

Molecular Cloning and Characterization of Canine Pre-B-Cell Colony-Enhancing Factor

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During our previous attempt to search for the candidate genes to acute lung injury (ALI), we unexpectedly identified PBEF as the most highly upregulated gene in a canine model of ALI by crosshybridizing canine lung cRNA to the Affymetrix human gene chip HG-U133A. The result suggested that PBEF may be a potential biomarker in ALI. To extend and translate that finding, we have performed the molecular cloning and characterization of canine PBEF cDNA in this study. Deduced amino acid sequence alignment revealed that the PBEF gene is evolutionarily highly conserved, with the canine PBEF protein sequence 96% identical to human PBEF and 94% identical to both murine and rat PBEF counterparts. Canine PBEF protein was successfully expressed both by in vitro transcription coupled with translation in a cell-free system and by transfection of canine PBEF cDNA into the human lung type II alveolar adenocarcinoma cell line A549. The expressed canine PBEF protein was visualized by either an anti-V5 tag peptide polyclonal antibody or an anti-canine PBEF peptide polyclonal antibody. RT-PCR assay indicates that canine PBEF is expressed in canine lung, brain, heart, liver, spleen, kidney, pancreas, and muscle, with liver showing the highest expression, followed by muscle. Isolation of the canine PBEF cDNA and expression of its recombinant protein may provide molecular tools to study the molecular

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mechanism of ALI in the canine model and to elucidate the potential role of PBEF as an ALI biomarker.

KEY WORDS: PBEF; cloning; canine; cDNA; gene expression.

Abbreviations: PBEF, pre-B-cell colony enhancing factor; ALI, acute lung injury; cDNA, complementary DNA; RT-PCR, reverse transcriptase–polymerase chain reaction.

INTRODUCTION

Acute lung injury (ALI) and its more severe form, acute respiratory distress syndrome (ARDS), represent important clinical diseases with mortality rates of 40–50% (Ware and Matthay, 2000; Goss *et al.*, 2003). The molecular pathogenesis of ALI is still incompletely understood. During our attempt to search for candidate genes of ALI in an animal model, we performed an extensive microarray expression profiling in a canine model (Marcucci *et al.*, 2001) of human ALI and unexpectedly found that the pre-B-cell colony enhancing factor (PBEF) gene was the most highly upregulated gene. That result suggested that PBEF may be a potential biomarker in ALI. To our knowledge, it represented the first observation that the PBEF gene is significantly upregulated in a lung injury model (Ye *et al.*, 2005).

PBEF is a novel cytokine first identified to be involved in the maturation of B-cell precursors (Samal *et al.*, 1994). Subsequently, it was found that expression of PBEF was upregulated by both mechanical force and inflammatory stimuli. Acute distention by mechanical force of WISH cells, a human amniotic epithelial cell line, and human fetal membrane induced significant upregulation of PBEF in both WISH cells and human fetal membranes (Nemeth *et al.*, 2000a,b). Several inflammatory stimuli, including lipopolysaccharide, interleukin (IL)-1 β , tumor necrosis factor (TNF) α and IL-6, all significantly increased the expression of PBEF in WISH cells, with dexamethasone significantly reducing the response of PBEF to these cytokines (Ognjanovic *et al.*, 2001). Recent evidence indicates that treatment of fetal membrane explants with recombinant human PBEF results in the significant upregulation of several key inflammatory cytokines: TNF α , IL-6, (IL)-1 β , macrophage inflammatory protein-1 α , macrophage inflammatory protein-1 β , macrophage inflammatory protein-3 α (Ognjanovic *et al.*, 2003). Such results suggest that PBEF may be a sensor and/or an amplifier of both mechanical forces and inflammatory stimuli, which are two key factors implicated in the pathogenesis of ALI (Brower, 2002; Groeneveld, 2002). In the canine model of ALI induced by both high tidal volume ventilation and injury from saline lavage (Ye *et al.*, 2005), upregulated PBEF expression may reflect the temporal and spatial response of the lung to mechanical forces and inflammatory stimuli. Since the dog

offers the advantage of larger physical size compared with murine and rat, the canine model is more suitable for our investigation of the lung-segment-specific responses to mechanical and biological stresses, which may be important to our understanding of ALI pathogenesis.

In this study, we report the molecular cloning and characterization of canine PBEF cDNA. Our objective for the isolation of the canine PBEF cDNA and expression of its recombinant protein is to provide molecular tools to study the molecular mechanism of ALI in the canine model and to elucidate the potential role of PBEF as an ALI biomarker.

