Bombesin-like peptides and receptors in normal fetal baboon lung: roles in lung growth and maturation

R. L. Emanuel, J. S. Torday, Q. Mu, N. Asokanathan, K. A. Sikorski, and M. E. Sunday. Bombesin-like peptides and receptors in normal fetal baboon lung: roles in lung growth and maturation. Am. J. Physiol. 21: L1003–L1017, 1999.—Previously, we have shown that bombesin-like peptide (BLP) promotes fetal lung development in rodents and humans but mediates postnatal lung injury in hyperoxic baboons. The present study analyzed the normal ontogeny of BLP and BLP receptors as well as the effects of BLP on cultured normal fetal baboon lungs. Transcripts encoding gastrin-releasing peptide (GRP), a pulmonary BLP, were detectable on gestational day 60 (ED60), peaked on approximately ED90, and then declined before term (ED180). Numbers of BLP-immunopositive neuroendocrine cells peaked from ED80 to ED125 and declined by ED160, preceding GRP-receptor mRNAs detected from ED125 until birth. BLP (0.1–10 nM) stimulated type II cell differentiation in organ cultures as assessed by [3H]choline incorporation into surfactant phospholipids, electron microscopy, and increased surfactant protein (SP) A- and/or SP-C-immunopositive cells and SP-A mRNA. BLP also induced neuroendocrine differentiation on ED60. Cell proliferation was induced by GRP, peaking on ED90. Similarly, blocking BLP degradation stimulated lung growth and maturation, which was completely reversed by a BLP-specific antagonist. The dissociation between GRP and GRP-receptor gene expression during ontogeny suggests that novel BLP receptors and/or peptides might be implicated in these responses.

INVESTIGATION OF HORMONAL REGULATION of lung maturation is of particular importance because it may reveal novel forms of treatment for preventing or minimizing the severity of respiratory distress syndrome (RDS) and bronchopulmonary dysplasia (BPD). Lung development, including morphogenesis, growth, and maturation, is a complex process involving interactions between multiple cell types and hormones (35). During development, the first epithelial cells to differentiate in both human and rodent fetal airways are the pulmonary neuroendocrine cells (PNECs) (11, 42). Mammalian bombesin (BN)-like peptide (BLP) was identified as the first neuropeptide immunoreactivity localized to PNECs (46), with the highest levels in human fetal lungs (39). Gastrin-releasing peptide (GRP) is the major endogenous pulmonary BLP implicated in promoting growth and maturation during fetal lung development in humans, rats, and mice (20, 36–38). In previous studies (33, 36), peak GRP transcript levels were demonstrated to occur in human and murine lungs during the canalicular period. Administration of BN to fetal mice during this time of development in utero or in organ culture leads to increased cell proliferation and differentiation (36). A blocking anti-BLP monoclonal antibody (2A11) blocks type II cell differentiation in fetal mice in utero (37) and in lung organ culture (36), indicating that BLP and hence PNECs can play a direct role in normal fetal lung development.

As a result of the collaborative program by the National Institutes of Health, the baboon model of BPD established by Coalson et al. (8) and Escobedo et al. (15) has been targeted as a tool in the investigation of chronic lung disease of newborns with interrupted gestation. Using this model, Sunday et al. (41) have recently demonstrated that BLP mediates lung injury in the hyperoxic baboon model of BPD. The present study was a comparative investigation of the ontogeny of BLP and BLP receptors in the developing baboon lung. We also compared the effects of BLP and other growth factors on cell proliferation and type II cell differentiation in fetal baboon lung organ cultures.

METHODS

Animals. Prematurely delivered baboons were provided by the Baboon Mononuclear Dysplasia Resource Core of the Southwest Foundation for Biomedical Research (San Antonio, TX). Normal fetal baboons were delivered by cesarean section at 60–180 days gestation (full term ~180 days). Only one animal was available at each of gestational ages 60, 80, and 90 days. Lung tissue was harvested aseptically: one portion (right upper lobe) was flash-frozen and stored at −70°C for RNA preparation, and another portion was fixed in buffer, processed, and cut into 4-μm sections and stained with hematoxylin and eosin for histological analysis. Both portions were used for RNA analyses. Total RNA was prepared from frozen fetal lung tissue with the TRI REAGENT (Molecular Research Center, Cincinnati, OH). Total RNA (10 μg/ml) was fractionated on 1% agarose-2.2 M formaldehyde denaturing gels and transferred to nitrocellulose by standard capillary transfer (12). After being baked, the blots were probed with cDNAs encoding human surfactant apoprotein (SP) A1 and SP-C. The cDNAs were obtained from non-small cell lung carcinoma line H441 mRNA by RT-PCR with the following primer pairs: SP-A 5′, CCCAGAGCCATGT, and 3′, TTCTCTCCAC-
GCTTCCA, and SP-C 5’; AGCAAGATGGATGTGGGCAG, and 3’; AGCTTAGCTAGGCACT. The resulting predicted cDNA fragments of 275 bp for SP-A1 (16, 22, 28) and 494 bp for SP-C (24, 48) were gel purified, eluted from agarose gel with Spin-X centrifuge tube filters (Corning Costar, Corning, NY), and labeled with the random-primed oligonucleotide method (Boehringer Mannheim, Indianapolis, IN). The blots were hybridized at 42°C overnight in 50% formamide, 2.5× Denhardt’s solution (0.2 g of Ficoll, 0.2 g of polyvinylpyrrolidone, and 0.2 g of BSA fraction V in 1 liter of diethyl saline-sodium citrate-0.1% SDS; and exposed to 50°C in 1× saline-sodium citrate-0.1% SDS; and exposed to Kodak Biomax film with an intensifying screen.

