Bombesin-like Peptides Modulate Alveolarization and Angiogenesis in Bronchopulmonary Dysplasia

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***Rationale*: The incidence of bronchopulmonary dysplasia (BPD), a chronic lung disease of newborns, is paradoxically rising despite medical advances. We demonstrated elevated bombesin-like peptide levels in infants that later developed BPD. In the 140-day hyperoxic baboon model of BPD, anti-bombesin antibody 2A11 abrogated lung injury.**

***Objectives*: To test the hypothesis that bombesin-like peptides mediate BPD in extremely premature baboons (born at Gestational Day 125 and given oxygen *pro re nata* [PRN], called the 125-day PRN model), similar to "modern-day BPD."**

***Methods*: The 125-day animals were treated with 2A11 on Postnatal Day 1 (P1), P3, and P6. On P14 and P21, lungs were inflation-fixed for histopathologic analyses of alveolarization. Regulation of angiogenesis by bombesin was evaluated using cultured pulmonary microvascular endothelial cells.**

***Measurements and Main Results*: In 125-day PRN animals, urine bombesin-like peptide levels at P2–3 are directly correlated with impaired lung function at P14. Gastrin-releasing peptide (the major pulmonary bombesin-like peptide) mRNA was elevated eightfold at P1 and remained high thereafter. At P14, 2A11 reduced alveolar wall thickness and increased the percentage of secondary septa containing endothelial cells. At P21, 2A11-treated 125-day PRN animals had improved alveolarization according to mean linear intercepts and number of branch points per millimeter squared. Bombesin promoted tubulogenesis of cultured pulmonary microvascular endothelial cells, but cocultured fetal lung mesenchymal cells abrogated this effect.**

***Conclusions*: Early bombesin-like peptide overproduction in 125-day PRN animals predicted alveolarization defects weeks later. Bombesin-like peptide blockade improved septation, with the greatest effects at P21. This could have implications for preventing BPD in premature infants.**

Keywords: bombesin; gastrin-releasing peptide; mechanical ventilation; prematurity; antibody treatment

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Bronchopulmonary dysplasia (BPD) is increasing in incidence despite medical advances. High bombesin-like peptide (BLP) levels are present in newborns who later developed BPD.

What This Study Adds to the Field

Our study demonstrates that BLP is an early mediator of arrested alveolar development and abnormal vascular morphogenesis in BPD.

Bronchopulmonary dysplasia (BPD) is a chronic lung disease of newborns, often following respiratory distress syndrome. The etiology of BPD remains enigmatic, although it has been associated with multiple factors, including mechanical ventilation, oxygen therapy, infection, inflammatory mediators, and immaturity (1–3). "Old BPD," described by Northway (4), was characterized by airway injury, inflammation, segments of atelectasis alternating with cystic overexpansion, and interstitial fibrosis. Such severe BPD is now less prevalent due to improved medical care. However, BPD is still a major burden in pediatric medicine, paradoxically due to success in improving acute survival. BPD affects approximately 30% of infants weighing less than 1,000 g. "Modern-day BPD," occurring in infants less than 30 weeks' gestation, seems to have a different pathophysiology and is characterized by arrested alveolar development and microvascular defects (3, 5–8).

Although many animal models of BPD have been described (9–15), BPD in preterm baboons is most similar to human BPD clinically and pathologically. The original hyperoxic BPD model was in animals at 140 days' gestation (term = 180 d) given 100% O_2 for 10 days (140-day 100%) (16, 17), which develop acute respiratory distress syndrome followed by severe BPD similar to old BPD (18). In this model, the gestational control (GC) 140-day baboons given O_2 *pro re nata* (PRN [as needed]) for 10 days to maintain arterial O_2 saturation, approximately 90% (140-day PRN) develop acute respiratory distress syndrome, but recover and do not develop BPD. Recently, a model more similar to the milder modern-day BPD has been developed in extremely premature baboons given surfactant and O_2 PRN (125-day PRN) (19), primarily characterized by decreased alveolarization (20).

Increased pulmonary neuroendocrine cells containing bombesin-like peptide immunoreactivity were found to occur in infants dying with BPD (21). In addition, we have observed elevated levels of bombesin-like peptides and increased pulmonary neuroendocrine

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cells shortly after birth in the 140-day 100% baboons (22) that develop severe BPD. We hypothesized that bombesin-like peptides could play a role in BPD because bombesin-like peptides can induce macrophage activation and fibroblast proliferation, inhibit alveolarization in mice, and cause bronchoconstriction in guinea pigs (23). Treatment with a blocking anti-bombesin antibody (2A11) abrogated evidence of lung injury in these hyperoxic animals, which usually develop severe changes similar to old BPD, indicating that bombesin-like peptides mediate lung injury (22, 24, 25).

Bombesin-like peptides include amphibian bombesin and its homolog, gastrin-releasing peptide (GRP), which have essentially identical immunogenicity and physiologic effects, because both bombesin and GRP bind to the bombesin/GRP-preferring receptor (26). In fact, amphibian bombesin functions at higher affinity at human GRP receptors than does human GRP itself (26), which is likely to be due to its smaller molecular weight and greater access to the receptor binding site. GRP is the major bombesin-like peptide present in mammalian lung (27). Bombesin-like peptides (bombesin and/or GRP) induce proliferation of bronchial epithelial cells and pulmonary fibroblasts (28) and promote fetal lung development, including branching morphogenesis, cell proliferation, and cell differentiation (29). GRP receptor gene expression was detected in mesenchymal cells around airways and blood vessels in fetal lung (30). Normally GRP and GRP receptor mRNA levels peak during the canalicular period and decline by alveolarization (30, 31). Thus, increased bombesin-like peptide–immunopositive pulmonary neuroendocrine cells and/or bombesin-like peptide levels in human infants and baboons that develop BPD may be part of the pathological process (22, 32).

Although we observed elevated bombesin-like peptide levels in premature human infants who later develop BPD (33), the role of bombesin-like peptides in modern-day BPD is unclear. In the present study, we have used the "interrupted gestation" 125-day PRN baboon model, which is clinically similar to modern-day BPD, to test the hypothesis that bombesin-like peptides mediate arrested alveolar development in primates with BPD.

METHODS

Detailed methods are given in the online supplement.

Animals

The National Research Council Guide for the Care and Use of Laboratory Animals was strictly adhered to throughout this study. The Animal Care Committee of the Southwest Foundation for Biomedical Research approved all protocols. Animal care details are published (19). No animals received antenatal steroids in the current investigation.

1. 125-day PRN (BPD animals): These were delivered at 125 days' gestation, given intratracheal beractant (Survanta; Ross Laboratories, Seattle, WA) (100 mg/kg), intravenous saline (vehicle control), and ventilated for 14 to 21 days to maintain Pa_{O_2} saturation of approximately 90%, as described (19, 34).
2. 125-day PRN+2A11: These BPD animals were given 2A11, a murine anti-bombesin blocking antibody (5 mg/kg in saline), intravenously from 2 to 4 hours after birth, and on Postnatal Day 3 (P3) and P6 (4 mg/kg), such as in 140-day animals (22).
3. 125-day PRN+MOPC21: MOPC21, a negative control murine IgG1, was given using the same dosage schedule as 2A11 (Sigma Chemical Co., St. Louis, MO).
4. 140-Day GCs: These are normal fetal animals at 140 days' gestation.
5. 125-Day GCs: These are normal fetal animals at 125 days' gestation.
6. 146-Day GCs: These are normal fetal animals at 146 days' gestation.

Urine Bombesin-like Peptide RIA

Bombesin-like peptide levels were measured (22) in 24-hour urine collections from individual baboons using a commercial RIA (IncStar Corp., Stillwater, MN), normalized for creatinine using a creatine assay kit (Sigma Chemical Co., St. Louis, MO) expressed as mg/100 ml. Details are given in the online supplement.

