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Original Article

Lung fluid restriction affects growth but not airway branching of embryonic rat lung

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ABSTRACT During the later stages of fetal life lung growth and development is dependent upon a variety of factors, including a normal amount of liquid within the lung's lumen. To investigate whether embryonic lung epithelium secretes fluid and whether lung liquid is essential for proper embryonic airway lung growth and branching, we incubated 12-day rat lung primordia (term= 22 days) in submersion culture in serum-free medium for 48 h in room air (21% O_2/5% CO_2). Under these conditions, lung growth and branching proceeded but at a slower rate when compared to growth and branching *in vivo*. Neither addition of serum nor incubation in a fetal O_2 concentration (≈3% O_2) changed the growth rate or the degree of branching *in vitro*. The luminal area of the explant increased progressively with time in culture. Inhibitors of active Cl^- secretion (200 µM bumetanide and 1 mM furosemide) significantly reduced the lumen size compared with control. A similar effect was noted with lung explants of 13-15 days of gestation. Branching morphogenesis was not impaired by lung fluid reduction. Reduction of luminal liquid significantly increased DNA synthesis of 12-day embryonic lung explants, but this effect of bumetanide and furosemide on DNA synthesis was reversed when 13-15 day lung explants were used. These data suggest that embryonic lung epithelium secretes fluid and that the secretion is chloride dependent. Lung fluid is involved in controlling lung growth but not branching of the embryonic rat lung

KEY WORDS: *lung fluid, lung growth, branching morphogenesis*

Introduction

A proper lung fluid and transpulmonary pressure balance appears to be essential for normal fetal lung growth during the later stages of fetal life. Studies with fetal lamb have demonstrated that distension of the lung by tracheal ligation stimulates (Alcorn *et al.*, 1977; Fewell *et al.*, 1983), while tracheal drainage inhibits (Alcorn *et al.*, 1977), lung growth and development. Obstruction of the trachea and bronchus in the human fetus has been associated with lung hyperplasia (Griscom *et al.*, 1969). The rate of lung fluid production also influences the amount of amniotic fluid volume. Clinically, the lack of amniotic fluid (oligohydramnios) has been associated with lung hypoplasia (Perlman and Levin, 1974; Thomas and Smith, 1974), while experimental introduction of oligohydramnios by amniotic drainage in fetal animals (Nakayama *et al.*, 1983; Moessinger *et al.*, 1986) reduces lung growth. Subsequently restoring amniotic fluid volumes reverses the observed changes (Nakayama *et al.*, 1983). Although the experiments of nature and whole animal experiments do demonstrate a relation between lung fluid and normal fetal lung growth, its influence on embryonic lung morphogenesis remains to be elucidated.

Several studies have shown that airway epithelial cells (Zeitlin *et al.*, 1988; McCray, Jr. and Welsh, 1991) can secrete fluid during development. Active Cl^- transepithelial transport appears to be the major force for fluid secretion (Gatzy, 1983; Welsh, 1986; Lahf *et al.*, 1987; Hanrahan and Tabcharani, 1989; O'Brodovich, 1991; Wagner *et al* , 1991). Micropipette electrophysiological studies of developing acinar tubules have suggested that the bronchopulmonary epithelium from the pseudoglandular stage of rat (Krochmal *et al.*, 1989) and human (McCray Jr. *et al.*, 1992) lung development actively secretes fluid by Cl^- dependent mechanisms. To our knowledge, there is no information regarding ion transport by embryonic lung epithelium.

To investigate the role of lung fluid in embryonic lung development, we used Cl^- transport inhibitors in cultures of 12-day embryonic rat lung explants in a serum-free, chemically defined medium. We report that embryonic lungs secrete lung fluid and that inhibitors of Cl^- secretion reduce lumen volume. Reduction of fluid volume does not affect the initial process of branching

Abbreviations used in this paper: DMSO, dimethyl sulfoxide; DNA, deoxyribonucleic acid; FBS, fetal bovine serum; LDH, lactate dehydrogenase; PDGF, platelet-derived growth factor; IGF, insulin-like growth factor.

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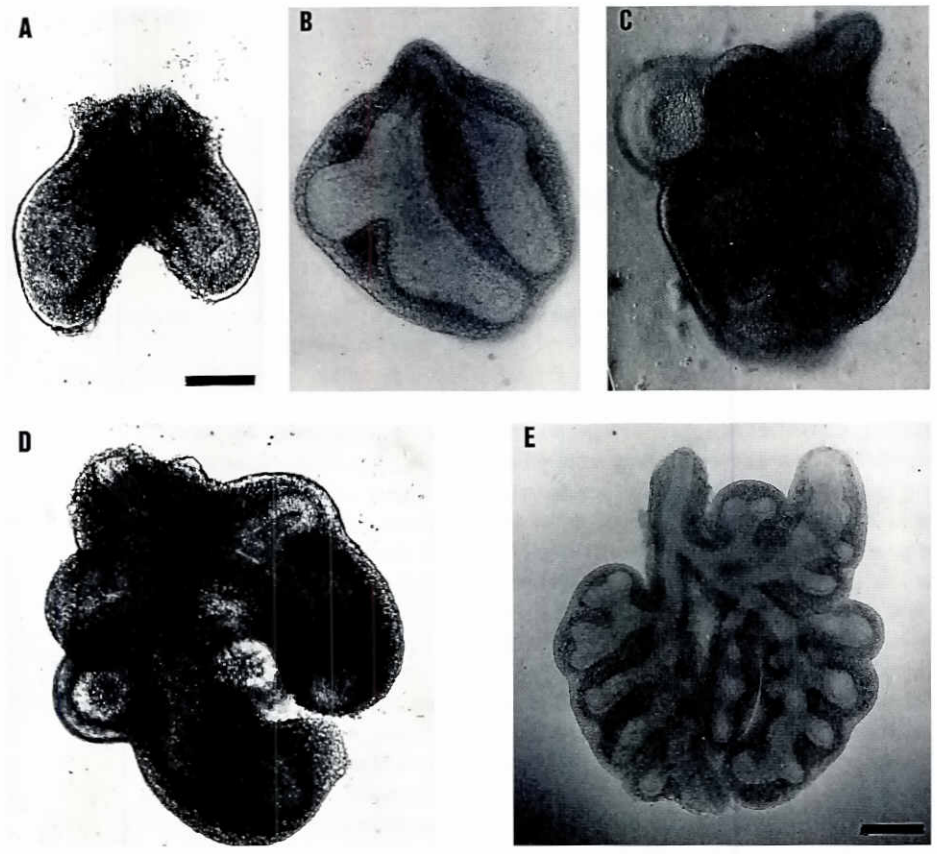