RT-PCR. RT-PCR was required to detect the low levels of mRNAs encoding GRP and GRP receptor (GRPR) in the available small tissue samples. cDNA was prepared, and semiquantitative RT-PCRs were carried out as previously described (40, 47) with 30 and 35 cycles of PCR for GRP and GRPR, respectively, and 18 cycles for 18S rRNA, each cycle including denaturation (0.5 min at 93°C), annealing (1.0 min at 55°C for GRP and 18S rRNA and 50°C for GRPR), and extension (3 min at 72°C). Synthetic oligonucleotide primers were designed to span at least one intron corresponding to the conserved sequences of human GRP (yielding a 194-bp product) (32), human GRPR (yielding a 388-bp product) (1), and human 18S rRNA (yielding a 567-bp product) (GenBank). Southern blots of the PCR products were probed with a corresponding end-labeled internal oligonucleotide specific for GRP, GRPR, or 18S rRNA. Primers were synthesized by Oligos Etc. (Wilsonville, OR). The sequences of the primers used were GRP 5’; AAGACACAACGTGGAGATG, GRP 3’; GAGAGTCTACCAACTTGGCC, and GRP probe, GAAGGTTTGGAACGTTTCGC; and 18S 5’; TAGCCTCTTGCTTACTCTCTG; GRPR 5’; TCTCCCGTGAACAGTACGGTTG, GRPR 3’; ATCTTCATACGAGGATGGAC, and GRPR probe, AGGTTTGGAAGCTTGGCC; and 18S 5’; TAGCCTCTTGCTTACTCTCTG; GTTATTGCTCAATCTCGGGTG. Total RNAs from human lung carcinoma cell lines were used as positive controls for RT-PCR: H128 (small cell lung carcinoma) for GRP, H720 (pulmonary carcinoid) for GRPR, and H441 (non-small cell lung carcinoma) for SP-A and SP-C. The human lung carcinoma cell lines were cultured as previously described (6). In compliance with guidelines for submission to the Journal of Physiology, it should be noted that specific testing for mycoplasma was not carried out in these short-term primary cultures.

In situ hybridization. Nonisotopic in situ hybridization was carried out to localize mRNAs in tissue sections. Three-millimeter-thick sections of fetal baboon lung were fixed for 3–4 h in 4% paraformaldehyde, and then transferred into 70% ethanol at 4°C overnight before being routinely processed into paraffin. A baboon GRPR cDNA was obtained by PCR with primers corresponding to human GRPR cDNA sequence (1). To prepare a template for cRNA probes, the resulting 388-bp PCR cDNA was subcloned into Bluescript SK+ and its identity was verified by dideoxy sequencing. cRNA probes were labeled with digoxigenin (Dig)-UTP with the Dig RNA labeling kit (Boehringer Mannheim) according to the manufacturer’s specifications. The remainder of the protocol was carried out as previously described (34) with the following modifications; hybridization was carried out at 55°C overnight with 25 ng Dig-labeled cRNA/µl hybridization buffer prepared according to the manufacturer’s specifications (Boehringer Mannheim). After being washed, the slides were developed for 4 h at 20°C with 1 µl/slide of anti-Dig antibody (alkaline phosphatase conjugated; Boehringer Mannheim), 5 µl normal goat serum, 1.5 µl 0.3% Triton X-100, and 0.5 ml 0.1 M Trizma base-0.15 M NaCl, pH 7.5 (TS). After a wash in TS for 5 min, 500 µl of nitro blue tetrazolium (NBT) color solution was applied to each slide from a mixture of 2.5 ml of TS-0.05 M MgCl2, 11 µl of 100 mg/ml of NBT, 9 µl of 5-bromo-4-chloro-3-indolyl phosphate (BCIP; Sigma, St. Louis, MO) and 2 drops (90 µl) of 50 mM levamisole. The NBT solution was incubated for 2–4 h in a dark humid chamber, the reaction was stopped with Tris-EDTA, and the sections were coated with aqueous mounting medium before placement of glass coverslips and viewing with a light microscope.