Morphometry

Right lower lobes were inflation-fixed and paraffin-embedded as described (22). Slides were viewed with a microscope (Nikon, Melville, NY) interfaced with a digitizer. Twenty ×20 fields were captured by stratified random sampling. ScionImage software (Scion Corp., Frederick, MD) was used to measure alveolar wall thickness, alveolar septa positive for α-smooth muscle actin (SMA) for myofibroblasts or CD31 for endothelial cells, and mean linear intercepts (31). The number of branch points per millimeter squared and surface density of secondary septa were calculated by computer-assisted image analysis as described (32, 33).

Immunohistochemistry

Paraformaldehyde-fixed tissues were processed into paraffin as described (34). Immunostaining was performed using our own rabbit anti-bombesin antisera (22) and monoclonal antibodies for SMA (1:100; Sigma) and CD31 (1:100; PharMingen, San Diego, CA) (35).

Capillary Tubulogenesis

Human pulmonary microvascular endothelial cells and human fetal lung mesenchymal cells were obtained from Cambrex Corp. (Baltimore, MD). The endothelial cells (10^5/0.1 ml) were plated on 24-well plates using BD Matrigel Matrix (BD Biosciences, San Jose, CA) (36). Some studies used Matrigel containing fetal lung mesenchymal cells (10^4/0.1 ml) cultured with overlying endothelial cells. Treatments included bombesin (0.1–10 nM), 2A11 (1 μg/ml), MOPC21 (1 μg/ml), or media alone, incubated 24 hours at 37°C in 5% CO_2. Tubule length was quantified using ScionImage. Branch points were counted manually and normalized for tubule length.

Reverse Transcriptase–Polymerase Chain Reaction Analyses

Total RNA, prepared using TriReagent (Molecular Research Center, Cincinnati, OH), was reverse transcribed and amplified with primers specific for GRP receptors (37).

Gene Arrays

Microarray analyses were performed as described (32). In brief, first-strand synthesis used 10 μg total RNA with polydeoxythymidine, T7-RNA polymerase adaptor, and Superscript-II reverse transcriptase (Invitrogen, Carlsbad, CA). Second-strand synthesis used DNA polymerase-I, RNase H, and T4-DNA polymerase. High-yield RNA transcripts were hybridized to HU-95Av2 GeneChip arrays (Affymetrix, Inc., Santa Clara, CA) for 16 hours; then data were processed using Affymetrix Microarray Analysis Suite version 5.0, and exported to the Siteman Cancer Center Bioinformatics Server online at http://www.siteman.wustl.edu.

Statistical Analyses

Statistical analyses used one-way analysis of variance. Results are given as mean ± SEM. Significance is defined as $P < 0.05$.

RESULTS

Higher Urine Bombesin-like Peptide Levels Predict Worse Oxygenation Index

Previously, we observed increased urine bombesin-like peptide levels in 125-day PRN baboons (n = 12), with elevations to 30% above the 24-hour (P1) baseline level at 48 hours and a doubling (100% increase) at 72 hours (22). To assess whether the magnitude of bombesin-like peptide levels in individual animals correlates with clinical severity, the oxygenation indices were determined for a group of 125-day PRN animals for which urine bombesin-like peptide levels had been measured (n = 14), using

the following formula: oxygenation index = [(FI_{O_2} × mean airway pressure) × 100/Pa_{O_2}]. Seven of these 14 baboons were killed at 6 days, and the remaining seven were killed at 14 days. Normally, oxygenation index is 1–2 or less, whereas values greater than 2.5 suggest impaired lung function. The mean oxygenation indices of 125-day PRN animals at 13 to 14 days of age directly correlate with urine bombesin-like peptide levels at 24 to 48 hours ($P < 0.0075$, n = 7, $R^2 = 0.789$, $R = 0.89$) (Figure 1). Table 1 gives the *P* values from comparisons of mean oxygenation indices of 125-day PRN animals, with urine bombesin-like peptide levels at 24-hour intervals from 1 to 24 hours up to 13 to 14 days of age (for oxygenation index) and from 1 to 24 hours up to 48 to 96 hours for urine bombesin-like peptide levels. Urine bombesin-like peptide levels from 1 to 24 hours up to 48 to 96 hours predict a decline of pulmonary function at 12 to 14 days. In contrast, there is no correlation between early urine bombesin-like peptide levels at 24 to 96 hours and early oxygenation index at 24 to 96 hours (Tables 1 and E1).

Increased Pulmonary Neuroendocrine Cells in 125-Day BPD Model

To evaluate relative numbers of pulmonary neuroendocrine cells, we immunostained lung sections from ventilated 125-day animals using the anti-bombesin antibody 2A11 that detects GRP in tissue sections (Figure 2). As shown in Figures 2a, 2c, and 2d, there were numerous clusters of GRP-positive neuroendocrine cells after 14 days. When antibody 2A11 was preabsorbed with human GRP, the immunostaining in immediately adjacent serial sections (Figure 2a) was completely abrogated (Figure 2b). At lower magnification, numerous GRP-positive cells are observed scattered throughout the small bronchioles and alveolar ducts (Figure 2c, *arrows*). For the lung tissue on every slide, all of the GRP-positive neuroendocrine cells were counted and normalized for the total area of lung tissue. The results of this morphometric analysis are given in Figure 2g. The legend to Figure 2g provides details about the numbers of animals per group and the significance of different statistical comparisons. In brief, 125-day baboons treated with O_2 for 14 days have a significant increase in relative numbers of neuroendocrine cells, and MOPC21 treatment had no significant effect on this increase. In contrast, 2A11 treatment abrogated the increase in neuroendocrine cells down to the level of 125-day GCs.

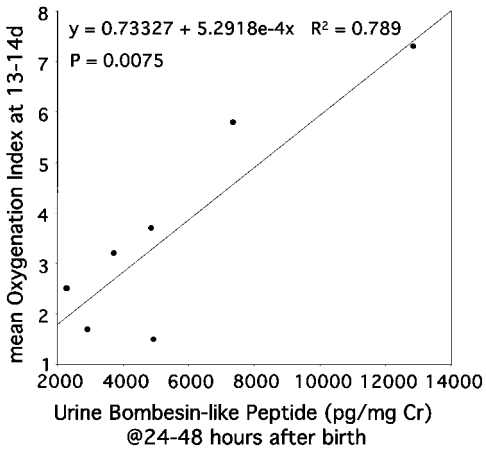

Figure 1. Elevated urine bombesin-like peptide levels in 125-day PRN (*pro re nata*) baboons. The 24-hour urine bombesin-like peptide levels at 24 to 48 hours are directly correlated with the mean oxygenation index at 13 to 14 days in 125-day PRN animals. The 24-hour urine specimens were collected at 24 to 48 hours from 125-day PRN animals without intervention (n = 7). A linear correlation was the best fit for the points, with $R^2 = 0.789$, $R = 0.89$, $P < 0.0075$.

Increased GRP mRNA Levels in Lungs of BPD Animals

We tested the hypothesis that GRP mRNA in the lung is up-regulated because we observed increased bombesin-like peptide levels in the urine at 48 to 72 hours after birth. We prepared lung RNA from GCs (125 d, n = 4; 140 d, n = 3) and ventilated 125-day PRN animals at P1 (n = 3), P2 (n = 3), P6 (n = 3), and P14 (n = 3). We used microarray analyses to compare 125-day PRN animals to 125-day GCs. The number of animals used for these studies was set by institutional review. The 125-day PRN animals had 2- to 16-fold increased GRP mRNA levels at all ages compared with 125-day GCs. Compared with 125-day GC animals, all 125-day ventilated animals had significantly elevated GRP mRNA levels: at P1, ventilated animals had a 7.8-fold increase in mean GRP mRNA levels ($P < 0.005$) (Figure 3 and Table 2); at P2, they had a 1.7-fold increase ($P < 0.05$); at P6, they had a 16.4-fold increase ($P < 0.001$); and at P14, they had a 3.8-fold increase ($P < 0.01$) (Figure 3). Increased GRP mRNA has been demonstrated previously in lungs of 125-day PRN baboons using semiquantitative reverse transcriptase–polymerase chain reaction (RT-PCR) (32). In addition to GRP, several other proinflammatory and developmentally important genes were up-regulated more than threefold at 24 hours of age in lungs of ventilated 125-day animals, as summarized in Table 2. However, not all developmentally important genes are up-regulated. Platelet-derived growth factor-AA (PDGF-AA), which plays a major role in alveolarization, was significantly altered in lungs developing BPD: at 125 days plus 2 days of mechanical ventilation, PDGF-AA gene expression was not up-regulated by 1.27-fold compared with 125-day GCs; at 125 days plus 6 days of mechanical ventilation, the level of PDGF-AA mRNA was 0.89-fold compared with 125-day GCs; and at 125 days plus 14 days of mechanical ventilation, the level was 1.4-fold compared with the age-matched 140-day GCs.

