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Mitogenic Activity of Tracheal Effluents from Premature Infants with Chronic Lung Disease

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ABSTRACT

Lung injury alters the expression and release of growth factors that disrupt postnatal pulmonary development in newborns and causes chronic lung disease (CLD). The effect of these factors, released into the airways of newborns with CLD, on cell proliferation and collagen production was characterized *in vitro*. Human fetal lung fibroblast and alveolar-epithelial-like cell lines (FHs 738Lu and A549, respectively) were exposed to tracheal effluents from infants with CLD (mean gestation, 24.7 ± 0.9 wk; birth weight, 666 ± 85 g; postnatal age, 0–62 d). In both cell types, proliferation was assessed by measuring [^{3}H]-thymidine uptake; in fibroblasts, collagen production was analyzed by measuring [^{3}H]-proline incorporation. The activity of specific growth factors in effluents was determined using anti-growth factor antibodies and the growth factors themselves. Growth factors in tracheal effluents promoted proliferation in a dose-dependent manner and caused up to a 10.2- and 3.1-fold increase in thymidine uptake by fibroblasts and epithelial cells, respectively. Collagen production by fibroblasts increased dose dependently, peaking at 177% of baseline. Antibody against transforming growth factor beta-1 (TGF-β_1) inhibited proliferation and the increase in collagen production by 31% ($p = 0.01$) and 14% ($p = 0.045$), respectively. Antibody against hepatocyte growth factor (HGF) inhibited proliferation of epithelial cells (25%, $p = 0.039$). The effects of exogenous TGF-β_1 on fibroblasts and HGF on epithelial cells resembled those of tracheal effluents. Potent mitogenic and differentiating substances are released into the tracheal effluents of newborns with CLD. TGF-β_1 may worsen CLD by inducing fibrosis whereas HGF may favor resolution by promoting epithelialization. **(*Pediatr Res* 55: 960–965, 2004)**

Abbreviations

CLD, chronic lung disease
EGF, epidermal growth factor
TGF-β_1, transforming growth factor-beta 1
HGF, hepatocyte growth factor
bFGF, basic fibroblast growth factor
VEGF, vascular endothelial growth factor

CLD of prematurity remains a major cause of morbidity and mortality in the preterm infant (1). Volutrauma, oxygen toxicity, infection, and edema all cause acute lung injury, resulting in emphysema-atelectasis, pulmonary hypertension, fibrosis, and decreased number of alveoli (2). However, the precise pathogenesis of CLD is uncertain. Moreover, effective therapy to prevent or ameliorate CLD has not been established. Better understanding of the pathogenesis of CLD is essential to improve treatment.

In recent years, several studies have suggested that growth factors play important roles in the pathogenesis of CLD and modulate cellular responses to newborn pulmonary injury. EGF, TGF-β_1, HGF, bFGF, and VEGF have been detected in the tracheal effluents of premature infants with CLD (3–8).

TGF-β_1 has been reported to induce proliferation and stimulate collagen production by human embryonic fibroblasts *in vitro,* (9) and HGF and VEGF promote growth of lung epithelial cells *in vitro* (10, 11). EGF induces proliferation of human fetal lung fibroblasts and enhances phospholipid synthesis in the fetal lung (9, 12). Furthermore, Toti *et al.* (13) reported that the numbers of fibroblasts are increased in the lungs of newborns with CLD, in association with increased TGF-β_1 expression. Although these studies raise the possibility that various growth factors contribute to the pathogenesis of CLD, the precise effects that these factors have on immature lung have not been investigated.

Lung injury alters the expression and release of growth factors that disrupt postnatal pulmonary development in newborns and causes CLD. Factors inducing lung fibrosis by increasing cellular proliferation and stimulating collagen production by lung fibroblasts might worsen CLD. On the other

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hand, factors inducing epithelialization by stimulating the proliferation of alveolar epithelial cells might favor the resolution of CLD. We therefore measured the effects of growth factors that modulate fetal lung fibroblast and lung epithelial cell proliferation and differentiation in the tracheal effluents of newborns with lung injury.