Fig. 1. Branching morphogenesis of embryonic rat lung *in vitro*. **(A)** *12-day unbranched lung immediately after dissection.* **(B)** *12-day lung after 24 h incubation in serum-free medium.* **(C)** *12-day lung after 48 h incubation in serum-free medium.* **(D)** *13-day and* **(E)** *14-day freshly isolated lungs. Scale bar, 100 μm (A-D); 250 μm (E).*

and stimulates growth of the early embryonic lung. However, the growth of lung explants obtained at later gestational ages is significantly reduced by inhibiting fluid secretion. These data suggest that maturational changes determine the interaction of lung fluid and lung growth.

Results

Branching morphogenesis

Serumless culture

We first examined whether 12-day embryonic lung rudiments branched in culture without serum supplementation. A 12-day embryonic rat lung consists of a primitive trachea and left and right main stem bronchi (Fig. 1A). No rudimentary lobar bronchi are present yet. As can be seen in Figure 1B and C, lobar and segmental bronchial branching were evident after 24- and 48-h culture in serum-free medium. However, the number of terminal buds of the cultured explants was significantly smaller than the developmentally stage-matched lungs *in vivo* (Fig. 1D and E and Table 1). This suggests that the initial process of branching takes place *in vitro* but at a slower rate than *in vivo*. Twelve-day embryonic lung explants cultured in the presence of 2 % (v/v) FBS were not different from explants cultured in serum-free medium (Fig. 2A and B), implying that serum factors do not influence the initial branching process. In addition, no significant morphological differences were observed between explants incubated in gas tensions mimicking the normal fetal oxygen concentration [3% O_2] and explants incubated in room air [21% O_2] (Fig. 2C and D). Furthermore, branching morphogenesis was not different between submersion and air-liquid interphase cultures (not shown).

The terminal branches (buds) of the 12-day lung explants, cultured as a submersion culture, had a "cystic-like" appearance (Fig. 1B and C). Morphometric analysis revealed that the lumen area of a 12-day lung explant increased with time in culture (Fig. 3). This suggests that fluid is secreted into the lumen. When lungs, in which the secondary process of branching had already started *in vivo* (14 and 15-day lung), were cultured as submersion cultures in serum-free medium the cystic appearance of the terminal branches was less evident (Fig. 4). This suggests that either the airway epithelium lining the secondary and tertiary bronchi is less active in fluid secretion compared to the epitheli-

TABLE 1

COMPARISON OF LUNG BRANCHING MORPHOGENESIS *IN VITRO* AND *IN VIVO*

Gestational age	number of terminal buds	
	in vitro	*in vivo*
12-day lung	2	2
13-day lung	5.95±0.16	8.05±0.14*
14-day lung	7.25±0.20	24.80±0.50*

Values are means ±SE of at least 8 separate experiments. A 13- and 14-day lung *in vitro* refers to a 12-day lung primordia in culture for 24 and 48 h, respectively. *$P<0.05$ compared with *in vitro*.