Histologic Analyses

Lungs from 125-day GC animals corresponded developmentally to the late canalicular/early saccular stage, comparable to 24 to 26 weeks of human gestation. The alveolar walls were thicker than in 140-day GCs and round distal saccules occurred with small protuberances along their walls, representing progenitor secondary crest ridges that evolve into alveoli. Ventilated 125-day PRN animals developed chronic lung disease, with an arrested number of enlarged primitive alveoli and blunted secondary alveolar septa giving the appearance of aborted development (Figures 4a,

TABLE 1. *P* VALUES FOR COMPARISON OF MEAN OXYGENATION INDEX VERSUS URINE BOMBESIN-LIKE PEPTIDE LEVELS AT DIFFERENT 24-HOUR TIME INTERVALS

	Urine Bombesin-Like Peptide Levels			
Time Interval of Mean OI (*h*)	1–24 h	24–48 h	48–72 h	72–96 h
1–24	0.6224	0.4707	0.3982	0.2966
24–48	0.4581	0.3185	0.5763	0.9165
48–72	0.9154	0.5665	0.8629	0.6356
72–96	0.5043	0.7088	0.9543	0.4329
96–120	***0.0477***	0.4118	0.1372	***0.0447***
120–144	***0.0306***	0.2136	***0.0263***	***0.0316***
144–168	**0.0884**	**0.0736**	***0.0244***	**0.0577**
168–192	0.1516	**0.0581**	**0.0967**	0.1986
192–216	***0.0010***	***0.0031***	***0.0067***	***0.0128***
216–240	0.1016	0.1526	0.1760	0.1952
240–264	***0.0286***	**0.0553**	**0.0659**	**0.0514**
264–288	***0.0425***	**0.0779**	**0.0534**	***0.0399***
288–312	***0.0072***	***0.0084***	***0.0024***	***0.0050***
312–336	***0.0214***	***0.0075***	***0.0075***	***0.0168***

Significant *P* values (≤ 0.05) are shown in bold italics. *P* values consistent with a trend (0.05 ≤ *P* ≤ 0.10) are shown in bold. *P* values > 0.10 are shown in normal type.

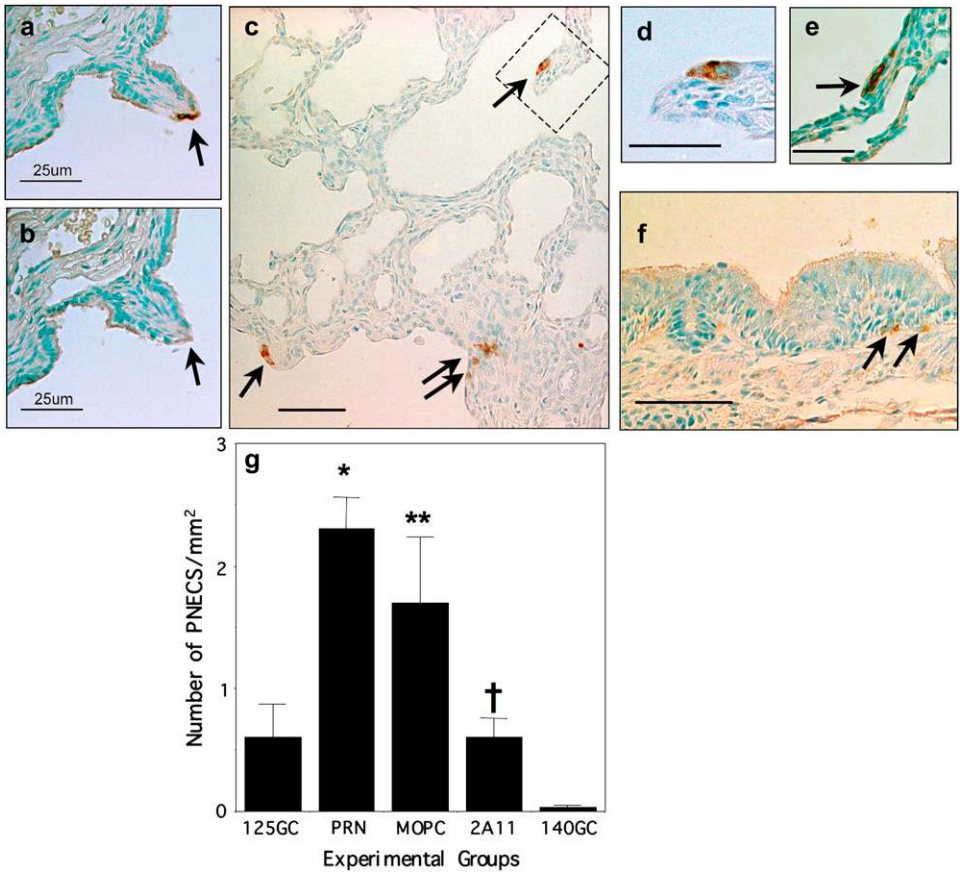

Figure 2. Increased gastrin-releasing peptide (GRP)–immunopositive pulmonary neuroendocrine cells in 125-day PRN baboons. In the 125-day baboon model of bronchopulmonary dysplasia, there is marked hyperplasia of pulmonary neuroendocrine cells. (*a*) Immunostaining for bombesin-like peptide demonstrated numerous GRP-positive neuroendocrine cells, which were mainly localized to small bronchioles and alveolar ducts. A small GRP-positive cluster of neuroendocrine cells is demonstrated in a bronchiole. *Bar* in lower left hand corner = 25 μm. (*b*) A serial section immediately adjacent to that shown in (*a*) was immunostained in parallel with the same anti-bombesin antibody (2A11) preabsorbed with human GRP as described in the online supplement METHODS. Note that the immunostaining shown in (*a*) was abrogated, indicating the antigen specificity of this staining (*arrow*). *Bar* in lower left hand corner = 25 μm. (*c*) In 125-day PRN baboons, numerous GRP-positive cells are observed scattered throughout the small bronchioles and alveolar ducts (*arrows*). *Bar* in lower left hand corner = 50 μm. (*d*) The *dotted-line box* in the upper right hand corner of (*c*) is shown enlarged in (*d*), with multiple nuclei and abundant cytoplasm observed in one representative cluster. *Bar* in lower left hand corner = 25 μm. (*e*) In contrast, in a 2A11-treated 125-day PRN baboon, GRP-positive cells are only infrequently identified in small bronchioles (*arrow*). *Bar* in lower left hand corner = 25 μm. (*f*) The majority of the GRP-positive positive neuroendocrine cells in 2A11-treated baboons occur in larger bronchioles and in cartilaginous airways (*arrows*). *Bar* in lower left hand corner = 50 μm. Cartilage is visible just below the *scale bar*. (*g*) GRP-positive pulmonary neuroendocrine cells were counted in entire tissue sections using computerized image analysis with Scion Image 6.2 (Scion Corp.), normalized for the area of lung tissue present because cells were scattered in small bronchioles and alveolar ducts in all groups. Results are expressed as the number of pulmonary neuroendocrine cells per millimeter squared of lung tissue. Numbers of animals quantified were: 125-day gestational controls (GCs; 125GC), n = 6; 125-day PRN, n = 6; 125-day PRN+MOPC21, n = 4; 125-day PRN+2A11, n = 5; 140-day GCs (140GC), n = 5. PNECs = pulmonary neuroendocrine cells. *$P < 0.0002$ compared with 125-day or 140-day GCs; †$P < 0.0003$ compared with 125-day PRN without any intervention; **$P < 0.02$ compared with 125-day GCs, 140-day GCs, or 125-day PRN +2A11. There was no significant difference between the 2A11 group and 125- or 140-day GCs ($P > 0.27$).