METHODS

Materials. A human fetal lung cell line, FHs 738Lu (line number HTB-157 ATCC), and a human lung epithelial cell line, A549 (line number CCL-185 ATCC), were purchased from the American Type Culture Collection (Bethesda, MD, U.S.A.). The morphologies of these cell lines were fibroblast-like and alveolar-epithelial-like, respectively. Dulbecco minimal essential medium and FCS were obtained from Nissui (Tokyo, Japan). Kaighn's modification of Ham's F12 medium was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). [^{3}H]-thymidine (4.0 Ci/mmol) was purchased from Valeant Pharmaceuticals (Costa Mesa, CA, U.S.A.), and [^{3}H]-proline (26.0 Ci/mmol) from Amersham Pharmacia Biotech (Little Chalfont, Buckinghamshire, U.K.). Genistein was purchased from Calbiochem (Tokyo, Japan). Recombinant human EGF, TGF-β_1 HGF, bFGF, and VEGF and anti-human EGF, TGF-β_1, and HGF were purchased from Genzyme/Techne (Minneapolis, MN, U.S.A.). Antibodies against human bFGF and VEGF were purchased from PeproTech EC Ltd. (London, U.K.).

Patient selection. Sixteen premature infants who were admitted to the neonatal intensive care units of Osaka City University Hospital or Osaka City General Hospital were included in the study. Inclusion criteria were: 1) gestational age $\leq$ 26 wk, and 2) a diagnosis of CLD. CLD was defined as an abnormal chest radiograph and a requirement for supplemental oxygen at 28 d of age (Table 1). The control group consisted of six premature infants who required mechanical ventilation for nonpulmonary reasons at 14 d of age and could be maintained on fraction of inspired oxygen (Fio_2) $\leq$ 0.25. Gestational age ranged from 27 to 32 wk, and birth weight from 1060 to 1630 g.

Method of obtaining tracheal effluents. Tracheal effluents were obtained twice weekly during the period of mechanical ventilation in a standardized manner. After 0.5 mL of 0.9% saline was instilled into the endotracheal tube, the infant was ventilated manually. A suction catheter was introduced into the airway distal to the tip of the tube, and any fluid in the airways was collected directly into the trap of the catheter. Samples were immediately centrifuged at 15,000 *g* for 10 min, and the supernatants were frozen and stored at −80°C. All tracheal effluents were obtained by the same operator to avoid interobserver variability. All analyses were done by the same technicians, and all results were evaluated by the same expert to exclude the possibility of variability due to technique or interpretation of results. Informed and written consent for participation was obtained from the parents. The protocol had institutional approval.

Table 1. *Clinical characteristics of premature infants in study group (*n = *16)*

Gestational age (wk)	24.7 ± 0.9 (23–26)
Birth weight (g)	666 ± 85 (521–785)
Oxygen therapy (d)	88.2 ± 25.0 (54–157)
Maximum Fio_2	0.52 ± 0.17 (0.3–1.0)
IPPV (days)	44.9 ± 20.4 (11–92)

IPPV, intermittent positive pressure ventilation.
Values are means ± SD; ranges are in parentheses.

Fibroblast culture. For the bioassay of mitogenesis and collagen production in human fetal lung fibroblasts, a uniform number of these cells (1.5×10^4/well) was added to Costar 96-well plates and cultured with 200 μL of Dulbecco minimal essential medium containing 10% (vol/vol) FCS for 4–5 d. All measurements were performed on subconfluent monolayers. Cells were cultured with 200 μL of serum-free Dulbecco minimal essential medium for 24 h and then with 200 μL of serum-free medium containing various concentrations of tracheal effluents or various concentrations of growth factors for 24 h, followed by 2 h incubation with [^{3}H]-thymidine (1 μCi/well) or 24 h incubation with [^{3}H]-proline (1 μCi/well).