Immunoperoxidase and morphometric analyses. Three-millimeter-thick sections of fetal baboon lung were fixed for 18–24 h in 4% paraformaldehyde before being routinely processed into paraffin. Three-micrometer paraffin sections were dewaxed, and immunoperoxidase analyses carried out with a mouse monoclonal anti-BN antibody (2A11) and the avidin-biotin complex immunoperoxidase technique with diaminobenzidine as the substrate as previously described (41). All of the BLP-immunopositive neuroendocrine cells were counted on every slide in which there was comparable representation of proximal cartilaginous airways and terminal bronchioles near the pleural surface. The number of BLP-positive cells was normalized by expressing the value as a function of the total length of intrapulmonary bronchial plus bronchiolar (columnar) epithelium present on each slide. Immunostaining for SP-B and proSP-C was carried out with 1:500 dilutions of antisera generously provided by Dr. Jeffry Whitsett (Children’s Hospital Medical Center, Cincinnati, OH) with the avidin-biotin complex immunoperoxidase technique as described (41).

Lung organ cultures. Fetal lung was harvested at the time of cesarean section and collected at 4°C in Waymouth medium (GIBCO BRL, Life Technologies, Frederick, MD) supplemented with 5% fetal calf serum (Atlanta Biologicals, Atlanta, GA). After overnight shipping at 4°C, the tissue was chopped into 0.5-mm cubes and cultured in six-well plates with the same medium with and without added BLP or other agents. Cultures were grown for 48 h or 6 days at 37°C in 5% CO2-air on a rocking platform at 5 oscillations/min (36). Tissue DNA and protein content. [3H]thymidine incorporation into acid-precipitable DNA, DNA, and protein contents. On the day of harvest (after 44 h), the tissue was incubated with [3H]thymidine (4 µCi/ml) or [3H]choline (16 µCi/ml; NEN-Dupont, Boston, MA) for 4 h at 37°C in 5% CO2-air on a rocking platform before being harvested and analyzed for [3H]labeled DNA, DNA content, [3H]-labeled saturated phosphatidylcholine (Sat PC), and protein content. [3H]thymidine incorporation into acid-precipitable counts was carried out in quadruplicate as previously described (36). DNA was assayed after trichloroacetic acid precipitation with the method of Burton (5). For determination of the rate of surfactant phospholipid synthesis, lipids were extracted from cell homogenates with chloroform-methanol, and [3H]cholesterol incorporation into Sat PC was determined with the method of Bradford (2) with BSA (Bio-Rad, Hercules, CA) as a standard. Experimental values were normalized by defining the mean of the control groups as baseline and expressing the values as percent change above or below this.

Statistical analyses. Numerical data were analyzed with unpaired Student’s t-test, with values expressed as means ± SE.
RESULTS

Ontogeny of GRP and GRPR gene expression. GRP transcripts were detected by semiquantitative RT-PCR analysis as early as ED60, peaked on ED90, and declined to low levels during the rest of gestation and to undetectable levels in adult animals (Fig. 1A). In most cases, when the lung tissue was subdivided, GRP transcripts were more abundant in the distal than in the proximal region of the developing lung (Fig. 1A). In one of two samples on ED125 and the one subdivided ED140 lung, there was approximately twofold more GRP proximally compared with distally, likely representing the relative predominance of conducting airways in proximal tissue versus those in the distal lung, which at this stage includes predominantly primitive alveoli; GRP was expressed in PNECs in bronchi and bronchioles but not in normal alveoli (see below). These kinetics of GRP gene expression preceded peak GRPR gene expression, which was first clearly detected on ED125, peaked on ED160, and decreased to undetectable levels in adult animals (Fig. 1A). We were unable to detect mRNA for the BN-related peptide neumedin B (NMB) or for the other two cloned mammalian BLP receptors, the NMB receptor (NMBR) and BN-receptor subtype 3 (BRS-3), with 30–35 cycles for RT-PCR despite good detection of positive control mRNAs in baboon brain (for NMB), esophagus (for NMBR), and testis (for BRS-3) (data not shown). For comparison to known parameters of lung maturation, levels of SP-A and SP-C mRNAs were determined by Northern blot analysis (Fig. 1B). SP-A mRNA was first detected as a 2.2-kb transcript late in gestation, peaked on approximately ED160, and was expressed throughout adulthood. SP-C mRNA expression was first detected as a 1.0-kb transcript on ED90, peaked from ED160 to