MATERIALS AND METHODS

Canine Tissue Total RNA Isolation

The protocol to obtain brain, heart, liver, spleen, kidney, pancreas, muscle, and lung tissues of normal male mongrel dogs (20–25 kg) was approved by the Animal Care and Use Committee of the Johns Hopkins University School of Medicine. The canine model of acute lung injury was produced by a unilateral lung injury and mechanical ventilation to allow the comparison of control and injured tissue samples from the same animal, as described previously (Marcucci *et al.*, 2001). The dissected canine tissues were immediately submerged in a RNA stabilization solution, RNA Later (cat. no. 7022, Ambion, Inc., Austin, TX), and stored at -20°C until use. Total RNA from each tissue was extracted using the TRI_{ZOL} reagent (Invitrogen), followed by cleanup with an RNeasy kit (Qiagen).

Cloning Canine PBEF cDNA

Total RNA from the canine lung tissue was isolated as described above. First-strand cDNA was synthesized from 1 μg total canine lung RNA at 42°C for 50 min using random hexamers as primers and SuperScript II reverse transcriptase (Invitrogen).

One-tenth of the first-strand cDNA product was used in the PCR reaction. The primers were designed based on the human PBEF cDNA reference sequence (GenBank accession number NM_005746): PBEFC-5: 5'-GCCACCATGAATCCTGCGGCAGAAGCCGA-3', PBEFC-3: 5'-CAAATGATGTGCTGCTTCCAGTTCAATAT-3'. The underlined hexamer in the primer PBEFC-5 was added to create a Kozak consensus sequence. In the primer PBEFC-3, a UAG stop codon was mutated into a UUG codon encoding leucine, thus cloning canine PBEF cDNA in frame with the V5 epitope and polyhistidine tag (C-terminal peptide) in the pcDNA3.1/V5-His-TOPO expression vector (Invitrogen) in order to detect or purify the canine PBEF protein. Platinum high-fidelity DNA polymerase (Invitrogen) was used for the PCR amplifications with the following cycling parameters: 30 cycles of 94°C for 30 s, 55°C for 45 s, and 72°C for 2 min with a final primer extension at 72°C for 5 min.

Since only one band with the predicted size can be seen on agarose electrophoresis, the PCR product was directly subcloned into the pcDNA3.1/V5-His-TOPO vector (Invitrogen) according to the supplier's instruction.

Sequencing and Analyzing Canine PBEF cDNA

Canine PBEF cDNAs of three clones were independently sequenced using the fluorescent dideoxy terminator method of cycle sequencing on an Applied Biosystems Division 3700 DNA Analyzer (DNA Analysis Facility, Johns Hopkins University School of Medicine). Sequencing primers were synthesized on a Perkin-Elmer Applied Biosystems Division 3948 DNA Synthesizer. All consensus sequences were generated using the Sequencher Software from Gene Codes. Alignments of canine PBEF cDNA and predicted amino acid sequences to its human, murine, and rat counterparts were done using the GCG package software, SeqWeb (Ver. 2) and the T-COFFEE program (Notredame *et al.*, 2000).

Transcription/Translation of Canine PBEF cDNA In Vitro

Canine PBEF cDNA in the pcDNA3.1/V5-His-TOPO vector was expressed in the TnT T7 Quick Coupled Transcription/Translation System (Promega) in vitro according to the supplier's instruction. Briefly, 1 μ g canine PBEF cDNA-pcDNA3.1/V5-His-TOPO recombinant circular double-strand DNA, which contains a T7 promoter, was added to an aliquot of the TnT Master Mix and incubated in a 50 μ L reaction volume for 90 min at 30°C. The synthesized canine PBEF protein was then separated by SDS-polyacrylamide gel electrophoresis and detected by either an anti-V5 tag peptide polyclonal antibody or an anti-human PBEF peptide polyclonal antibody raised in rabbit against the human PBEF peptide (N412/S431), which shares 100% identity with the canine PBEF amino acid sequence. Canine PBEF cDNA in reverse orientation in the pcDNA3.1/V5-His-TOPO vector was included as control. Alternatively, canine PBEF was labeled with the biotinylated lysine using the Transcend Biotinylated tRNA (Promega) and detected by the streptoavidin (SA) coupled enzymatic reaction.