Lung epithelial cell culture. For bioassay of mitogenesis of human lung epithelial cells, a uniform number of these cells (1.5×10^4/well) was added to Costar 96-well plates and cultured with 200 μL of Kaighn's modification of Ham's F12 medium containing 10% (vol/vol) FCS for 4–5 d. All measurements were performed on subconfluent monolayers. Cells were cultured with 200 μL of serum-free Kaighn's modification of Ham's F12 medium for 24 h and then with 200 μL of serum-free medium containing various concentrations of tracheal effluents or various concentrations of growth factors for 24 h, followed by 2 h incubation with [^{3}H]-thymidine (1μCi/well).

Proliferation and collagen production assays. Cells were collected by a cell harvester and deposited on discs of filter paper, and radioactivity was measured with a scintillation counter. In both cell types, proliferation was assessed by measuring the radioactivity of [^{3}H]-thymidine. Collagen production was analyzed by assessing the incorporation of [^{3}H]-proline by FHs fibroblasts into newly synthesized collagen, according to the method by Fenwick *et al.* (14). The assays were performed in triplicate.

Blocking study. Genistein, an inhibitor of tyrosine kinase, was dissolved in DMSO and used at a final concentration of 50 μg/mL. Cells were preincubated with this inhibitor for 1 h and then washed free of inhibitor before adding test samples. To study the blocking of antibodies to growth factors, cells were incubated with antibodies against EGF, TGF-β_1, HGF, bFGF, or VEGF (5 μg/mL), together with or without the tracheal effluents.

Data analysis. The significance of difference between means was determined using *t* test, and correlations were determined using Pearson's test. Statistical significance was set at $p < 0.05$.

RESULTS

One hundred forty-two tracheal effluent samples were obtained from 16 infants between 0 and 62 d of age. The mean number of samples per patient was 8.9 ± 4.3 (range, 5–15).

Figures 1 and 2 show [^{3}H]-thymidine incorporation into cultured lung fibroblasts and lung epithelial cells incubated with various concentrations of tracheal effluent. Exposure to tracheal effluent from newborns with CLD produced a dose-dependent increase in thymidine uptake in fibroblasts and epithelial cells. The responses were dose-dependent regardless of patient age at the time the samples were obtained. Accordingly, we used tracheal effluents at a final concentration of 50% in subsequent assays. Tracheal effluents from control infants at a final concentration of 50% produced no increase in thymidine uptake in either type of cell.

For the blocking study, tracheal effluent samples obtained from six patients between 14 and 28 d of age were used. Genistein inhibited tracheal effluent stimulation by 44% (mean) in cultured fibroblasts ($p < 0.0001$) and by 62% (mean) in cultured epithelial cells ($p < 0.0001$). Stimulation of fibroblasts by tracheal effluent from newborns with CLD was partially inhibited by antibodies against TGF-β_1 (31%, $p = 0.010$), bFGF (30%, $p = 0.001$), and VEGF (25%, $p = 0.002$) (Fig. 3), and by antibodies against HGF (25%, $p = 0.039$) in cultured epithelial cells. On the other hand, activity was increased by antibodies against TGF-β_1 in cultured lung epithelial cells (Fig. 4). Cell incubation with antibodies against growth factors, without tracheal effluents, yielded no change in thymidine uptake in either type of cell.

When cultured fibroblasts were incubated with EGF, TGF-β_1, bFGF, or VEGF, [^{3}H]-thymidine incorporation into cells increased in a dose-dependent manner (Fig. 5). In contrast, when cultured lung epithelial cells were incubated with growth factors, only EGF and HGF exhibited a trophic response, whereas TGF-β_1 decreased [^{3}H]-thymidine incorporation into epithelial cells (Fig. 6).

Fibroblastic stimulation by tracheal effluent of newborns with CLD increased with neonatal age up to 40 postnatal days. However, effluent stimulation of epithelial cells was independent of age and activity was much lower than for fibroblasts (Fig. 7). The maximum fibroblastic stimulation in infants with CLD correlated with the duration of supplemental oxygen therapy ($p = 0.032$; Fig. 8).