![Fig. 1. Ontogeny of gastrin-releasing peptide (GRP) and GRP-receptor (GRPR) transcripts compared with surfactant protein (SP) A and SP-C in fetal baboon lungs. A: lungs were harvested from fetal baboons [embryonic day 60 (E60) to E175] and adult animals. Total RNA was prepared, and RT-PCR was carried out with GRP, GRPR, and 18S rRNA primers as described in METHODS. P, proximal; D, distal; T, term; AD, adult; +Con, positive control. RNAs obtained from human cell lines [small cell lung carcinoma H128 and pulmonary carcinoid cell line H720] were used as positive controls for GRP and GRPR gene expression, respectively. Nos. on right, size of transcripts. B: total RNA prepared from baboon lungs (E60 to adult animals) was used for Northern blot analyses as described in METHODS. RNA used for SP-A-positive control was obtained from cell line H441. 18S rRNA was estimated from ethidium bromide photograph of Northern gel. Nos. on right, size of SP-A and SP-C bands; nos. on left, location of RNA markers.](http://ajplung.physiology.org/doi/10.220.32.247/10.1152/ajplung.00005.2005)
ED175, and continued to be expressed abundantly during adulthood.

In situ hybridization analyses with antisense cRNA probes localized GRPR transcripts primarily to airway epithelium (Fig. 2A, black arrows), to undifferentiated (loose) mesenchymal cells surrounding developing blood vessels and Airways (Fig. 2, A and C, yellow asterisks) and throughout the alveolar interstitium (Fig. 2, A, between white arrows, and C, alv), and to alveolar macrophages (data not shown). In contrast, there was no mRNA signal with the corresponding sense control slides in immediately serial sections (Fig. 2, B and D).

BLP-immunopositive cells were detectable between ED60 and ED140 (Fig. 3). At ED60, single BLP-positive cells were localized only to large proximal airways that had begun to branch (data not shown). By ED80–90, BLP-positive PNECs occurred predominantly in small clusters of two to four cells within the basement membrane of the conducting airway epithelium (Fig. 3, A and B). A few larger PNEC clusters containing 8–14 PNECs were also present, usually at airway bifurcations (Fig. 3C). By ED125, most PNEC clusters were in bronchioles (Fig. 3D, arrows). The same cells immunostained for both BLP and protein gene product 9.5 immunoreactivity (data not shown). GRP mRNA in situ hybridization on thin serial tissue sections colocalized GRP mRNA in over half of the BLP-immunopositive PNEC clusters (Fig. 3E, thin arrow). GRP mRNA was additionally present in longer stretches of airway epithelium that was devoid of BLP or protein gene product 9.5 immunostaining (Fig. 3E, double-headed arrow). The corresponding GRP sense control section was negative (Fig. 3F).

Morphometric analysis of BLP-immunopositive cells in fetal lungs demonstrated the maximal number of BLP-positive PNECs per square centimeter of lung tissue at approximately ED80–90 (Fig. 3G), corresponding to the time of peak GRP gene expression (Fig. 1A). However, when the number of PNECs was normalized for the total length of airway epithelium per slide, the relative number of PNECs peaked on approximately ED125 (Fig. 3G). By ED160, the number of PNECs detected immunocytochemically declined to near zero.

Role of BLP in type II cell differentiation in fetal baboon lung. The role of BLP in fetal baboon lung maturation was explored with a simple in vitro system with lung tissue collected at various gestational ages and grown in culture for 48 h. Lung organ maturation, defined as type II pneumocyte differentiation with surfactant phospholipid synthesis, was measured as the amount of \(^{3}H\)choline incorporation into Sat PC. \(^{3}H\)choline uptake was optimized after 24 h in culture with 1 nM BN or GRP (Fig. 4, A and B, respectively). There was significant stimulation of choline uptake by GRP as early as ED60 (one animal harvested; data not shown). The maximal effect of BN was on ED140 and of GRP on ED90 (31 \pm 240 \pm 45\% increase over baseline, respectively; P < 0.001; Fig. 4, A and B, respectively). The BN-related peptide NMB (not present in normal lung; Table 1) had minimal effects on lung maturation (<15\%) at all gestational ages (data not shown).

Another amphibian BN-related peptide, \([\text{Leu}^8\text{-phyllolitorin}](L8\text{P})\), that King et al. (21) previously found to stimulate branching morphogenesis in murine embryonic lung buds also stimulated lung maturation in 48-h cultures at gestational ages ED125 and ED140, with the maximal effect observed with 10 nM on ED125 and 0.1 nM on ED140 (55 \pm 2 and 49 \pm 6\% increase over baseline, respectively; P < 0.001) as shown in Fig. 5A. The CD10 neutral endopeptidase (NEP) 24.11 inhibitor Sch-32615, which blocks hydrolysis of BLP and thus potentiates tissue levels of endogenous BLP (31), increased \(^{3}H\)choline incorporation at all gestational ages tested, with the maximal effect seen with 5 nM in ED90 cultures (46 \pm 2\% increase over baseline; P < 0.05; Fig. 5B). The specific BLP-receptor antagonist \([\text{Leu}^{13-\Psi(\text{CH}_2\text{NH})}\text{Leu}^{14}]\text{BN} (\text{L13BN})\) alone inhibited lung maturation maximally at 100 nM in ED125 cultures (31 \% compared with negative controls; P < 0.001; Fig. 5B). At ED140, the combination of L13BN and Sch-32615 resulted in net inhibition of \(^{3}H\)choline incorporation (28 \% compared with baseline; P < 0.001; Fig. 5B).