Expressing Canine PBEF Protein in Human Lung Epithelial Cells

A549 cells (passage 75; cat. no. CCL-185, ATCC, Manassas, VA), a pulmonary type-II epithelial cell line derived from an individual with alveolar cell carcinoma, were cultured in Ham's F-12 medium supplemented with 10% bovine fetal serum (Sigma, St. Louis, MO), penicillin (50 units/mL; GIBCO, Grand Island, NY), streptomycin (50 μ g/mL; GIBCO), and fungizone (2 μ g/mL; GIBCO). For the transfection experiment, A549 cells were plated on glass coverslips to grow to 50–60% confluence and transfected with the recombinant plasmid canine PBEF

cDNA-pcDNA3.1/V5-His-TOPO vector using fugen 6 as a transfection reagent. Briefly, cells were incubated with 1 μg plasmid DNA and 3 μL of fugen 6 in 1 mL of OPTI-MEM for 4 h. The solution was then replaced by 1 mL of complete culture medium and the whole incubated for 48 h. The canine PBEF expression in the transfected A549 cells was visualized by immunofluorescent microscopy as described below.

The transfected A549 cells on glass coverslips were fixed in 3.7% formaldehyde solution in PBS for 10 min at 4°C, washed three times with PBS, permeabilized with 0.2% Triton X-100 in PBS-Tween (PBST) for 5 min at 4°C, and blocked with 2% BSA in PBST for 20 min. Incubation with rabbit anti-V5 antibody, diluted 1:100 with blocking solution, was performed for 1 h at room temperature. After three washes with PBS, cells were incubated with anti-rabbit secondary antibody conjugated with the fluorescent dye Alexa⁴⁸⁸ (Molecular Probes) for 1 h at room temperature. Actin filaments were visualized by staining cells with Texas red phalloidin (Molecular Probes) for 1 h at room temperature. The coverslips were mounted and analyzed with a Nikon video-imaging system consisting of a phase-contrast inverted microscope equipped with objectives and filters for immunofluorescence and connected to a digital camera and image processor. The images were recorded and saved on a Pentium II personal computer as TIFF files compatible with Adobe Photoshop 5.0 and National Institutes of Health image analysis programs.

Preparation of Rabbit Anti-Human PBEF Peptide Polyclonal Antibody

The synthesis of the canine PBEF peptide N412/S431 (CNVFKDPVADPNKR SKKGRLS) and the preparation of a rabbit anti-canine PBEF peptide N412/S431 polyclonal antibody were carried out by Bethyl Laboratories, Inc. (Montgomery, TX). Briefly, a cysteine residue was added to the N-terminus of this peptide during the peptide synthesis. The peptide was purified by HPLC, verified by mass spectrometry, and stored as a lyophilized material. The peptide was then conjugated via maleimide chemistry to link its sulfhydryl group to the carrier keyhole limpet hemocyanin (KLH). This conjugated prep was injected into the rabbit as an immunogen. The produced antibody was affinity-purified against the peptide N412/S431 coupled to agarose using a cKhorramogen bromide method. The titer of the affinity-purified antibody was determined by ELISA.

Western blotting was used to examine the specificity of the prepared anti-canine PBEF antibody in human pulmonary artery endothelial cell (HPAEC). Primary cultures of HPAEC were obtained from Clonetics (California). HPAEC cells were maintained in endothelial cell growth medium: a modified MDCB 131 formulation supplemented with 5% heat-inactivated fetal calf serum (FCS), 10 ng/mL human recombinant epidermal growth factor, 1 $\mu\text{g}/\text{mL}$ hydrocortisone, 50 ng/mL amphotericin-B, 50 $\mu\text{g}/\text{mL}$ gentamycin and bovine brain extract

containing 12 $\mu\text{g}/\text{mL}$ protein and 10 $\mu\text{g}/\text{mL}$ heparin. Confluent EC monolayers were harvested in lysis buffer containing 50 mM Tris (pH 7.5), 1% Triton, 5 mM EGTA, 150 mM NaCl, and protease/phosphatase inhibitors and incubated on ice for 20 min. Then the crude cell lysates were centrifuged in a microcentrifuge at 10,000 g for 15 min and the cell supernatants subjected to immunoblot analysis. Total protein (equivalent to 30 μg for each sample) was loaded and resolved by 7.5% SDS-PAGE and then electroblotted to nitrocellulose (0.2- μm pore size) membrane (Schleicher and Schuell, Dassel, Germany). Rainbow prestained molecular weight markers (Amersham Biosciences UK, Ltd.) were run concurrently. For signal generation the membrane was incubated at room temperature first for 2 h with 5% nonfat dried milk in Tris-buffered saline with 0.1% Tween 20 (TBST, 0.1 M, pH 7.4) and then for 16 h at 4°C with the rabbit anti-human PBEF peptide N412/S431 polyclonal antibody diluted 1:5000 in TBST. Then the membrane was washed three times in TBST and further incubated for 1 h at room temperature with HRP-conjugated anti-rabbit IgG antibody (Dako, Cambridge, UK) diluted 1:5000 in TBST. The membrane was finally washed six times with TBST and the chemiluminescence signal generated (Super Signal Ultra, Pierce, Chester, UK) and recorded onto Hyperfilm ECL (Amersham Biosciences UK, Ltd.). Image acquisition and band quantitation were undertaken on GS-700 densitometry with Molecular Analyst software (Bio-Rad).