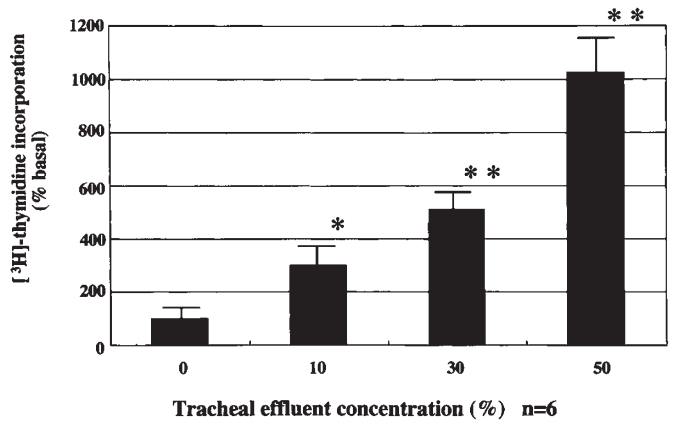

Figure 1. Incorporation of [^{3}H]-thymidine into cultured fetal lung fibroblasts incubated with tracheal effluent from infants with chronic lung disease. Samples were obtained from six patients between 14 and 28 d of age. Bars show means ± SD. * $p = 0.003$, ** $p < 0.0001$, compared with the concentration of 0%, respectively.

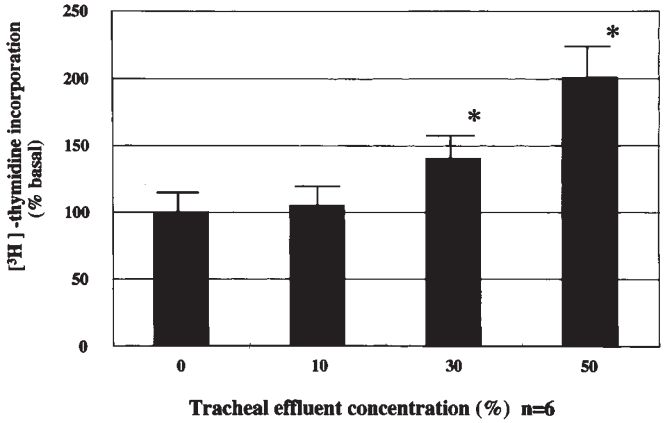

Figure 2. Incorporation of [^{3}H]-thymidine into cultured human lung epithelial cells incubated with tracheal effluent from infants with chronic lung disease. Samples were obtained from six patients between 14 and 28 d of age. Bars show means ± SD. * $p < 0.0001$, compared with the concentration of 0%.

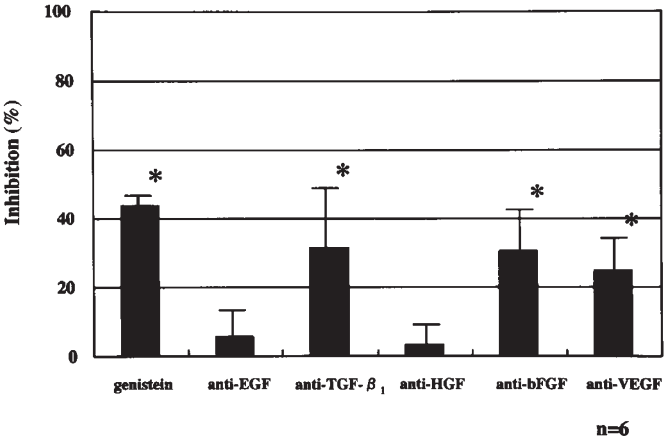

Figure 3. Growth factor activity in tracheal effluent is blocked by genistein and antibodies. Human fetal lung fibroblasts were incubated with genistein or with one of several antibodies against growth factors. Stimulation by tracheal effluent was inhibited by genistein ($p < 0.0001$), antibodies against TGF-β_1 ($p = 0.010$), bFGF ($p = 0.001$), and VEGF ($p = 0.002$). Samples were obtained from six patients between 14 and 28 d of age. Asterisks indicate significant inhibition. Bars show mean ± SD.