Dexamethasone (Dex), used as the positive control for choline uptake assays, increased choline uptake in lung explants at all gestational ages tested. The maximal effect of Dex was observed with 10 nM in cultures of ED140 lung (121 \% compared with baseline; Fig. 6A). The combination of low-dose BN (0.1 nM) and low-dose Dex (0.1 nM) resulted in the inhibition of the Dex effect at both ED125 and ED140 (Fig. 6B).

Epidermal growth factor (EGF), a second positive control for choline uptake assays, stimulated choline uptake in culture only on ED125, with 10 nM resulting in a maximal effect (39 \% increase over baseline; P < 0.001) as indicated in Fig. 6A. In combination, low doses of EGF (0.1 nM) and BN (0.1 nM) synergistically increased \(^{3}H\)choline incorporation in ED140 explants by 27 \% over baseline (P < 0.001) as indicated in Fig. 7B.

Additional effects of BLP on cell differentiation. To provide additional supporting evidence for the effects of BLP (BN or GRP) on cell differentiation in the fetal baboon lung, immunoperoxidase analyses were carried...
Fig. 3. Immunohistochemical analyses for bombesin-like peptide (BLP) and GRP-positive cells in normal fetal baboon lungs. A–C: immunoperoxidase staining was carried out on E80–90 fetal baboon lung with anti-bombesin antibody 2A11 (10) as described in METHODS. Most BLP-positive cells occurred in small clusters of 2 (A)–4 (B) cells. Infrequent (<10%) larger clusters of ~8–14 cells were also present on E80–90, some of which protruded into airway lumen (AW), mostly at airway bifurcations (C). Serial sections incubated with irrelevant control IgG1 MOPC21 were devoid of signal (data not shown). Original magnification, ×200. D–F: by E125, most BLP-positive clusters (D, arrows) occurred immediately adjacent to GRP mRNA-positive cell clusters (E, small arrow) in thin serial sections. Occasional BLP-positive cell clusters did not correspond to GRP mRNA-positive cells in serial sections (D: top arrow). In addition, many epithelial cells containing GRP mRNA did not contain detectable BLP immunoreactivity (E, double-headed arrow). There was no GRP mRNA signal in mesenchyme. No hybridization signal was observed with GRP sense cRNA probe (F). Original magnification, ×50. G: epithelial cells positively immunostained for BLP were quantitated and normalized, then plotted vs. gestational age. Two different methods for normalization were carried out: 1) for total amount of airway epithelium present (on E60–90; this included all primitive airways lined by columnar to cuboidal epithelium, in which it was not possible to distinguish between primitive alveoli and future conducting airways) and 2) for total area of lung tissue present (noninflated). PNEC, pulmonary neuroendocrine cell.
out after 6 days of culture with and without added BLP or Dex. Although 2-day cultures were optimal for the choline uptake assay, 6-day cultures were necessary to elicit a differentiated phenotype as detected by immunoperoxidase analyses. Increased PNEC differentiation was observed only in the youngest fetal lung available, of which we were able to obtain just one animal. Results from this one ED60 lung are given in Fig. 8. The most striking observation was an increased number of PNECs in the presence of GRP (peak at 0.1 nM; Fig. 8, A and C) or Dex (1 nM; Fig. 8C) compared with negative control cultures (Fig. 8, B and C). The total number of BLP-positive PNECs was quantitated and normalized for the total area of tissue present on each slide (with proximal and distal portions of the lung equally represented in every group) and for the total number of PNECs per airway (which was significantly increased only for 0.1 nM GRP; Fig. 8C). There was no effect of either GRP or Dex on the number of immunostainable PNECs between ED80 and ED160.