Tissue Distribution of Canine PBEF mRNA

A semi-quantitative duplex RT-PCR was carried out to examine the tissue distribution of canine PBEF mRNA. Reverse transcription was performed at 42°C for 50 min in a final volume of 20 μL , containing 0.5 μg total RNA, 11 pmol random hexamer, 500 μM each dNTP, 20 U RNasin ribonuclease inhibitor (cat. no. N2111, Promega, Madison, WI), 200 U Superscript II RNase H reverse transcriptase (cat. no. 18064-014, Life Technol., Inc., Rockville, MD) and 4 μL five \times first-strand buffer, which came along with the reverse transcriptase. The primers for canine PBEF and human ribosomal protein S18 (RPS18) genes were designed using Primer 3 software (Primer 3.cgi, V0.2b, Whitehead Institute/MIT Center for Genome Research) based on our isolated canine PBEF sequence and the published GenBank mRNA sequence X69150, respectively. The primer sequences for the canine PBEF were as follows: PBEF-5: 5'-AAGCTTTTTAGGGCCCTTTG-3', and PBEF-3: 5'-AGGCCATGTTTTATTGCTGA-3'. The PCR product size was 319 bp. The primer sequences for the human RPS18 were: RPS18-5: 5'-GCAGACATTGACCTACCAA-3', and RPS18-3: 5'-GGACCTGGCTGTATTTTCCA-3'; PCR product size 154 bp (GenBank accession number X69150). The RPS18 gene was used as a housekeeping gene control. The primers were synthesized on a Perkin-Elmer Applied Biosystems Division (PE/ABd) 3948 DNA Synthesizer in the DNA Analysis Facility of Johns Hopkins

University School of Medicine. A semi-quantitative duplex PCR was carried out in 50 μL final volume containing 1 μL cDNA (one-twentieth of RT products), 0.5 μM of each primer, 0.2 mM of each dATP, dTTP, dCTP, and dGTP, 50 mM KCl, 20 mM Tris-HCl, pH 8.0, 1.5 mM MgCl_2 , 2.5 U of Taq DNA polymerase (cat. no. N808-0160, Perkin-Elmer, Foster City, CA). Thermal cycling was done in a Perkin-Elmer GeneAmp PCR System 9600 Thermal Cycler with an initial 2 min denaturation at 94°C followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 1 min, and a final extension of 5 min at 72°C.

One-fifth of the PCR product was separated by 2% agarose gel electrophoresis for 1.5–2 h at constant voltage of 110 V. The gels were stained by 0.5 $\mu\text{g}/\text{mL}$ of ethidium bromide. The image was captured on thermal paper using the Eagle Eye II Still Video System (Stratagene, La Jolla, CA). The gel images on the thermal paper were scanned and quantitatively analyzed using densitometry software. Statistical analysis of the PBEF mRNA level was performed using the SigmaStat program (Ver. 3.0, SPSS, Chicago, IL).

RESULTS

Deduced Canine PBEF Protein Amino Acid Sequence and Its Alignment to Human, Murine, and Rat Counterparts

Since the crosshybridization of canine lung RNA to the Affymetrix oligonucleotide-based human GeneChip yielded the present call on the PBEF gene and suggested that the PBEF gene is highly conserved, we designed a pair of primers based on the human PBEF cDNA sequence to amplify the canine PBEF cDNA from the canine lung tissue total RNA. The canine PBEF cDNA was successfully amplified, and its coding sequence consists of 1476 bp nucleotides. The open reading frame of canine PBEF encodes polypeptides of 491 amino acids (Fig. 1). The canine PBEF protein is 96% identical to human PBEF protein and 94% identical to both murine and rat PBEF proteins. There were 12 conserved amino acid changes and 5 nonconserved amino acid changes. Although the conserved amino acid changes nearly spread throughout the whole sequence, three out of five nonconserved amino acid changes are located toward the C-terminal region. In the canine PBEF protein, there are 2 predicted *O*-glycosylation sites (S270 and T304), 1 *N*-glycosylation site (N29), 14 serine phosphorylation sites (S17, 35, 47, 155, 165, 199, 200, 275, 314, 374, 425, 431, 470, 472), 3 threonine phosphorylation sites (T30, 233, 304), and 7 tyrosine phosphorylation sites (Y18, 23, 54, 123, 240, 281, 403). All these modification sites are identical among canine, human, murine, and rat PBEF except that in the canine PBEF, there are mutated serine 303, threonine 220, and tyrosine 87 phosphorylation sites and an added tyrosine 123 phosphorylation site. The functional implications of those