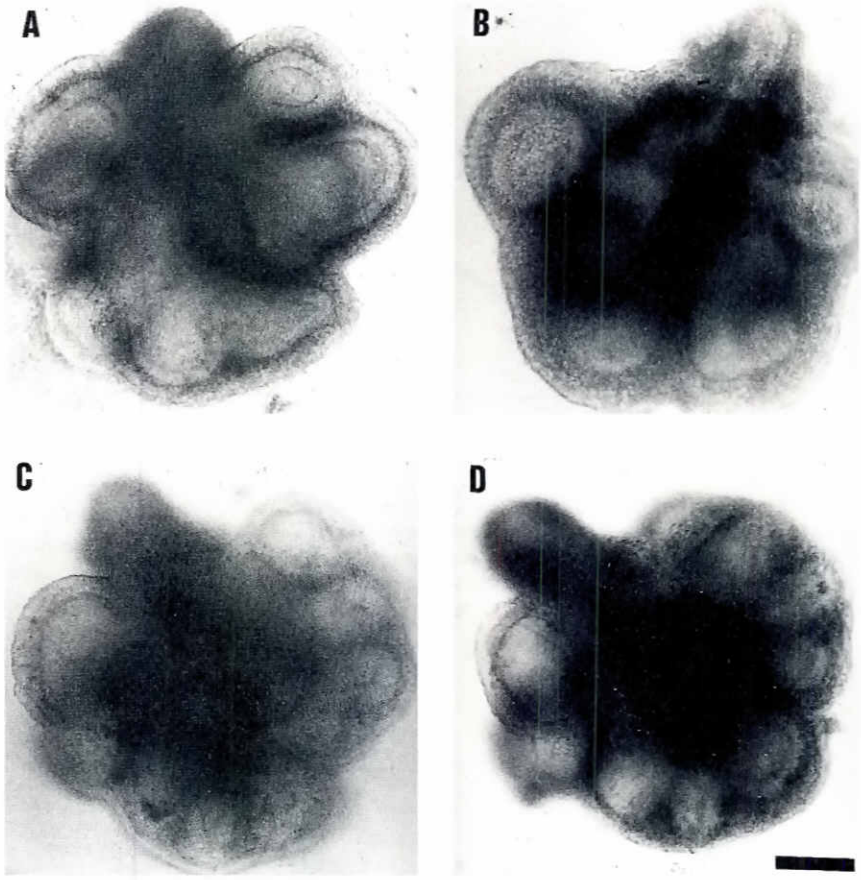

Fig. 2. Effect of serum and oxygen on branching morphogenesis *in vitro*. *Twelve-day lung explants were cultured for 48 h in the absence* **(A)** *or presence* **(B)** *of 2% (v:v) FBS or in serum-free medium in an atmosphere containing 3%* **(C)** *or 21% O_2* **(D)**. *Scale bar, 100 μm.*

um lining the main bronchi or that these more distal airways are less distensible.

Inhibition of Cl^- secretion

To determine whether luminal liquid secretion had an influence on the early lung branching process, lungs were incubated with bumetanide or furosemide. These agents stop active Cl^- secretion by inhibiting Na^+-coupled Cl^- co-transport across the basolateral cell membrane (Widdicombi *et al.*, 1983; Cassin *et al.*, 1986). Bumetanide reduced lumen patency in a dose-dependent manner (not shown). The lumen area of 12-day lung explants was significantly reduced when cultured in the presence of 200 μM bumetanide (Figs. 3 and 5A). Morphometrical analysis showed that the mesenchymal and epithelial tissue mass were not affected by bumetanide treatment (Fig. 5B). A similar dosage of bumetanide (200 μM) also reduced lumen patency in lungs of later gestational ages (Fig. 6). Similar results were seen using 1 mM furosemide, another inhibitor of Cl^- secretion (Cassin *et al.*, 1986). Furosemide treatment reduced lumen area size to a similar extent as 200 μM bumetanide (not shown). Both inhibitors did not increase the LDH release into the medium (<5% of control lung explants), indicating that the inhibitory effect on luminal fluid secretion was not due to cytotoxicity of the Cl^- inhibitors. The reduction in luminal area by bumetanide did not affect branching of 12-day embryonic lung *in vitro* (Fig. 5A and Table 2). Bumetanide treatment of lung rudiments of 13-15 days of gestation also did not visibly influence the branching of these lungs (Fig. 6).

Lung growth

[^{3}H]Thymidine incorporation into DNA

To evaluate whether lumen patency affected lung growth, we measured DNA synthesis by [^{3}H]thymidine incorporation. The reduction in luminal area of 12-day lung rudiments induced by 200 μM bumetanide or 1 mM furosemide was accompanied by a significant increase in DNA synthesis when compared to control explants (Fig. 7A). In contrast, DNA synthesis significantly decreased in bumetanide-treated lung explants of 13-15 days of gestation (Fig. 7B). Furosemide treatment had similar effects on DNA synthesis as bumetanide (not shown).

Autoradiography

To determine the cell type affected by bumetanide, the number of cells labeled with thymidine was assessed for control and bumetanide treated explants. There was a significant increase (>40%) in the number of labeled cells in the bumetanide-treated 12-day lung explant cultures (Table 3). Although the labeling index (LI) increased for both epithelial cells and mesenchyme, the increase in the number of labeled epithelial cells was greater than that of mesenchymal cells (increase in LI: 80 vs. 27 %, epithelial cells vs. mesenchyme). The labeling index of 15-day

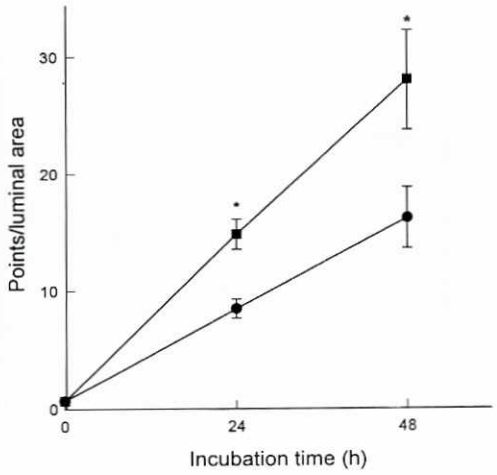

Fig. 3. Morphometrical analysis of the luminal area of 12-day lung primordia after 24 and 48 h culture in the presence of vehicle (control) or 200 μM bumetanide. *Results are expressed as mean ±SE of point counts per lung from 6 separate experiments.:* ■, *control;* ●, *bumetanide. * P<0.05 compared with control.*

lung explants significantly decreased (>30%) by bumetanide treatment. Again, epithelial cells appeared to be more affected by the lumen reduction than mesenchymal cells (decrease in LI: 35 vs. 25 %, epithelial cells vs. mesenchyme).

Histology

Independent of gestational age and treatment, many mitotic figures were present in the lung explants after a 48 h culture (Fig. 8). No necrotic tissue was observed. Thus, the tissue appears viable throughout the culture period. It can also be seen that bumetanide-treated explants had a reduced luminal area size when compared with control explants.