5a, and 6a). In contrast, 2A11-treated 125-day PRN animals had more normally developed secondary septa (Figures 4b, 5b, and 6b). We performed morphometry to quantify alveolar wall thickness and the proportion of secondary septa containing capillaries (CD31 positive) and myofibroblasts (SMA positive) at septal tips.

Alveolar wall thickness was measured (Figures 4a and 4b) as described (35). At P14, 125-day PRN animals (Figure 4a) have significantly thicker alveolar septal walls compared with 2A11-treated 125-day PRN animals (Figure 4b). The wall thickness of 2A11-treated animals was similar to that of age-matched 140-day GCs, both of which were thinner than MOPC21-treated 125-day PRN animals (Figure 4c) ($P < 0.003$).

Alveoli form by division of terminal air sacs with secondary septa during development (36). Localization of myofibroblasts to the tips of secondary septa is critical for alveolar development. Without myofibroblasts, secondary septa do not develop, resulting in abnormally large airspaces (37). Immunostaining for SMA, a marker of myofibroblasts (Figure 5), demonstrated that compared with MOPC21-treated and untreated 125-day PRN animals (Figure 5a), 2A11 increased secondary septation in 125-day PRN animals (Figure 5b) ($P < 0.005$). Elevated SMA-positive septal tips occurred only in the 2A11-treated group and the age-matched 140-day GC animals (Figure 5c). The difference in SMA-positive cells was seen only in the septal tips. There was no significant difference in the overall volume percentage of SMA positivity in the alveoli. The volume percentage of SMA positivity refers to the relative proportion of the tissue volume occupied by SMA-positive cells, in this study between any of the 125-day groups (data given in the online supplement). The number of secondary crests was not altered at either 14 or 21 days, according to independent determinations by two of our investigators (C.B. and L.C.).

New capillary development along terminal septa was assessed by CD31 immunostaining because CD31 (also known as PECAM) is a well-characterized marker of capillary endothelial cells. CD31-positive cells were present in many secondary septa consistent with capillary migration along developing septa. Compared with control 125-day PRN BPD animals (Figures 6a and 6c), there were significantly more CD31-positive septa in the 2A11-treated animals (Figures 6b and 6c). In 125-day PRN animals, the CD31-positive septa were blunt and appeared to have arrested development (Figure 6a). In contrast, 2A11-treated animals had septal capillary growth that closely approached the developmental level of 140-day GCs (Figure 6c). There were significant differences in the numbers of CD31-positive septa between 2A11-treated 125-day PRN animals and 125-day GCs, and MOPC21-treated or untreated 125-day PRN animals ($P <$

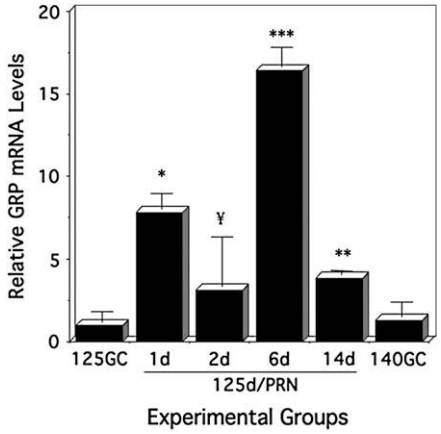

Figure 3. Elevated gastrin-releasing peptide (GRP) mRNA levels in lungs of 125-day PRN baboons. Lung GRP mRNA levels in different groups, as determined by mRNA microarray analyses: 125GC treated with mechanical ventilation PRN for 1 day (n = 3), 125GC treated with mechanical ventilation PRN for 2 days (n = 3), 125GC treated with mechanical ventilation PRN for 6 days (n = 3), 125GC treated with mechanical ventilation PRN for 14 days (n =3), and 140GC (n = 3). *$P < 0.005$, **$P < 0.01$, ***$P < 0.001$, $^{¥}P < 0.05$.

TABLE 2. ALTERED GENE EXPRESSION IN LUNGS OF 125-DAY PRN BABOONS AT 24 HOURS AFTER BIRTH

mRNA up-regulated to levels greater than threefold control values in 3 of 3 animals.
Proinflammatory
Gastrin-releasing peptide
Complement protein 3
FK506 binding protein
Histamine H3 receptor
Cathepsin L
Tissue inhibitor of metalloproteinases-1
Matrix metalloproteinase-14
Plasminogen activator inhibitor-1
Thromboplastin
Leukotriene A4 hydrolase
Metabolic
Glutathione peroxidase 3
Cytochrome c oxidase assembly protein
Catalase
C-EBP-δ
Differentiation
Surfactant proteins B and C
Programmed cell death-5
Cyclophilin B
mRNA down-regulated to levels less than one-third control values in 3 of 3 animals.
Nicotinic cholinergic receptor α-5
Soluble IL-6 receptor

0.0002). There was no visible difference in the relative proportion of CD31 immunostaining in the alveolar tissue between the different groups.

Capillary Tubulogenesis and Branching

We became interested in mechanisms of development of CD31-positive secondary septa in 2A11-treated animals and explored effects of bombesin-like peptides on capillary development using pulmonary microvascular endothelial cells. We chose to use the amphibian peptide bombesin for these *in vitro* studies rather than mammalian GRP because bombesin is more effective than GRP at eliciting physiologic responses at the mammalian bombesin/GRP-preferring receptor, possibly due to the smaller molecular