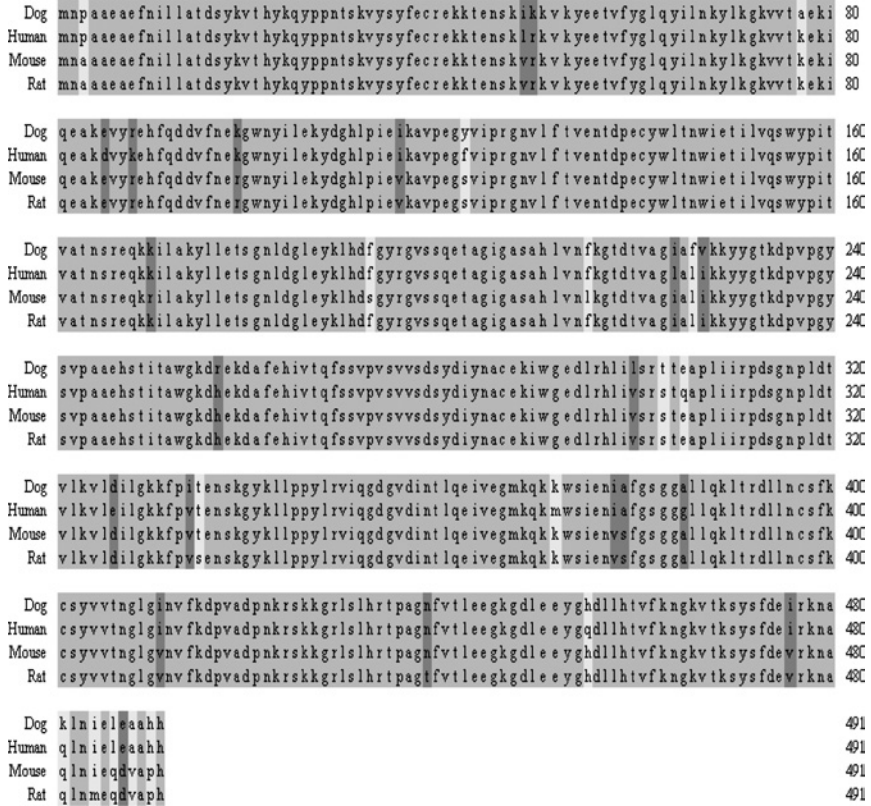


Fig. 1. Alignment of the deduced canine PBEF amino acid sequence with homologues in human, murine, and rat. Light blue-shaded regions indicate identical amino acid(s) across all species. Light red-shaded regions indicate functionally conserved similar amino acids. Light yellow-shaded regions indicate neither identical nor similar amino acids. Species, sequence ID/% identity compared to canine, and reference are as follows: human, NM_005746.1/96% (Samal *et al.*, 1994); mouse, NM_021524.1/94% (Rongvaux *et al.*, 2002); rat, NM_177928.2/94% (Kitani *et al.*, 2003). See the color version of this figure on the Biochemical Genetics Web site at www.kluweronline.com.

amino acid changes in canine PBEF protein remain to be investigated. Overall, this result indicates that the PBEF sequence is highly conserved.

Expression of Recombinant Canine PBEF Protein In Vitro and in Human A549 Cell Line

Although there was the predicted open reading frame of canine PBEF cDNA from the determined DNA sequence after analyzing three different sequenced

clones, we used the TnT T7 Quick Coupled Transcription/Translation System (Promega) *in vitro* to examine whether the cloned canine cDNA indeed express the PBEF protein. The *in vitro* coupled transcription/translation reactions were carried out either in the absence or presence of biotinylated lysine. As expected, both methods of detection showed that the cloned canine PBEF cDNA (cPBEF) expressed a protein of 52 Kd (Fig. 2), the size of the PBEF protein, whereas canine PBEF cDNA in reverse orientation (rPBEF, 3'–5') in the pcDNA3.1/V5-His-TOPO vector failed to express. A positive control, human PBEF cDNA (hPBEF), also expressed a human PBEF protein of equivalent size. This detection was performed using either anti-V5 antibody, since the canine PBEF cDNA was cloned in frame with a V5 epitope (top panel, Fig. 2), or a streptavidin (SA) coupled enzymatic reaction (lower panel, Fig. 2) when the synthesis was carried out in the presence of biotinylated lysine. These results indicate that the cloned canine PBEF cDNA had an open reading frame and expressed canine PBEF protein *in vitro*.