Discussion

Our findings suggest that the amount of fluid within the embryonic lung's lumen affects its growth but not branching. Embryonic lung rudiments of 12-day rat fetus were cultured in a simple serum-free and chemically-defined medium. Under these experimental conditions, lung branching occurs but at a slower rate than *in vivo*. Addition of 2% FBS to the culture medium did

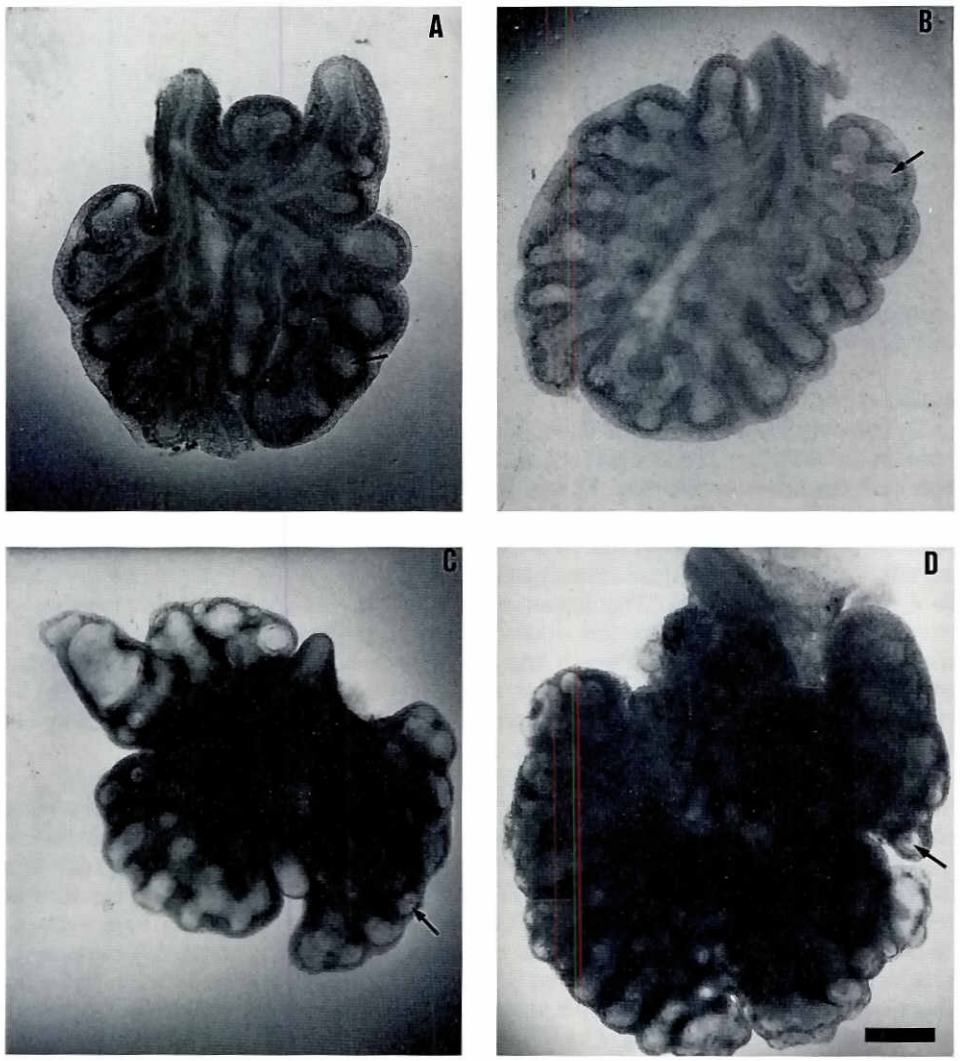

Fig. 4. Branching morphogenesis of 14- and 15-day fetal rat lung *in vitro*. **(A)** *14-day lung immediately after dissection.* **(C)** *14-day lung after 48 h incubation in serum-free medium.* **(B)** *15-day lung immediately after dissection.* **(D)** *15-day lung after 48 h incubation in serum-free medium. Arrows show lumen in terminal buds. Scale bar, 200 μm.*

