between PBEF, the inflammatory cytokines, the chemokines, and PGHS-2 that was derived from the Affymetrix microarray data. This shows how PBEF, by stimulating the expression of the cytokines and PGHS-2, may drive the myometrial contraction, cervical ripening, and fetal membrane rupture that leads to labor induction.

mediators of labor has been shown,[13] although their roles in infection-induced labor are well recognized.[14] Their expressions are all increased in both normal term and preterm labor,[13,14] and they induce the expression of the MMPs.[3] The increased expression of the chemokines MIP-1α, MIP-1β, and MIP-3α, a subclass of cytokines that attract and activate monocytes and macrophages, is also important. MIP-lα has been shown to play a role in the physiologic condition of both normal labor and in the pathogenesis of infection-associated preterm labor.[15,16] A further subgroup of chemokines that are stimulated by PBEF, included GRO-γ and IL-8, which both attract and activate neutrophils,[11,14,17] an activity of particular relevance to infection-induced labor. The action of PBEF to cause the up-regulation of these cytokines therefore would initiate an influx of neutrophils and macrophages into the tissues and their activation and the activation of the resident immune cells of the decidua. These interactions are shown in Fig 4. The up-regulation of the IL-8 gene was not 1 of the 8 genes that were up-regulated by PBEF in the tissues of all three patients that were used here. It was up-regulated in two of the tissues that were used; this response of IL-8 is clearly more variable than that of the other cytokines. The prostaglandins (E_2 and $F_{2\alpha}$) are produced in the gestational tissues and have been shown to be of central importance to the initiation and maintenance of labor in humans.[18] It is possible that PBEF is involved in the modulation of the prostaglandins, either before or during labor. As shown in Fig 4, the increase in PGHS-2 would increase the synthesis of the prostaglandins, presumably from both the amnion and decidua, because PBEF and the prostaglandins are products of these cells on either side of the cytotrophoblast .[9,19] This, in turn, would increase the production of the matrix metalloproteinases[3]; in the 4 hours of PBEF treatment, we showed that the genes for two key metalloproteinase genes, MMP-1 and MMP-3 (collagenase-1 and stromelysin-1) were significantly elevated. These enzymes are central to the degradation of the collagen types I and III, which are present in the connective tissues of the amniotic membrane[2] and the cervix[20] (Fig 4).

Finally, we showed that the treatment of the fetal membrane explants with rhPBEF statistically increased the expression of the PBEF gene itself in two of the three pairs of tissues, which shows the PBEF response to be as variable as that of the IL-8 response. Further work must be done to study the linkage between PBEF and IL-8 and their variability in expression.

In summary, this study shows that PBEF may be an important part of the normal pathway to labor induction in the human, which may involve a distension-responsive component.[7] In addition, the infection-induced expres-

sion of PBEF likewise would cause a similar, but more flamboyant secretion of this cytokine, which would initiate the events that lead to labor: cervical ripening, fetal membrane rupture, and myometrial contractions.

We thank the staff and nurses at Kapiolani Medical Center for their help with the tissue collections; Dr J. Killeen for the histologic examination of each tissue that was used in this study, and Drs Gabor Mocz and Paul Armstrong at the University of Hawaii Biotech and Molecular Biology Facility for processing the Affymetrix gene chips.

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