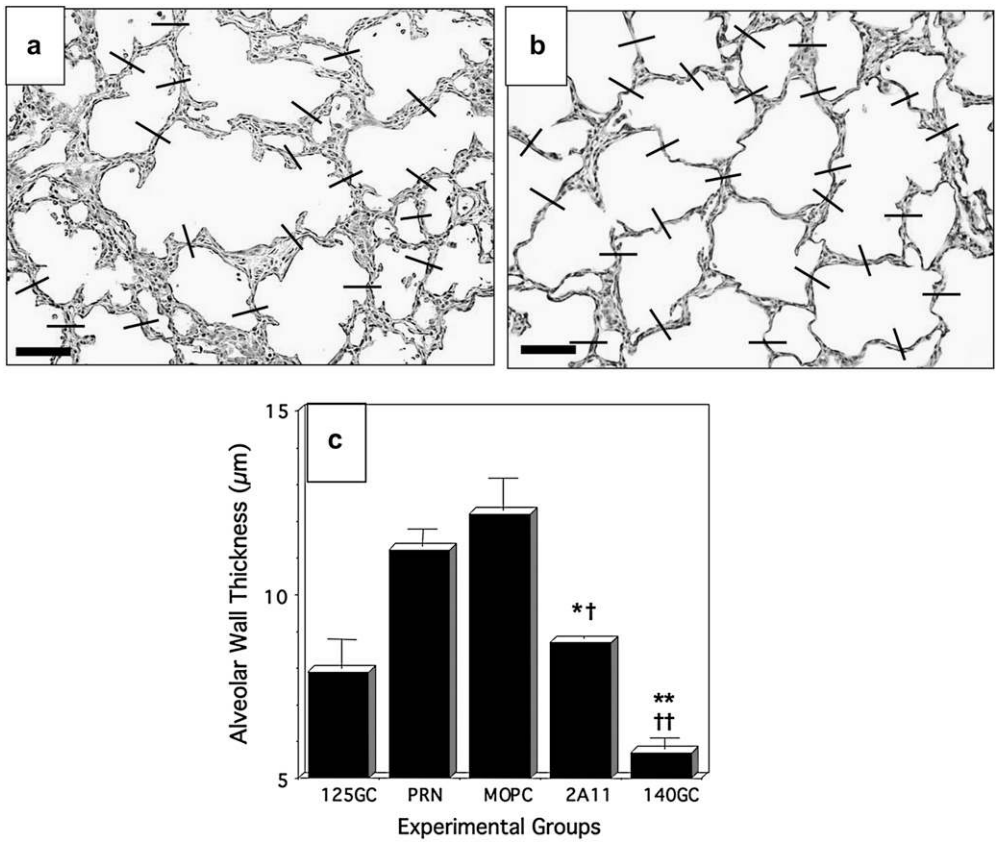

Figure 4. Alveolar wall thickness in 125-day PRN baboons maintained for 14 days without or with 2A11 or MOPC21. *See* Figure E4 for a color version of this figure. (*a*) Section of 125-day PRN animal maintained on mechanical ventilator for 14 days without pharmacological intervention. *Lines* were drawn perpendicular to alveolar septa. *Scale bar* in lower left corner, 40 μm. (*b*) Section of 125-day PRN animal maintained on mechanical ventilator for 14 days treated with 2A11. *Lines* were drawn perpendicular to alveolar septa. *Scale bar* in lower left corner, 40 μm. (*c*) Results of morphometry of alveolar septal wall thickness. Number of animals per group were: 125-day gestational controls (125GC), n = 5; 125-day PRN without intervention, n = 9; 125-day PRN+MOPC21, n = 6; 125-day PRN+2A11, n = 5; 140-day gestational controls (140GC), n = 5. *$P < 0.002$ compared with 125-day PRN+MOPC21; †$P < 0.01$ compared with 125-day PRN without intervention; **$P < 0.0001$ compared with 125-day PRN+MOPC21 or 125-day PRN without intervention; ††$P < 0.02$ compared with 125-day PRN+2A11.

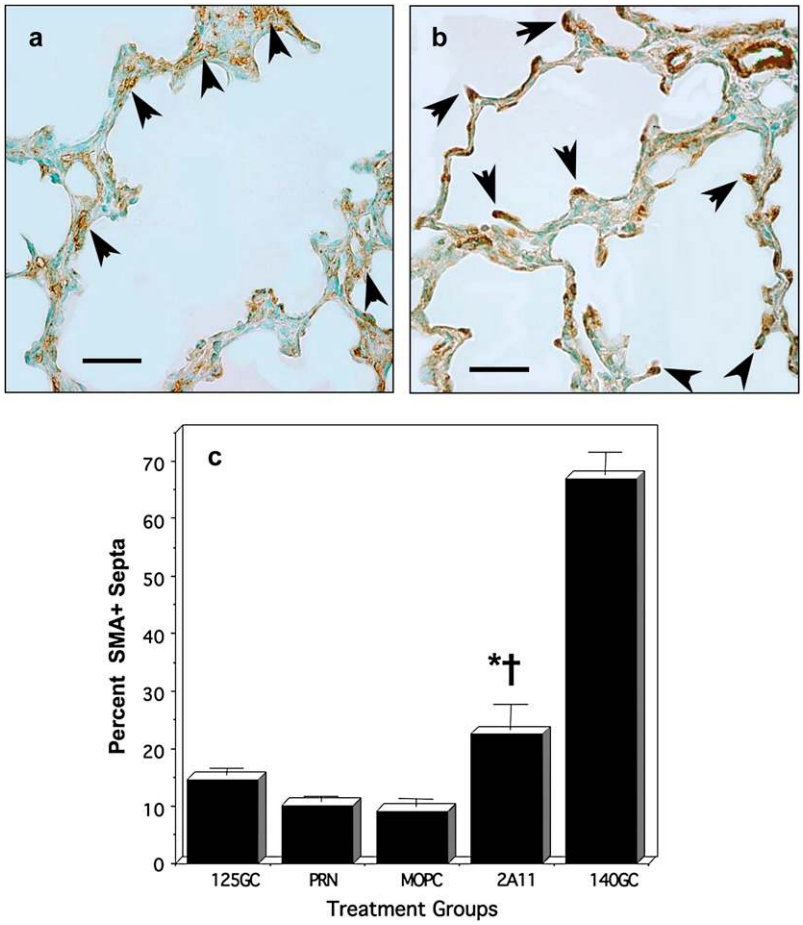

Figure 5. α-Smooth muscle actin (SMA)–positive immunostaining of secondary alveolar septal tips. (*a*) SMA immunostaining of lung sections from a 125-day PRN animal treated with MOPC21 (negative control IgG1). Myofibroblasts (SMA positive) are mostly in the interstitium (representative SMA-positive cells are indicated by *arrowheads*). *Scale bar*, 20 μm. (*b*) SMA immunostaining of lung from a 2A11-treated 125-day PRN animal. Myofibroblasts were concentrated at alveolar septal tips (*arrowheads*). *Scale bar*, 20 μm. (*c*) Pooled morphometric analyses of percentage of SMA-positive secondary septa in: 125-day gestational controls (125GC) (n = 5); 125-day PRN, n = 9; 125-day PRN+MOPC21, n = 6; 125-day PRN+2A11, n = 5; 140-day gestational controls (140GC), n = 5. $^{*}P < 0.0002$ compared with 140GC; $^{\dagger}P < 0.001$ compared with 125GC, PRN, or PRN +MOPC21.

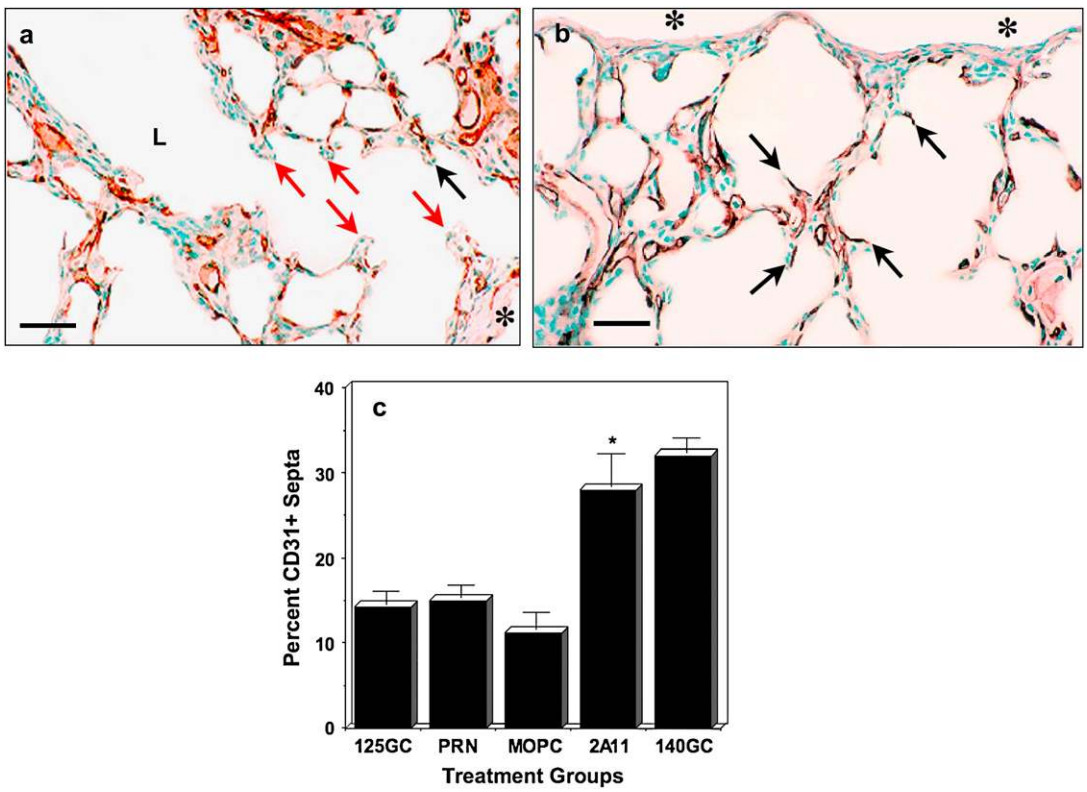

Figure 6. CD31 immunostaining in developing secondary septa. (*a*) CD31 immunostaining of lung from a 125-day PRN animal treated with MOPC21. Secondary septa were mostly CD31 negative (*red arrows*), with occasional CD31-positive septa (*black arrow*). *Scale bar*, 30 μm. *Asterisk* indicates pleural surface; L = airway lumen. (*b*) Lung from a 2A11-treated 125-day PRN animal has CD31-positive cells concentrated at alveolar septal tips. (*arrows*). *Scale bar*, 30 μm. *Asterisk* indicates pleural surface. (*c*) Numbers of CD31-positive secondary alveolar septal tips was determined as given in Methods. Pooled analyses of percentage of CD31-positive secondary septa are shown for: 125-day gestational controls (125GC), n = 5; 125-day PRN without intervention, n = 9; 125-day PRN+MOPC21, n = 6; 125-day PRN+2A11, n = 5; 140-day gestational controls (140GC), n = 5. $^{*}P < 0.0002$ compared with 125GC, 125-day PRN, or 125-day PRN+MOPC21.