We next examined expression of the cloned canine PBEF cDNA in eukaryotic cells. A representative immunostaining image in Fig. 3 (V5 antibody staining)

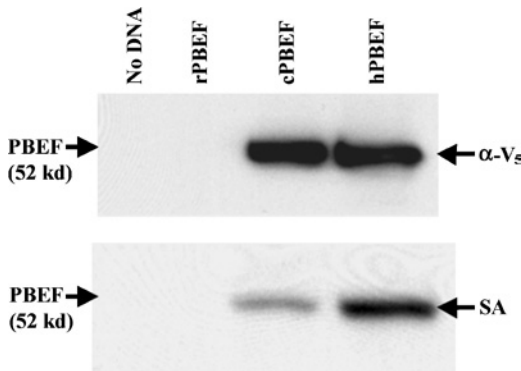


Fig. 2. Transcription/translation of canine and human PBEF *in vitro*. Canine PBEF cDNA in the pcDNA3.1/V5-His-TOPO vector was expressed in the TnT T7 Quick Coupled Transcription/Translation System (Promega) either in the absence or in the presence of biotinylated lysine. *In vitro* synthesized PBEF proteins were separated on SDS-PAGE, electroblotted on a nitrocellulose membrane, and visualized either with the anti-V5 antibody (top panel) or with the streptavidin (SA) horseradish peroxidase (lower panel) detection system. A control lane without DNA added shows no PBEF protein synthesized; in the rPBEF lane, the canine PBEF cDNA in 3'–5' reverse orientation did not demonstrate canine PBEF protein synthesized; the canine PBEF (cPBEF) lane shows the canine PBEF protein synthesized; and the human PBEF (hPBEF) lane shows the human PBEF protein synthesized.

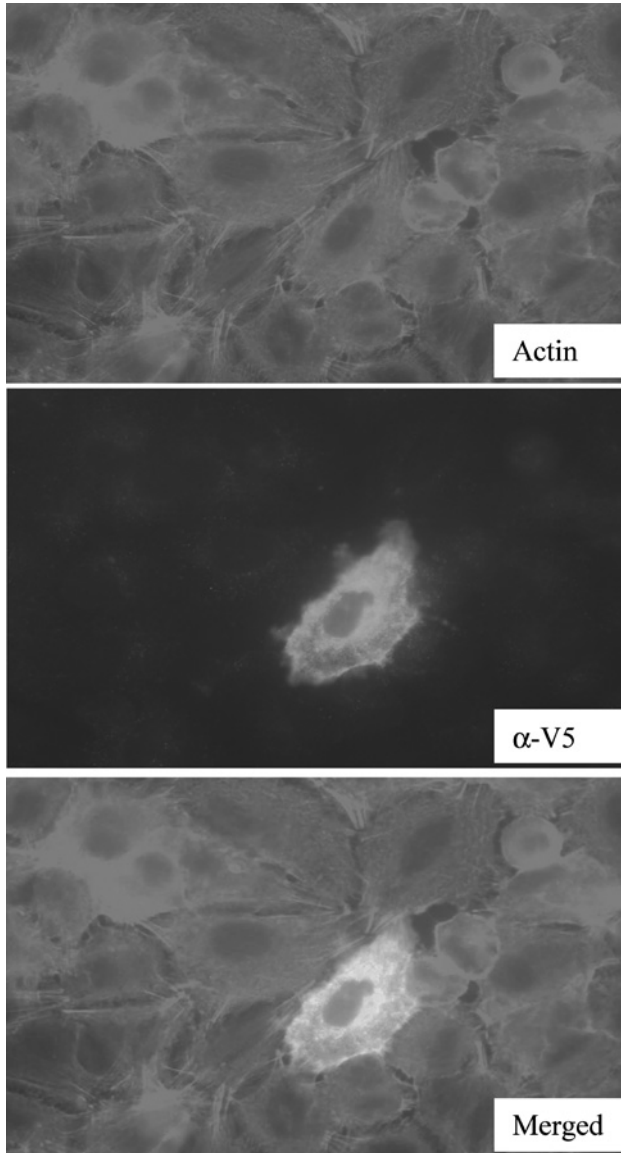


Fig. 3. Expression of the canine PBEF in human A549 cell line. Canine PBEF cDNA transfected A549 cells grown on glass coverslips were fixed and stained for F-actin with Texas red phalloidin (top panel) and for the anti-V5 epitope antibody (middle panel). Panel 3 is the merged photo of top panel and middle panel. See the color version of this figure on the Biochemical Genetics Web site at www.kluweronline.com.

displayed that transiently transfected A549 cells indeed expressed canine PBEF, although the transfection efficiency was low. V5-positive cells were not identified after transfection with canine PBEF cDNA in reverse orientation (3′–5′) in the pcDNA3.1/V5-His-TOPO vector (data not shown).