Table 1. The 3 major groups of bombesin-related peptides

<table>
<thead>
<tr>
<th>Group</th>
<th>Peptide Sequence</th>
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<tbody>
<tr>
<td>Bombesin family</td>
<td>1 Glu-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH₂</td>
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<tr>
<td></td>
<td>14 Met-Tyr-Pro-Arg-Gly-Asn-His-Trp-Ala-Val-Lys-His-Leu-Met-NH₂</td>
</tr>
<tr>
<td>Ranatensin family</td>
<td>1 Glu-Val-Pro-Gln-Trp-Ala-Val-Gly-His-Phe-Met-NH₂</td>
</tr>
<tr>
<td></td>
<td>11 Gly-Asn-Leu-Trp-Ala-Thr-Gly-His-Phe-Met-NH₂</td>
</tr>
<tr>
<td>Phyllolitorin family</td>
<td>9 [Leu8]Pl Glu-Leu-Trp-Ala-Val-Gly-Ser-Leu-Met-NH₂</td>
</tr>
<tr>
<td></td>
<td>9 [Phe8]Pl Glu-Leu-Trp-Ala-Val-Gly-Ser-Phe-Met-NH₂</td>
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GRP, gastrin-releasing peptide; RN-C, ranatensin C; NMB, neuromedin B; PL, phyllolitorin. Key COOH-terminal amino acid similarities are underlined. Nos. above amino acids, amino acid no.
Fig. 5. Effect of BLP-related peptides on fetal baboon lung maturation. Lungs collected at various gestational ages were cultured with and without BN-related peptide [Leu10]phyllotorin (L8PL; A) or CD10/neutral endopeptidase (NEP) inhibitor Sch-32615 and BN-receptor antagonist [Leu13, ψ(CH2)3Leu14]BN (L13BN; B) before being harvested for measurement of [3H]choline incorporation into Sat PC (see METHODS). Values are means ± SE representing pooled data for 1, 7, and 4 animals at 90, 125, and 140 days of gestation, respectively.

Fig. 6. Effect of dexamethasone (Dex) with and without BN on lung maturation. Lungs from various gestational ages were cultured for 48 h with varying doses of Dex (A) or low-dose (1 nM) Dex plus low-dose BN (B) before being harvested for measurement of [3H]choline incorporation into Sat PC (see METHODS). Values are means ± SE for 1, 7, 4, and 2 animals at 90, 125, 140, and 160 days of gestation, respectively.
Electron microscopy was also carried out on the same lung cultures, and morphometric analyses were carried out for early type II cell differentiation, defined as the proportion of lamellar body-positive cells per primitive alveolus (Fig. 8D). Both GRP (1 nM) and Dex (1 nM) induced a significant increase in the relative number of lamellar body-positive cells on ED60 (Fig. 8D) but not on ED140 (data not shown). There was no difference in the number of lamellar bodies per lamellar body-positive cell on either ED60 or ED140 (data not shown).

We also evaluated the effect of BLP on immunostaining for SP-A (Fig. 9, A and B) and SP-C (Fig. 9, C and D). BLP (1 nM) induced a marked increase in immunostaining for SP-A (Fig. 9B) and SP-C (Fig. 9D) compared with the same lungs cultured with medium alone (Fig. 9, A and C). The results in Fig. 9 are from one representative experiment of five separate experiments conducted at different times (each from a different ED125 fetal baboon), all with similar results. There was also increased SP-A and SP-C immunostaining from ED60 to ED90, but by ED140, the baseline levels were high and no appreciable further increase was apparent.

To determine whether the observed increase in SP-A and SP-C immunostaining was in part due to transcriptional regulation, RNA was prepared from three of five experiments of the same 6-day ED125 lung organ cultures. Compared with the negative control lung (Fig. 9E, lanes 1 and 4), there was a marked induction of SP-A mRNA in the presence of BLP (10 nM; lane 3) or Dex (10 nM; lane 5). In other experiments, the effective dose of BLP was between 1 and 10 nM BN or 1 and 10 nM L8PL (data not shown). There was no consistent effect of BLP or Dex on levels of SP-C mRNA in these cultures (data not shown).

Role of BLP in cell proliferation in fetal baboon lung. Lung growth was determined with [3H]thymidine incorporation into DNA, normalized for the total DNA content of the tissue samples. In organ cultures, BN and GRP induced cell proliferation on ED140, with a maximal effect at 0.01 nM (53 ± 36% increase over baseline; P < 0.05) as given in Fig. 10, A and B, respectively. However, the peak effect elicited by GRP occurred in the one ED90 lung available, at which time 0.1 nM GRP stimulated increased thymidine uptake (64 ± 7% over baseline; P < 0.001; Fig. 10B). In contrast, high-dose Dex (100 nM) treatment of the lung explants from the same animals resulted in inhibition of DNA synthesis by >70% on ED125 and ED160, but there was stimulation of thymidine uptake with low-dose Dex (1 nM) on ED140 (36 ± 4% increase over baseline; P < 0.001) as given in Fig. 10C. EGF, the positive control, stimulated thymidine uptake on ED140 and ED160, the maximal effect occurring on ED140
with 10 nM (52 ± 12% increase over baseline; P < 0.001) as summarized in Fig. 10D. Proliferating cell nuclear antigen immunostaining of tissue sections indicated a widespread induction of both epithelial and mesenchymal cell proliferation (data not shown), consistent with earlier observations by Sunday et al. (36) in fetal mouse lung.