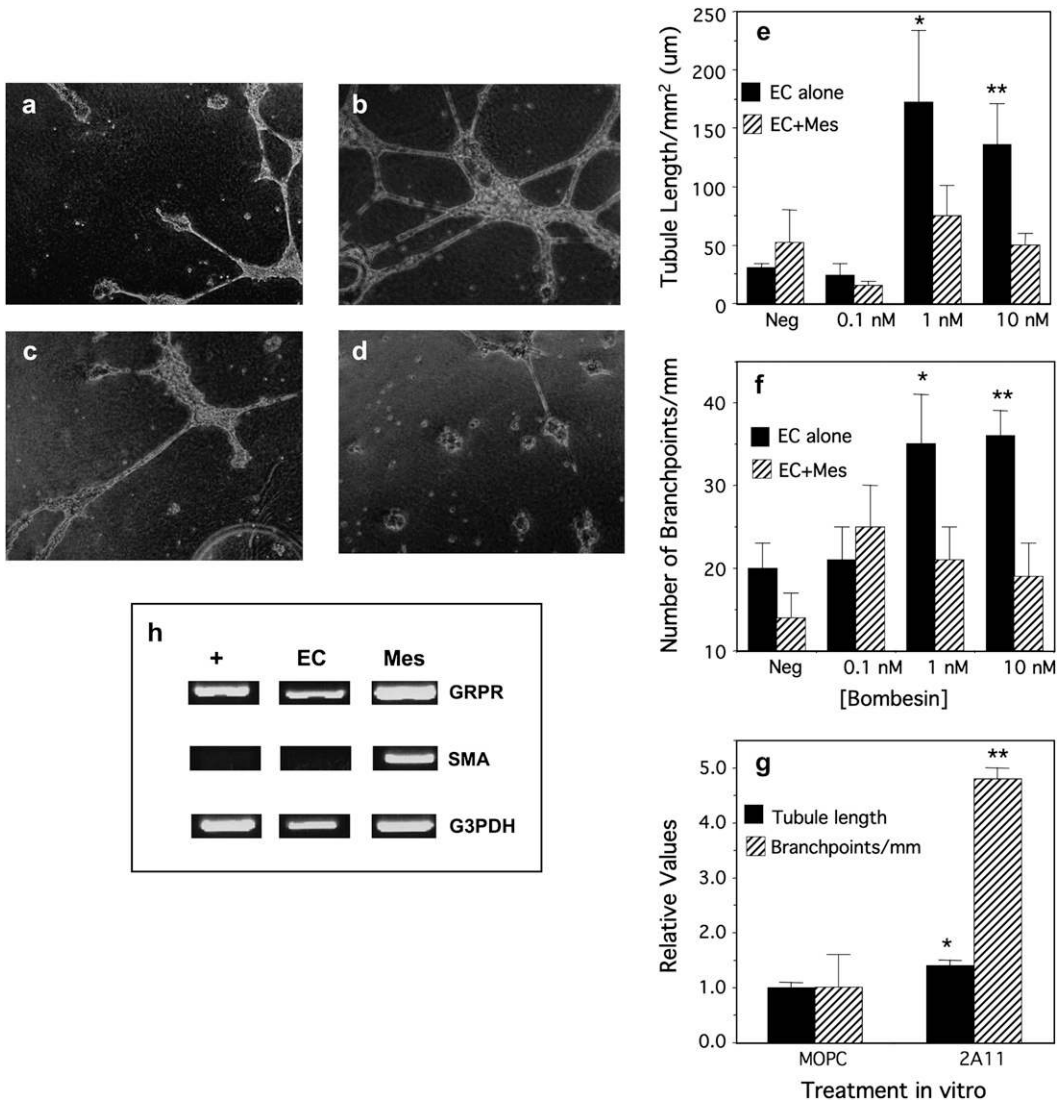

Figure 7. Bombesin regulates capillary tubulogenesis. (*a*) Pulmonary microvascular endothelial cells alone on Matrigel (BD Biosciences, San Jose, CA) for 24 hours had scant tubule formation with few branch points. (*b*) Pulmonary microvascular endothelial cells alone + 1 nM bombesin. Note increased total length of tubules and many branch points. (*c*) Pulmonary microvascular endothelial cells on Matrigel with fetal lung mesenchymal cells were similar to (*a*). (*d*) Pulmonary microvascular endothelial cells on Matrigel with fetal lung mesenchymal cells + 1 nM bombesin had no significant response to bombesin. (*e, f*) Pooled results of three experiments with pulmonary microvascular endothelial cells (EC) alone or pulmonary microvascular endothelial cells + fetal lung mesenchymal cells (EC + Mes). Bombesin induced greater than fivefold increased capillary tubule length; $*P <$ 0.003 and $**P < 0.01$ compared with negative control ("Neg" in figure). Bombesin increased branch points per millimeter by more than 50% (*f*); $*P < 0.008$ and $**P <$ 0.0005 compared with negative control. All bombesin-induced responses of EC were abrogated by cocultured fetal lung mesenchymal cells (EC + Mes). (*g*) Pooled results of three assays with pulmonary microvascular endothelial cells plus mesenchymal cells (EC + Mes) ± 2A11 or MOPC21. Values obtained with 2A11 are normalized for the mean values with MOPC21 for each experiment. Numbers of capillary tubule branch points per millimeter of tubule length were increased more than fourfold ($**P < 0.0001$) and tubule length was increased 40% ($*P < 0.05$). (*h*) Results of semiquantitative reverse transcriptase–polymerase chain reaction of pulmonary microvascular endothelial cells (EC) and fetal lung mesenchymal cells (Mes) for gastrin-releasing peptide receptor (GRPR), α-smooth muscle actin (SMA), and glyceraldehyde phosphate dehydrogenase (GAPDH).

weight of bombesin (26). The bioactive C-terminal amino acid sequence of GRP is identical to the C-terminal of bombesin (38). Compared with controls cultured alone on Matrigel (Figure 7a), microvascular endothelial cells demonstrated a dose-dependent increase in capillary tubulogenesis with bombesin (Figures 7b, 7e, and 7f). Bombesin increased both the total length of the capillary tubules (Figure 7e) and the number of branch points per millimeter of tubule (Figure 7f). The length of tubules peaked at 1 nM bombesin (more than fivefold increased, $P < 0.003$) and remained significantly elevated at 10 nM bombesin (more than fourfold increased, $P < 0.01$) (Figure 7e). Also, the number of branch points per millimeter of tubule increased approximately 75% at 1 nM ($P < 0.01$) and at 10 nM bombesin ($P < 0.0005$) (Figure 7f). However, pulmonary microvascular endothelial cells *in vivo* are in a complex multicellular environment that includes mesenchymal cells (SMA positive). When fetal lung mesenchymal cells were cocultured in Matrigel, the proangiogenic effect of bombesin on pulmonary microvascular endothelial cells was lost (Figures 7e and 7f). Conversely, treatment of cocultures with 2A11 resulted in a fivefold increase in the number of capillary branch points per millimeter of tubule ($P < 0.0001$), as well as a 50% increase in the length of tubules formed ($P < 0.05$) (Figure 7g). These data suggest that bombesin-like peptides induced production of antiangiogenic factors from fetal lung mesenchymal cells.