Rabbit Anti-Canine PBEF Antibody Is Specific to the PBEF in Human Pulmonary Artery Endothelial Cell Lysates

To examine further the PBEF gene's function at the protein level, we prepared and determined the specificity of a rabbit anti-canine PBEF peptide N412/S431 polyclonal antibody. This antibody was crossreactive to human PBEF since the canine PBEF peptide N412/S431, against which the anti-canine PBEF polyclonal antibody was raised in rabbits, shares 100% identity to the deduced human PBEF amino acid sequence. As expected, the antibody is crossreactive against a single band in three different human pulmonary artery endothelial cell lysates (Fig. 4, lanes 1–3) by Western blotting, which displayed the predicted size of the PBEF protein (52 Kd). The preimmune sera failed to show any specific reactivity to PBEF (Fig. 4, lanes 4–6). When preincubated with an 800-molar-fold excess of immunogen peptide N412/S431, the antibody's reaction to the PBEF protein was abolished (Fig. 4, lanes 7–9). These results support the contention that the rabbit anti-human PBEF antibody is specific to PBEF protein.

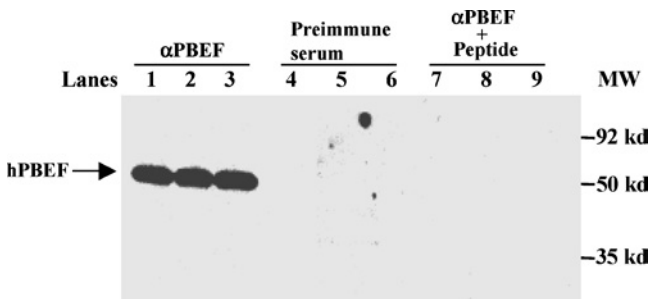


Fig. 4. Rabbit anti-canine PBEF antibody is specific to PBEF in human pulmonary artery endothelial cell lysate. Twenty micrograms of protein isolated from human HPAEC lysates was applied to each lane (lanes 1–9), then run on 10% SDS-PAGE and transferred to a nylon membrane. The membrane was blotted with rabbit anti-canine PBEF peptide N412/S431 (1:5000 dilution, 0.5 μ g protein, lanes 1–3) polyclonal antibody, with preimmune serum (1:5000 dilution, 225 μ g protein, lanes 4–6), and with rabbit anti-canine PBEF peptide N412/S431 antibody preblocked by PBEF peptide N412/S431 (800-fold excess, 50 μ g, lanes 7–9). Signals detected by ECL plus Western blotting detection reagents.

Tissue Distribution of Canine PBEF mRNA

The distribution of canine PBEF mRNA expression in canine tissues was analyzed by a semi-quantitative duplex RT-PCR (Fig. 5). This revealed that PBEF mRNA was expressed in all tissues we examined: brain, heart, liver, spleen, kidney, pancreas, muscle, and lung. If the level of lung PBEF mRNA is set at 1, the relative levels in other tissues, given as mean \pm SD, are 1.09 ± 0.25 (brain), 1.03 ± 0.08 (heart), 4.86 ± 0.10 (liver), 0.98 ± 0.06 (spleen), 0.99 ± 0.16 (kidney), 0.80 ± 0.01 (pancreas), and 3.06 ± 1.27 (muscle). Liver showed the highest expression, followed by muscle. The rest of the tissues showed comparable expressions.

DISCUSSION

In this report we present the predicted amino acid sequence, expression, characterization, and tissue distribution of the canine PBEF gene. Amino acid sequence alignment revealed that the canine PBEF protein sequence is 96% identical to