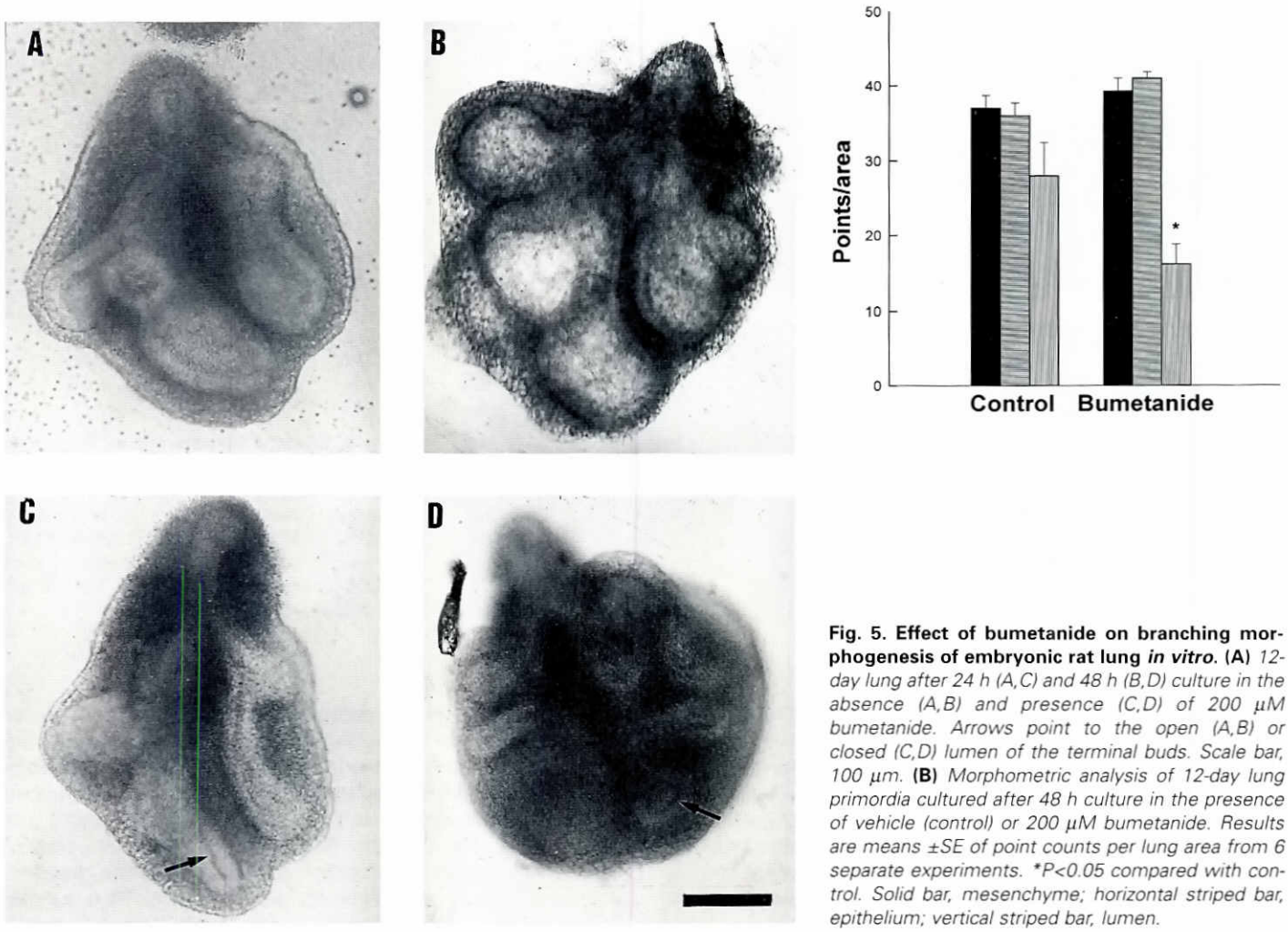

Fig. 5. Effect of bumetanide on branching morphogenesis of embryonic rat lung *in vitro*. **(A)** *12-day lung after 24 h (A,C) and 48 h (B,D) culture in the absence (A,B) and presence (C,D) of 200 μM bumetanide. Arrows point to the open (A,B) or closed (C,D) lumen of the terminal buds. Scale bar, 100 μm.* **(B)** *Morphometric analysis of 12-day lung primordia cultured after 48 h culture in the presence of vehicle (control) or 200 μM bumetanide. Results are means ±SE of point counts per lung area from 6 separate experiments. *P<0.05 compared with control. Solid bar, mesenchyme; horizontal striped bar, epithelium; vertical striped bar, lumen.*

not significantly alter either the pattern or rate of the branching process in 12-day lung rudiments compared to those cultured in serumless medium. This suggests that the initial airway branching process is controlled by intrinsic factors. Recent studies have implicated growth factors as intrinsic regulators of the branching process (Snead *et al.*, 1989; Ganser *et al.*, 1991; Warburton *et al.*, 1992, 1993; Souza *et al.*, 1994). The observation that *in vitro* branching morphogenesis of 12-day lung rudiment took place at a slower rate than *in vivo* suggests that additional factors of either fetal or maternal origin are required to sustain the normal rate of early lung branching. The fetal O_2 concentration did also not appear to influence the initial process of branching. *In vitro* branching morphogenesis under serum-free conditions has previously been reported for 11-day embryonic mouse lung (Jaskoll *et al.*, 1988; Ganser *et al.*, 1991; Warburton *et al.*, 1992). However, the process of secondary bronchial branching has already started in 11-day embryonic mouse lung rudiments while it is not evident in 12-day embryonic rat lung rudiments. When we used rat lung explants of 14-15 days gestation, we observed that *in vitro* branching was similar to that *in vivo*. This suggests that the early branching process is regulated in a different way than the branching process taking place at later gestation.

Under the experimental conditions used in this study, we found that the amount of embryonic rat lung luminal fluid decreases markedly by the Na^+-coupled Cl^- transport inhibitors, bumetanide and furosemide. This finding supports the idea that lung liquid secretion is driven by Cl^- transepithelial transport (Olver and Strang, 1974; Olver, 1977, 1983). The possible side effect of these agents, a weak inhibition of carbonic anhydrase activity, is quite unlikely considering that similar results were obtained with both inhibitors. Furosemide was used at a 5-fold higher dosage than bumetanide and the latter has a very low affinity for carbonic anhydrase (Vogh and Langham, 1981). Although active ion transport and fluid secretion has also been reported for fetal human lung epithelium (McCray *et al*, 1992), to our knowledge this is the earliest *in vitro* demonstration of lung fluid secretion.

Experimental evidence and experiments of nature have demonstrated the importance of an adequate lung fluid balance within the respiratory tract during fetal lung development (Kitterman, 1988; Nicolini *et al.*, 1989). Lung liquid appears to act as an internal factor around which the lung develops and its expansion or reduction in the ovine fetus has been shown to impair lung development (Alcorn *et al.*, 1977). In accordance