Finally, combinatorial effects between low doses of BN and Dex or EGF on cell proliferation were analyzed on ED125 and ED140 as shown in Fig. 11. Dex alone (1 nM) slightly inhibited growth on ED125 and modestly increased thymidine uptake on ED140 (Fig. 11A), consistent with observations in Fig. 10 from a different set of animals. The combination of low-dose BN plus Dex was synergistic in inhibiting or augmenting thymidine incorporation on ED125 or ED140, respectively (Fig. 11B). BN alone at 0.1 nM or EGF alone at 0.1 nM had only marginal effects on thymidine uptake in these cultures (Fig. 11A). However, there was synergism between BN and EGF for either inhibition of proliferation on ED125 or stimulation on ED140 (Fig. 11B).

DISCUSSION

In our previous studies, we observed that BLP promotes fetal lung development in rodents and humans but mediates postnatal lung injury in hyperoxic baboons (20, 21, 36-38, 41). The present study demonstrates that BLP induces cell proliferation and type II cell differentiation in organ cultures of normal fetal
baboon lung. Lung organ maturation was evaluated with multiple approaches: [3H]choline incorporation into Sat PC, the rate-limiting step in surfactant phospholipid synthesis; electron microscopy for lamellar bodies; immunostaining for SP-A and SP-C; and mRNA levels for SP-A. Cell proliferation was assessed with [3H]thymidine incorporation into DNA. We correlate these effects with the kinetics of gene expression for GRP, GRPR, SP-A, and SP-C.

GRP gene expression was detectable in the one ED60 baboon lung available for study, then peaked at midgestation (ED80–90) and became undetectable after ED140 (Fig. 1). GRP expression was more abundant in distal than in proximal lung tissue, consistent with the major localization of BLP-immunopositive neuroendocrine cells in small bronchioles scattered throughout the distal parenchyma. These kinetics of GRP gene expression, peaking at midgestation (Fig. 2), match the time of a peak number of BLP-immunopositive cells per square centimeter of lung tissue. These results are essentially identical to previous reports on fetal lung from humans (33) and rhesus monkeys (23). Thus baboons demonstrate transient developmental expression of the GRP gene during the canalicular phase of
lung development, the same as all other species studied to date (23, 33, 36, 37). It should also be noted that GRP gene expression from ED60 to ED125 precedes that of SP-A and SP-C, two markers of type II cell differentiation, both of which peak closer to birth (approximately ED160). Our results on the ontogeny of SP-A mRNA are consistent with observations from another laboratory (22).

Treatment of fetal baboon lung explants with exogenous BLP results in increased parameters of both maturation and growth. Maturation or type II cell differentiation, evaluated with \[^{3}H\]choline incorporation into Sat SPC, was stimulated by BN or GRP (collectively termed BLP as shown in Table 1), with maximal effect occurring on approximately ED90. GRP-induced maturational effects were also observed in ED60 and ED80 lungs. These observations are especially intriguing considering the fact that GRPR transcripts were extremely low in the same ED60–90 lung specimens. GRPR gene expression was absent or only marginally detectable in fetal baboon lungs before ED125, attaining maximal levels on ED160 and becoming undetectable after birth. These data suggest the possible presence of a novel mammalian BLP receptor in the midgestation baboon lung mediating BLP effects during the canalicular period. We did not detect transcripts encoding NMBR or BRS-3 in these normal baboon fetal lung samples using the given RT-PCR conditions. It is unlikely that RNA degradation was a factor in reducing detectable levels of these mRNAs because all tissue was snap-frozen at the time of harvest and no RNA degradation was evident on ethidium gels.

It is also interesting that there was a lack of GRP effects in cultures of ED125–160 lungs despite significant effects of the amphibian peptide BN, suggesting that there might be a novel mammalian BN-related peptide better capable of interacting with the GRPR expressed during this time period. We did not detect transcripts encoding NMB, the only other cloned mammalian BN-related peptide, in the normal fetal baboon lung. There remains the possibility that there could be another bioactive peptide such as a mammalian phyllolitorin acting at the GRPR (21), especially considering the immunological cross-reactivity of phyllolitorins with anti-BN antibodies. Phyllolitorins are able to elicit potent responses in the mammalian lung (14, 21). This possibility is even more feasible considering our observation that fetal baboon lung maturation was significantly stimulated from ED125 to ED140 by the amphib-
ian peptide L8PL, with a maximal effect occurring with 10 nM L8PL on ED140.