Bombesin-like Peptide Receptors on Fetal Lung Mesenchymal Cells and Endothelial Cells

We previously used *in situ* hybridization to localize GRP receptor mRNA in lungs of hyperoxic baboons with BPD (32) in the loose mesenchyme surrounding developing airways and blood vessels. In the present study, we used semiquantitative RT-PCR analysis to investigate expression of the cloned bombesin-like peptide receptor by fetal lung mesenchymal cells and pulmonary microvascular endothelial cells. We demonstrated that GRP receptor mRNA is expressed by both fetal lung mesenchymal cells and pulmonary microvascular endothelial cells (Figure 7h). The presence of SMA mRNA in the mesenchymal cells ("Mes" in Figure

7h) but not in the pulmonary microvascular endothelial cells ("EC" in Figure 7h) confirmed the identities of these cell populations (Figure 7h). The positive control for GRP receptor mRNA ("+" in Figure 7h) was the H345 small cell carcinoma cell line (39). The negative control lacking reverse transcriptase had no detectable GRP receptor mRNA (Figure 7h). These results shown in Figure 7h were replicated in three independent experiments with different RNA preparations from different passages of the cells. All experiments yielded identical results. In most studies of the GRP receptor, mRNA levels are determined by RT-PCR and the presence of functional receptor protein is inferred from the ability of GRP or bombesin to elicit physiologic responses at 0.1 to 1.0 nM.

Mean Linear Intercept

Computerized image analysis of alveolar development was performed on lungs of 125-day GC, 140-day GC, and 125-day PRN animals treated with MOPC21 or 2A11 after 14 or 21 days of O_2 PRN. The results of morphometry are given in Figure 8. Compared with untreated 125-day PRN animals (Figure 8a), there was a 30% increase in the number of air–tissue interfaces per millimeter at 21 days in the 2A11-treated animals (Figure 8b), which is directly proportional to alveolar surface area. We translated these data into "mean linear intercept." In simple terms, this is the average distance between two alveolar walls. Results are given in Figure 8c. Mean linear intercept was significantly reduced in the 2A11-treated 125-day PRN animals compared with the untreated animals at 21 days, consistent with 2A11-induced improvement of the arrested septation that occurs in 125-day PRN animals. The difference between untreated and 2A11-treated groups was less striking at 14 days, although the trend could still be observed. However, at 14 days, the mean linear intercept of the 2A11-treated group was significantly smaller than that of the MOPC21-treated animals.

In view of the more significant effect of 2A11 on mean linear intercept at 21 days, additional morphometric analyses were performed on a small group of 125-day PRN lungs after 21 days of ventilation, comparing untreated animals with 2A11-treated animals and the age-matched 146-day GCs. The number of

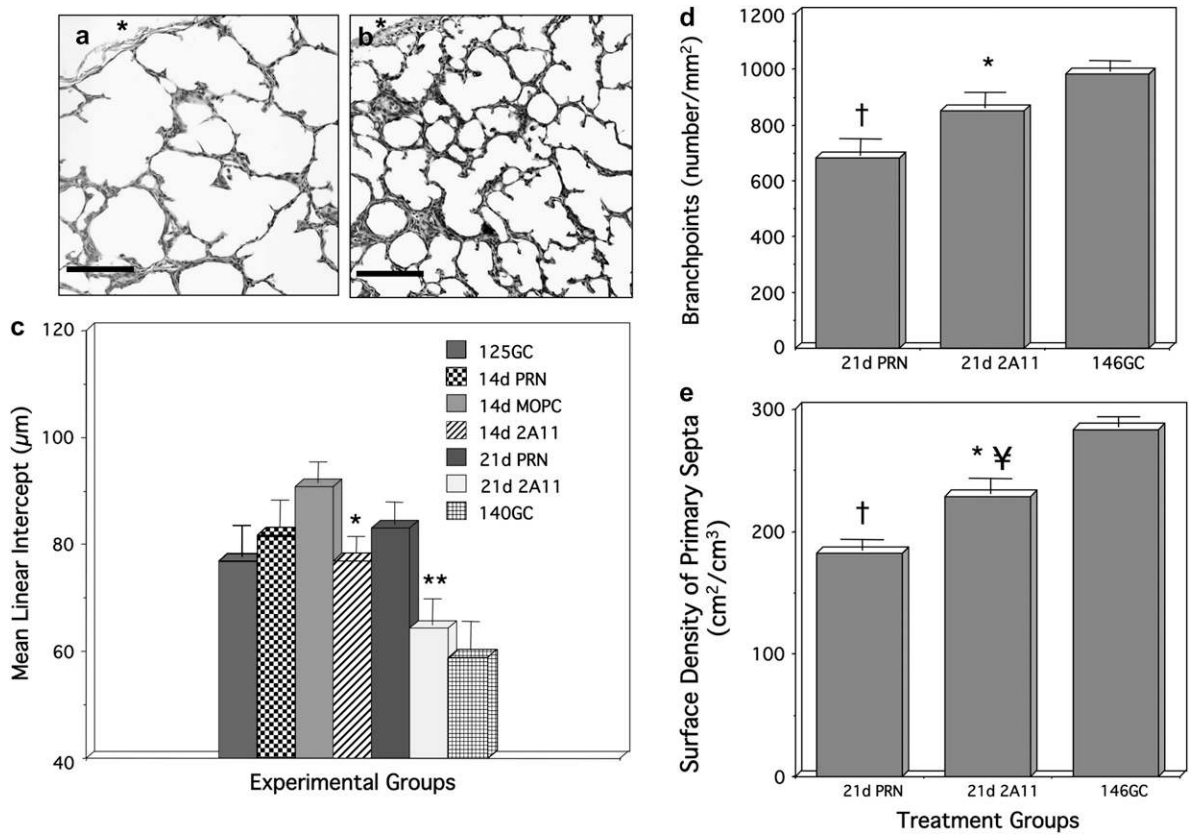

Figure 8. Morphometric analyses of alveolar development in 125-day PRN baboons after 14 or 21 days without, or with, 2A11 or MOPC21. *See* Figure E8 for a color version of this figure. (*a*) Hematoxylin-and-eosin–stained lung section from a 125-day PRN × 21-day baboon without any intervention. *Bar* in lower left hand corner = 100 μm. (b) Hematoxylin-and-eosin–stained lung section from a 125-day PRN × 21 days + 2A11. *Bar* in lower left hand corner = 100 μm. *Asterisks* (*) indicate pleural surface. (*c*) Summary of mean linear intercepts for: 125-day gestational controls (125GC), n = 5; 125-day PRN × 14 days without any intervention (14d PRN), n = 9; 125-day PRN × 14 days + MOPC21 (14d MOPC21), n = 6; 125-day PRN × 14 days + 2A11 (14d 2A11), n = 5; 125-day PRN × 21 days without any intervention (21d PRN), n = 7; 125-d PRN × 21 days + 2A11 (21d 2A11), n = 6; 140-day gestational controls (140GC), n = 5. *$P < 0.01$ compared with 14-day MOPC21; **$P < 0.001$ compared with 21-day PRN. (*d*) Pooled data giving the number of branch points per millimeter squared of alveolar surface area for the following groups: 21-day PRN (n = 9), 21-day 2A11 (n = 6), and 146-day GC (age-matched control) (n = 5). *$P < 0.05$ compared with 21-day PRN. There was no significant difference between 21-day 2A11 and 146-day GC; $^{\dagger}P < 0.005$ compared with 146-day GC. (*e*) Pooled data for surface density of primary septa (cm^2/cm^3) for 21-day PRN (n = 9), 21-day 2A11 (n = 6), and 146-day GC (age-matched control) (n = 5). *$P = 0.004$ compared with 21-day PRN; $^{\dagger}P < 0.0001$ compared with 146-day GC. $^{¥}P < 0.01$ compared with 146-day GC.

alveolar branch points per millimeter squared of lung tissue was increased in 2A11-treated lungs compared with untreated animals (Figure 8d) ($P = 0.05$). The value for 2A11-treated animals was close to the 146-day GC value ($P > 0.16$). In contrast, the number of alveolar branch points per millimeter squared of the untreated 125-day PRN group was significantly lower than the 146-day GC group ($P < 0.0025$). Similarly, the surface density of primary septa was significantly increased with 2A11 treatment compared with no intervention (Figure 8e) ($P < 0.003$), which was midway toward the 146-day GC value ($P < 0.003$). In contrast, the surface density of primary septa of the untreated 125-day PRN group was lower than the 146-day GC group ($P < 0.0001$).