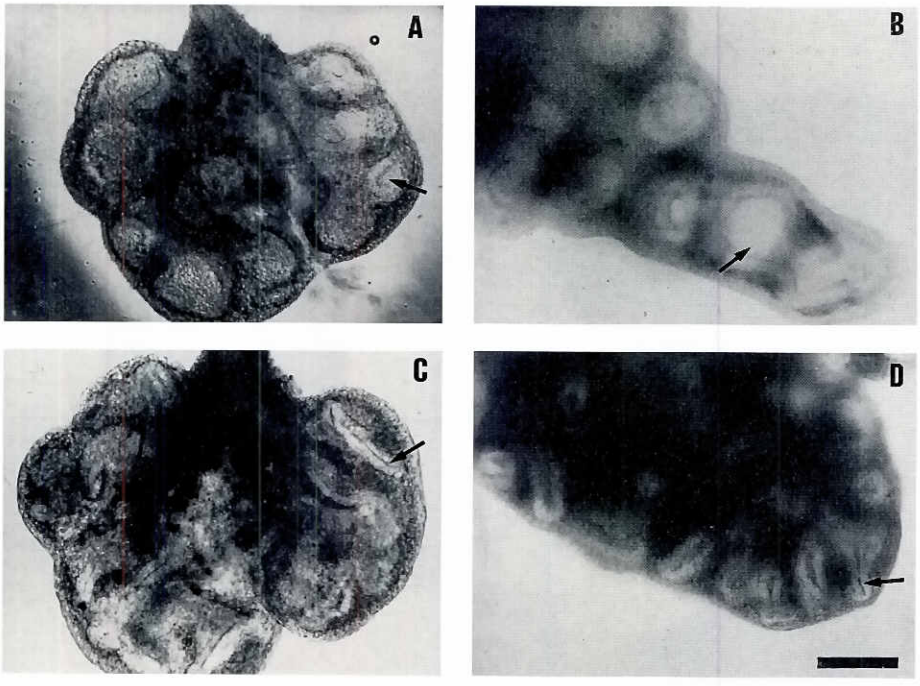

Fig. 6. Effect of bumetanide on branching morphogenesis of 13-day and 15-day *in vitro*. **(A,C)** *13-day and* **(B,D)** *15-day lung explants cultured for 48h in the presence of vehicle* **(A,B)** *or 200 μM bumetanide* **(C,D)**. *Arrows point to open* **(A,B)** *or closed lumen* **(C,D)** *of the terminal buds. Scale bar, 120 μm (A and C); 100 μm (B and D).*

with these previous studies, *in vitro* luminal liquid restriction of 13-15d lung explants with bumetanide resulted in a significant reduction of DNA synthesis. In contrast, DNA synthesis of a 12-day lung explant was stimulated by luminal liquid reduction, suggesting that overdistension of the lung at this early gestation is negatively affecting lung growth. We, however, cannot rule out the remote possibility that furosemide and bumetanide directly stimulate DNA synthesis at this specific stage of lung development. Since the 12-day lung explants had a "cystic-like" appearance, however, it is also possible that the control explants were overextended, which has been shown to stimulate lung growth at later gestation (Kitterman, 1988; Hooper *et al.*, 1993). Branching morphogenesis was not affected by the reduction in lung liquid pressure, implying that lung liquid mainly affects growth, but not the degree of branching, of embryonic rat lung. This suggests that intrinsic factors, such as growth factors, which control either lung growth or branching, are affected by distending forces in a different way. Several lines of evidence suggest that growth factor expression during fetal lung development is regulated by physical forces. We have recently demonstrated that fetal rat lung cell proliferation is stimulated by mechanical strain (Liu *et al.*, 1992) and that mechanical strain induces the production of platelet-derived growth factor B (Liu *et al.*, 1995). Hooper *et al.* (1993) have reported that altering lung expansion in fetal sheep has a rapid effect on DNA synthesis and IGF-II gene expression. In addition, shear stress has been shown to induce PDGF-B expression in endothelial cells (Hsieh *et al.*, 1992; Resnick *et al.*, 1993). The mechanism by which distending forces affect gene expression is not known. Recently, Resnick *et al.* (1993) identified a cis-acting fluid shear-stress-responsive element on the PDGF-B promoter. The putative transcription binding site is also present in the promoter region of TGFß1, which is also induced in endothelial cells by shear stress. It is possible that distending forces act on similar response elements on the growth factor promoters. Another possibility is that distending forces alter the configuration of the genome leading to alterations in accessibility of transcriptional binding sites on the growth factor promoters. However, distending forces may also affect RNA stability and protein translation. Recently, Singhvi *et al.* (1994) have demonstrated that limiting cell extension reduces hepatocyte growth in a defined medium saturated with growth factors. Thus, luminal liquid restriction may reduce the shape of fetal lung cells, thereby decreasing DNA synthesis.

In summary, the results of our studies indicate that embryonic rat lung secretes liquid by Cl^- dependent mechanisms and that lung liquid is essential for embryonic lung growth but not for lung branching. The mechanism(s) by which the distending forces are transduced is unknown and its elucidation should provide insights in normal and abnormal fetal lung growth.

TABLE 2

EFFECT OF BUMETANIDE ON 12-DAY LUNG BRANCHING MORPHOGENESIS *IN VITRO*

Groups	n	culture duration	number of terminal buds
12-day *in vivo*	8	—	2
Control	10	24 h	5.50±0.23
Bumetanide	6		5.33±0.71
Control	9	48 h	8.50±0.53
Bumetanide	8		8.44±0.44

Values are means ±SE; n= number of separate cultures.