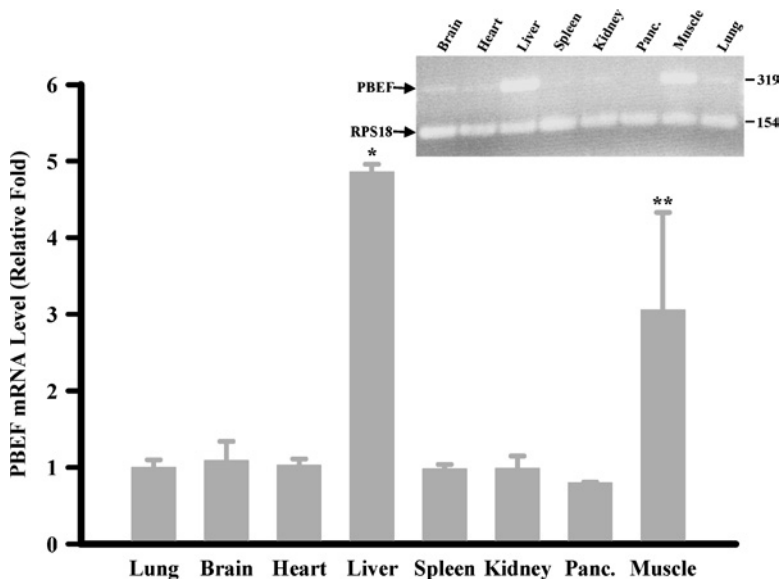


Fig. 5. Tissue distribution of canine PBEF mRNA. Total RNAs ($1 \mu\text{g}$) from canine brain, heart, liver, spleen, kidney, pancreas, muscle, and lung were reverse transcribed. One-tenth ($2 \mu\text{L}$) of the first-strand cDNA product ($20 \mu\text{L}$) was subjected to a semi-quantitative duplex PCR amplification. One-fifth ($10 \mu\text{L}$) of the PCR product ($50 \mu\text{L}$) was separated by 2% agarose gel electrophoresis and visualized by $0.5 \mu\text{g}/\text{mL}$ ethidium bromide staining. A representative gel pattern of PBEF mRNA semi-quantitation by RT-PCR is presented in the insert. Vertical bar graph represents the change (mean \pm SD) of PBEF expression in different tissues of two dogs relative to their lungs (lung value arbitrarily set at 1).

human and 94% identical to murine and rat PBEF counterparts. This suggests that the PBEF gene is evolutionarily highly conserved. The recombinant canine PBEF protein was successfully expressed both by *in vitro* transcription coupled with translation in a cell-free system and by transfected human lung type II alveolar adenocarcinoma cell line A549. An anti-canine PBEF peptide polyclonal antibody was successfully prepared in rabbit. This antibody is specific to the PBEF protein and is crossreactive to the human PBEF. It is also crossreactive to murine and rat PBEF (data not shown). RT-PCR assay indicates that canine PBEF is expressed in canine brain, heart, liver, spleen, kidney, pancreas, muscle, and lung. The liver showed the highest expression, followed by muscle. Although PBEF was expressed at modest levels in the uninjured lung, its expression increased more than five-fold in acute lung injury (Ye *et al.*, in press).

The PBEF gene came to our attention when we unexpectedly found that PBEF was the most highly upregulated gene in a canine model of ALI using genomic technology to search for candidate genes in ALI. Compared with murine and rat, the dog is more suitable as an animal model to investigate mechanical heterogeneity, which is a fundamental property of acute lung injury in man, since mechanical and biological phenomena that contribute to ventilator-associated lung injury vary widely throughout the lung and with interventions (Lachmann, 1992; Verbrugge and Lachmann, 1999). Such heterogeneity is not captured in cellular and rodent models, which are not on the same scale in terms of the effects of gravity, regional interdependence, chest wall/abdominal interactions, hemodynamics, or fluid dynamics (Kolobow *et al.*, 1987; Tsuno *et al.*, 1990). Thus, a necessary step in the translation of basic science findings in ALI research to clinical studies and practice is their testing and validation in mechanically relevant large animal models. Isolation of the canine PBEF cDNA and expression of its recombinant protein in this study have provided tools that will allow future elucidation of the potential role of PBEF as an ALI biomarker and the molecular pathogenesis of ALI in the canine model. The derived knowledge may be more relevant to human pathophysiology and clinical application.

The canine model is also a particularly good model for genetic studies because of its small founding population, high levels of inbreeding, and the creation and maintenance of multigenerational pedigrees. Many of the ~360 known genetic disorders in dogs resemble human conditions, and their causes may be more tractable in large canine pedigrees than in small, outbred human families (Kirkness *et al.*, 2003). Although we focused our studies on PBEF in the pathogenesis of ALI, isolation of canine PBEF cDNA and protein provides tools that will also help to study the molecular mechanism of PBEF involvement in other diseases in the canine model. PBEF gene expression appears to be dysregulated in several diseased conditions, including TALL-1-treated RPMI-8226 B lymphoma cell line (Xu *et al.*, 2002). Severely infected fetal membranes (Ognjanovic *et al.*, 2001), human amnion and choriondecidua membrane derived from preterm labor

with chorionamnionitis (Marvin *et al.*, 2002), and colorectal cancer tissue (Van Beijnum *et al.*, 2002) exhibit significant PBEF upregulation. The PBEF gene was downregulated in Hyper-IgE syndrome (Chehimi *et al.*, 2001) and in denervated muscle (Tang *et al.*, 2000). Thus, isolation of the canine PBEF cDNA and expression of its recombinant protein may have broad applicability in the elucidation of pathogenesis of human diseases in addition to ALI.

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