DISCUSSION

In the present study, we have demonstrated that anti-bombesin blocking antibody 2A11 can protect against pathologic evidence of BPD in the most clinically relevant animal model. Cumulatively, our observations suggest that GRP secreted by pulmonary neuroendocrine cells either mediates lung injury or is a necessary cofactor for lung injury, in combination with other factors such as IL-8 and/or leukotrienes (40). This "interrupted gestation" 125-day PRN model was designed to be closely similar to the clinical experience with premature human infants currently being treated in the intensive care unit, who require surfactant to survive and also treatment with gentler ventilation techniques and lower levels of oxygen than had been administered 20 years ago (19). Previously, we demonstrated high levels of urine bombesin-like peptides in 125-day PRN animals at 24 to 72 hours after birth compared with the first 0- to 24-hour urine collection (22). Similarly, in human infants born at or less than 28 weeks' gestation, elevated urine bombesin-like peptide levels confer a 10-fold increased risk of developing BPD (29). We now provide functional evidence that elevated urine bombesin-like peptide levels at 24 to 96 hours of age predict significantly worse pulmonary status as assessed by mean oxygenation index at 12 to 14 days of age in the 125-day PRN model. Thus, elevated bombesin-like peptide levels predict a more severe decline in pulmonary function, presumably reflecting stiffer airways and/or diminished alveolar gas exchange.

Microarray analyses of 125-day baboon lung showed that 125-day PRN animals had eightfold increased levels of GRP mRNA at 24 hours compared with 125-day GCs (Figure 3). At all subsequent time points, the 125-day PRN animals continued to have significantly elevated GRP mRNA in the lung, mostly of lower magnitude than the 24-hour values, except for a peak at the 6-day time point. The timing of these expression data would be consistent with high urine bombesin-like peptide levels at 24 to 72 hours of age, with a time lag potentially required for peptide synthesis and excretion in the urine (22).

Cumulatively, these observations are consistent with the increased numbers of bombesin-like peptide–positive pulmonary neuroendocrine cells in the 125-day PRN baboons at 14 days postnatal age, consistent with observations in ventilated infants dying with BPD (21, 41). Neuroendocrine cell hyperplasia in the lung can be induced at least in part by oxidant injury because numbers of bombesin-like peptide–positive pulmonary neuroendocrine cells were greatly reduced in hyperoxic BPD baboons treated with a catalytic antioxidant (42). Furthermore, although there was no significant difference in numbers of mast cells between 125-day PRN baboons and 125-day GCs, there was a significant decrease in mast cells in 125-day PRN animals treated with 2A11 (data given in the online supplement). This observation supports a widespread effect of 2A11 on reducing numbers of several cell types that can be implicated in lung injury (22, 24). Interestingly, treatment with the negative control IgG, MOPC21, resulted in a worsening of mean linear intercepts in 125-day PRN baboons at 21 days (Figure 8). This nonspecific worsening of parameters of BPD by MOPC21 treatment in the 125-day model is similar to effects of MOPC21 in the 140-day model (22). This effect could be related to nonspecific activation of macrophages by IgG binding to Fc receptors and triggering an exacerbation of proinflammatory and/or alveolar growth–arresting signaling cascades (43). These observations indicate that the beneficial effects of 2A11 are related to its specific GRP-blocking function.

Untreated 125-day PRN animals had lung histopathology similar to modern-day BPD, with a reduced number of larger alveoli, blunted secondary alveolar septa, and thicker alveolar walls (19). The 2A11 (but not MOPC21) normalized these parameters of lung injury, reversing the arrested alveolarization and leading to thinner septal walls. Conversely, newborn mice treated with bombesin or GRP developed reduced alveolarization and thicker alveolar septal walls (44). An interesting feature of 2A11-induced alveolarization was widespread development of septal capillaries together with increased myofibroblasts at the tip of growing secondary septa. Although overall there was no significant difference in the volume percentage of SMA-positive cells in the alveoli, there was a significant increase in the relative numbers of septal tips that were positive for SMA or CD31 in 2A11-treated animals. The septa are important during alveolar formation because the secondary septa divide the terminal sacs into alveoli (36, 45). The localization of alveolar myofibroblasts to the septal tips is important because this is where the myofibroblasts synthesize elastin (46). Elastin-null mice have defective secondary septation and alveolarization (46). Similarly, PDGF-A–null mice had arrested alveolarization due to lack of septal myofibroblasts (37). We did not observe any changes in PDGF-AA gene expression in the 125-day PRN baboon lung at any time point, supporting the concept that multiple independent pathways are likely to regulate angiogenesis during BPD (7).

Impaired angiogenesis has been observed in many studies of infants and baboons with BPD (3, 7, 19, 47). In contrast, De Paepe and colleagues have described an increase in total pulmonary microvascular endothelial cell volume in long-term ventilated infants (8). However, the observations of "short-term ventilated preterm infants" by De Paepe and colleagues are completely consistent with the results of our current investigation. We have demonstrated that 125-day BPD animals have abnormal directed growth of capillary endothelial cells into developing secondary septa *in vivo*, although there is no appreciable difference in the volume of microvasculature in the lung tissue. Some studies have reported that angiogenesis may be an integral part of alveolarization (6, 48), and alveolarization is inhibited in BPD (5). To explore the possible regulation of angiogenesis by bombesin-like peptides, we used the Matrigel assay of capillary tubulogenesis. We demonstrated that bombesin increases capillary tubulogenesis of pulmonary microvascular endothelial cells cultured alone, consistent with other reports of proangiogenic effects of GRP (49). However, cocultured mesenchymal cells reverse bombesin-induced angiogenesis. GRP receptor gene expression in both pulmonary microvascular endothelial cells and in mesenchymal cells and the bombesin dose response suggest that GRP receptors mediated these effects. The effect of 2A11 in cocultures is believed to be due to GRP present in the Matrigel or in the fetal calf serum used in these cultures. The *in vivo* data are also consistent with this interpretation: the baseline block in angiogenesis occurs at the point of branching into the secondary septa. The defect in capillary outgrowth into secondary septa in BPD is coupled with the presence of SMA-positive cells throughout the interstitium but not at the septal tips. These diffusely distributed myofibroblasts in the interstitium are a likely source of antiangio-

genic factor(s) capable of inhibiting capillary sprouting into the developing secondary septa. A candidate antiangiogenic factor induced by GRP in fetal lung mesenchymal cells may be tissue inhibitor of metalloproteinase (TIMP)-1 (50), which has been implicated in the etiology of BPD (51). In our microarrays, TIMP-1 mRNA is increased in parallel with GRP in 125-day PRN lungs 24 hours after birth (Table 2). TIMP-1 is up-regulated by bombesin (52) and is also known to be antiangiogenic (53). Other potential antiangiogenic molecules that are up-regulated in the lung of 125-day PRN animals at 24 hours after birth include matrix metalloproteinase-14 (54) and plasminogen activator inhibitor (55).

In summary, 2A11 treatment improves alveolarization and capillary development in preterm baboons with modern-day BPD. The 2A11 did not have any adverse events in the present study, which provides important preclinical data leading up to trials with bombesin-like peptide blockade in premature infants. Cumulatively, our observations represent a paradigm shift in understanding the pathophysiology of BPD, and open new avenues for molecule-specific preventative therapies. In conclusion, GRP overproduction by pulmonary neuroendocrine cells could be an early maladaptive response of infants during the first days after birth that plays a key role in mediating the earliest stages of BPD. GRP blockade may provide a useful approach for preventive therapy of BPD in high-risk infants.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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