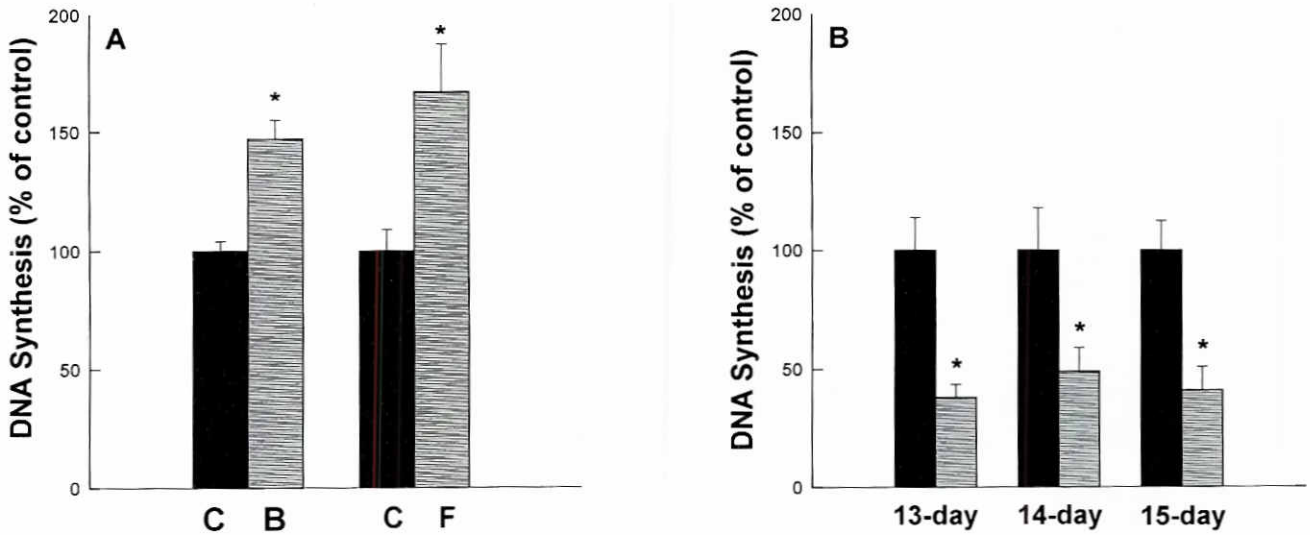

Fig. 7. Effects of bumetanide and furosemide on DNA synthesis. (A) *12-day and* **(B)** *13-15 day lung explants were cultured for 48 h in the presence of either vehicle (control), 200 μM bumetanide or 1 mM furosemide. DNA synthesis was measured by [³H]thymidine incorporation into DNA. Data are measured as dpm/μg protein and expressed as % of control. Results are mean ±SE of n= 9 (bumetanide) and n= 3 (furosemide) separate experiments. *P<0.05 compared with control. Solid bar, control; striped bar, bumetanide (B) or furosemide (F).*

Materials and Methods

Materials

Female (200-250 g) and male (250-300 g) Wistar rats were obtained from Charles River (St. Constant, Quebec, Canada) and were bred in the animal facilities of the Hospital for Sick Children, Toronto. Culture media, fetal bovine serum and antibiotics were purchased from Gibco (Grand Islands, NY, USA). Ascorbic acid, bumetanide and furosemide were from Sigma (St. Louis, MO, USA). Four well culture dishes were from Nunc (Intermed, Denmark) and culture inserts from Millipore (Bedford, MA, USA). [³H]thymidine was from Amersham (Arlington Heights, IL, USA).

Explant submersion cultures

At designated days of pregnancy (vaginal plug= day 0), dams were killed by diethylether excess. Embryos were aseptically removed from the uterine horns and developmentally staged by external features. Lung rudiments were microsurgically dissected from the embryos under a dissection microscope. For explant culture, lungs were transferred to culture inserts (pore size 0.4 μm) and incubated as submersion cultures in serum-free medium consisting of DMEM/F-12 supplemented with 100 μg/ml streptomycin, 100 units/ml penicillin and 0.25 mg/ml ascorbic acid, pH 7.4. Lung rudiments were incubated for 48 h at 37°C in 95% air/5% CO_2 in the presence of bumetanide, furosemide or vehicle (1:1000 (v/v) DMSO). In separate experiments, explants were cultured in a gas phase of 3% O_2:5% CO_2:92% N_2, to mimic the normal fetal arterial oxygen concentration of ≈20 mm Hg. To assess the effect of serum on branching morphogenesis, 12-day embryonic lung explants were also cultured in the presence of 2% (v/v) FBS. Medium was changed daily. Each culture insert contained 6 explants of 12-day lung, 4 explants of 13- and 14-day lung and 3 explants of 15-day lung.

Morphometry and branching morphogenesis

To asses lumen patency and branching morphogenesis, explants were daily monitored by phase contrast microscopy. Explants were randomly photographed at x62 magnification. Each image was printed on photographic paper at a final magnification of x2046. Morphometry was performed on each photomicrograph using a hexagonal lattice of points as described by Weibel (1962). Branching morphogenesis was quantitatively assessed by counting the number of terminal buds visible in the photographs.

[³H]Thymidine incorporation into DNA

To evaluate growth, lung explants cultured for 48 h were incubated in the presence of 1 μCi of [³H]thymidine per milliliter of medium. After 5 h of incubation, explants were washed 3 times in PBS, homogenized and DNA extracted as described by Greenstein *et al.* (1984). Protein content was measured according to Bradford (1976) and thymidine incorporation

TABLE 3

[³H]THYMIDINE LABELING OF LUNG CELLS AFTER BUMETANIDE TREATMENT OF LUNG EXPLANTS

Explant	tissue layer	treatment	% labeled cells	P
12-day	whole lung	control	45.8±2.0	<0.001
		bumetanide	66.0±2.9	
12-day	epithelium	control	38.8±1.7	0.008
		bumetanide	69.9±2.9	
12-day	mesenchyme	control	50.7±2.6	<0.001
		bumetanide	64.5±3.9	
15-day	whole lung	control	35.8±2.2	<0.001
		bumetanide	25.4±1.2	
15-day	epithelium	control	39.0±2.2	<0.001
		bumetanide	25.3±1.9	
15-day	mesenchyme	control	33.9±2.7	0.014
		bumetanide	25.4±1.5	

Values are means ±SE; n= 10 high power fields per treatment.