BLP induced differentiation of multiple epithelial cell types in the fetal lung. In the earliest lung sample collected (ED60), BLP induced differentiation of single PNECs, supporting the concept that this peptide can act as an autocrine differentiation factor. In addition to stimulation of choline uptake, BLP (both BN and L8PL) induced other parameters of type II cell differentiation. There was increased immunostaining for SP-A (in Clara cells and type II cells) and SP-C (type II cell specific) as well as increased mRNA encoding SP-A on ED125. These data suggest that upregulation of SP-C genes is occurring primarily at the posttranslational level and/or that increased SP-C mRNA levels are not sustained for the full 6 days in culture. Divergent regulation of SP-A and SP-C genes is a frequent observation (20). The timing of BLP-induced cell differentiation according to different assays appears to depend on the status of endogenous cell differentiation in lung tissue at different gestational ages. Thus differentiation of PNECs normally precedes differentiation of all other epithelial cell types in the lung, and SP-C gene expression precedes that of SP-A in vivo. BLP appears to function more to induce the expression of several differentiated phenotypic features rather than simply enhancing the level of established features.

In previous experiments with murine and human fetal lungs, King et al. (20) and Sunday et al. (38) observed increased cell proliferation and differentiation using the CD10/NEP inhibitor Sch-32615. CD10/NEP hydrolyzes BLP, leading to a loss of BLP bioactivity (4, 31). In the present study, specific CD10/NEP inhibition by low-dose Sch-32615 (5 nM) stimulated thymidine and choline uptake in fetal baboon lungs. These effects were neutralized by the specific BN-receptor antagonist L13BN, similar to observations in human and murine fetal lungs (20, 38). Treatment with L13BN alone resulted in decreased baseline choline incorporation at 125 days gestation, suggesting that the GRPR is likely to play a role at this time in ontogeny. These observations indicate that endogenous BLP or closely related peptides such as potential mammalian phyllolitorins are required for the observed potentiation of cell proliferation and type II cell differentiation resulting from CD10/NEP inhibition. In murine experiments with in utero inhibition of CD10/NEP, King et al. (20) observed increased mRNA encoding SP-A, similar to the present data on BLP-treated fetal baboon lung.

Consistent with previous work in primates and rodents (17, 25, 30), the positive controls EGF and Dex augmented baboon lung maturation. It was previously reported (23) that BN can induce DNA synthesis in rhesus monkey fetal lung cultures at midgestation. We similarly observed significant stimulation of thymidine incorporation with low-dose GRP (<1 nM) in the ED90 baboon lung; 1 nM GRP inhibited growth at this gestational age in the baboon, likely due to receptor downregulation by the higher dose. In contrast, at later ages of gestation, BN and/or GRP either inhibited growth (on ED125 and ED160) or had marginal effects on stimulating proliferation (on ED140). Such diverse effects argue for the presence of additional BLP receptors in addition to the GRPR functioning in these systems. Alternatively, signal transduction between ED125 and ED160 could favor cell differentiation over proliferation. It should be noted that observed small effects on lung growth are of questionable clinical significance, even the inhibitory effects of higher doses of Dex (18, 19), because this appears to be reversible inhibition (27). It is lung immaturity rather than lung size per se that appears to present the major clinical problem in preterm infants with RDS as evidenced by the dramatic improvement in infant survival with the advent of exogenous surfactant replacement therapy (7).

The synergistic effect of low-dose BLP with low doses of either EGF or Dex suggests that all three factors act via distinct receptors to stimulate the same common final pathway. In contrast to the cloned seven-transmembrane-spanning, G protein-coupled BLP receptors (9), the EGF receptor is a protein tyrosine kinase (13), whereas glucocorticoid receptors are present in the cytoplasm and translocate to the nucleus after ligand binding (29). It appears that EGF (30) and Dex (26)
induce type II pneumocyte differentiation by stimulating production of fibroblast-derived factors, which, in turn, trigger type II cell differentiation. Consistent with the hypothesis that BLP similarly acts via a fibroblast intermediary, we localized GRPR transcripts primarily to interstitial fibroblasts in the developing alveoli (Fig. 2). In a separate study of fetal rat lungs, Brimhall et al. (3) have recently reported that fibroblasts are required for the effect of BLP on type II cell maturation. It is likely that a similar mechanism of action is operating in the developing baboon lung.

The present study demonstrates that pulmonary BLP plays an important role in promoting growth and maturation of the fetal baboon lung, especially during the canalicular period at midgestation. This is important because baboons provide the only animal model of RDS that is clinically and pathologically similar to acute RDS in human infants followed by BPD (9, 15). Postnatally, BLP appears to act as a proinflammatory mediator in the hyperoxic baboon model of BPD in which anti-BLP-blocking monoclonal antibodies greatly diminish the severity of chronic lung disease (41). In conclusion, these observations indicate that BLP functions to promote lung embryogenesis during stages preceding clinical viability but could, in fact, be harmful in premature infants when sustained postnatally.

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REFERENCES