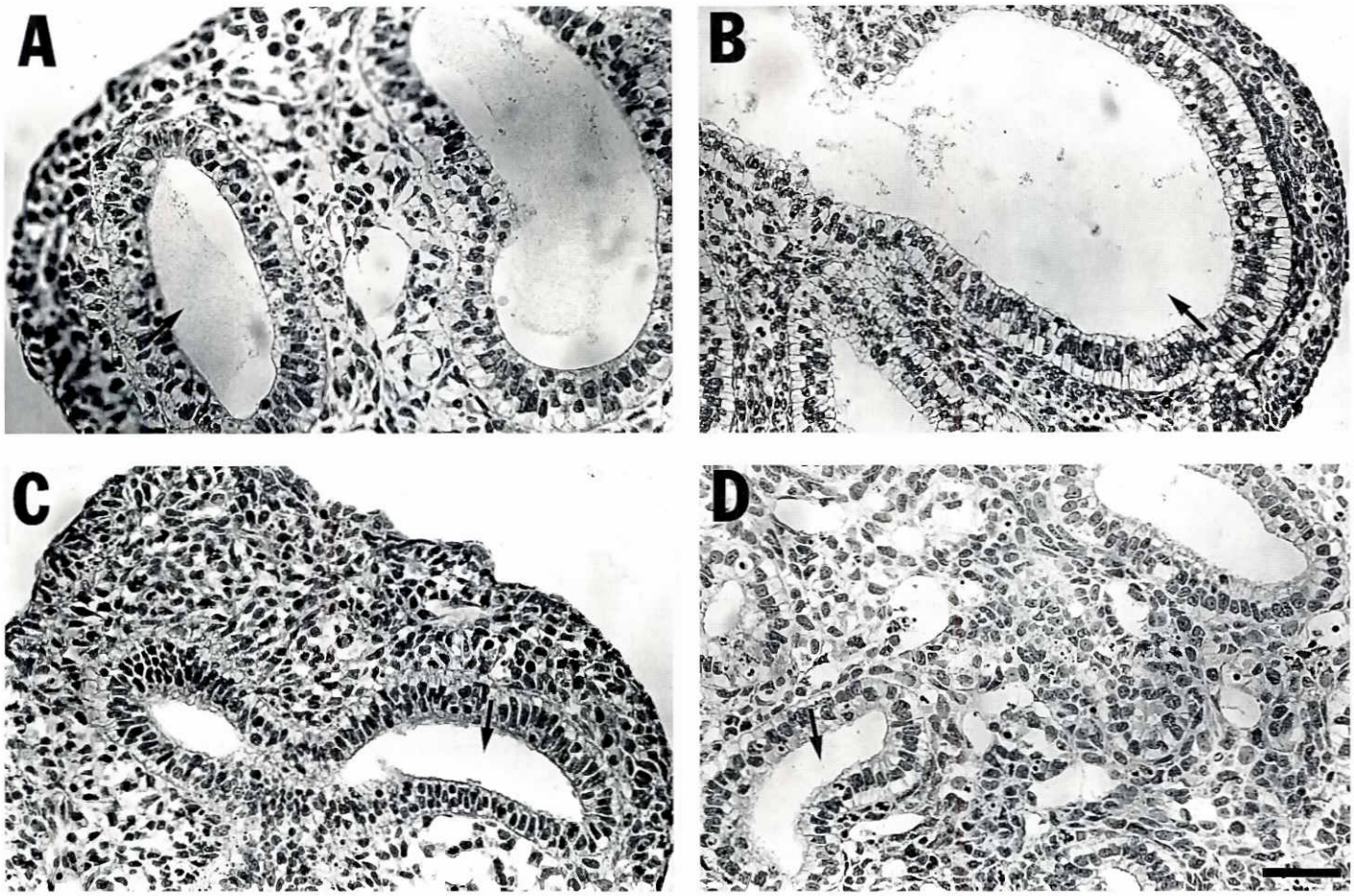

Fig. 8. Sections of 12-day and 15-day lung explants stained with Carazzi hematoxylin after 48 h culture in the presence of vehicle (control) or bumetanide. (A and B) *Control 12-day* **(A)** *and 15-day* **(B)** *lung explants.* **(C and D)** *Bumetanide-treated 12-day* **(C)** *and 15-day* **(D)** *lung explants. Arrows point the lumen of the terminal buds. Scale bar, 20 μm.*

into DNA was calculated as dpm/μg protein and is expressed as a percentage of control.

Histology and autoradiography

Lung explants were fixed in 2% (w/v) paraformaldehyde, embedded in paraffin, cut in 5 μm sections and mounted on α-aminopropyltriethoxysilane-coated slides. Tissue sections were deparaffinized in 100% ethanol, rehydrated in a graded series of ethanol dilutions, stained with Carazzi hematoxylin, dehydrated and mounted. For autoradiography, explants labeled for 5 h with 1 μCi/ml [^{3}H]thymidine, were fixed in 2% (w/v) paraformaldehyde, embedded in paraffin and cut in 5 μm thick sections. Sections were mounted on α-aminopropyltriethoxysilane-coated slides, deparaffinized, air dried and coated with NBT-2 emulsion (Eastman Kodak, Rochester, NY, USA). After 5 days of exposure, slides were developed, counterstained with Carazzi hematoxylin and examined by bright-field and dark-field microscopy. The labeling index was determined using the criteria of Chwalinski *et al.* (1988). A total of 10 random microscopic fields per group were counted.

Data presentation

All data are presented as means ±SE. Statistical significance was determined by Student' s *t*-test for paired groups and by one-way of analysis of variance followed by assessment of differences using Student-Newman-Keuls test for non-paired groups. Significance was defined as $P<0.05$.

Acknowledgments

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