Figure 9 shows [^{3}H]-proline incorporation into cultured fetal lung fibroblasts incubated with tracheal effluents from infants with CLD. Culture with tracheal effluent produced a dose-dependent increase in collagen production to a maximum of a 1.8-fold increase at 50% concentration. Stimulation was inhibited by antibodies against TGF-β_1 (14%, $p = 0.045$). In contrast, tracheal effluents from controls at a final concentration of 50% did not increase collagen production. Incubation with TGF-β_1 increased [^{3}H]-proline incorporation into cultured fibroblasts in a dose-dependent manner (Fig. 10).

DISCUSSION

To clarify the roles growth factors play in the pathogenesis of CLD, the effects of tracheal effluents on cultured human fetal lung fibroblasts (FHs 738) and human lung epithelial cells (A549) were studied. Because FHs 738 is derived from normal second-trimester lung, it is a suitable model for the study of lung fibroblast in extremely low birth weight infants (15).

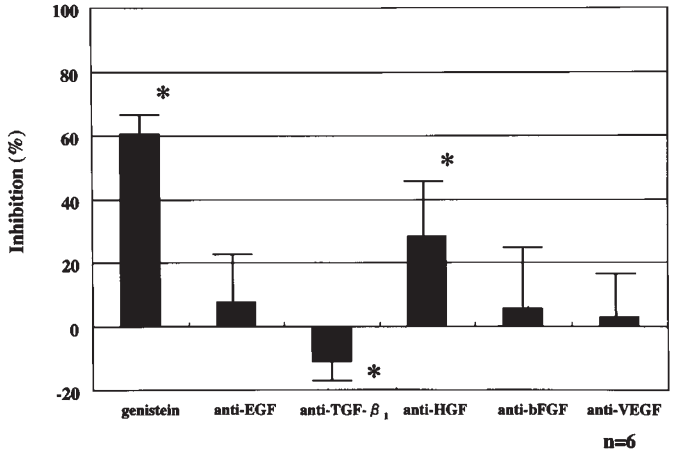

Figure 4. Growth factor activity in tracheal effluent is blocked by genistein and antibodies. Human lung epithelial cells were incubated with genistein or with one of several antibodies against growth factors. Stimulation by tracheal effluent was inhibited by genistein ($p < 0.0001$), and antibodies against HGF ($p = 0.039$). Samples were obtained from six patients between 14 and 28 d of age. Asterisks indicate significant inhibition. Bars show mean ± SD.

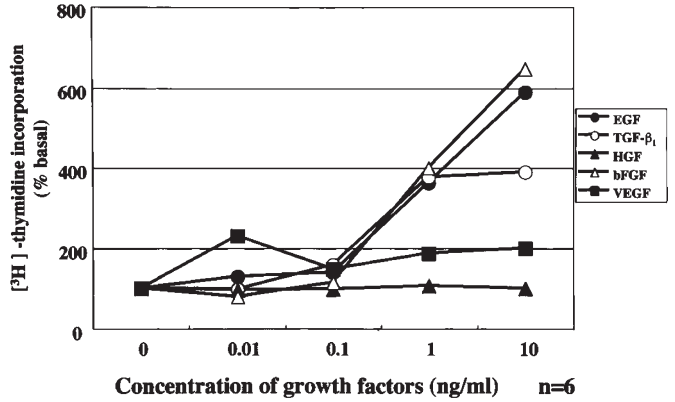

Figure 5. Incorporation of [^{3}H]-thymidine into cultured human fetal fibroblasts incubated with recombinant human EGF, TGF-β_1, HGF, bFGF, or VEGF.

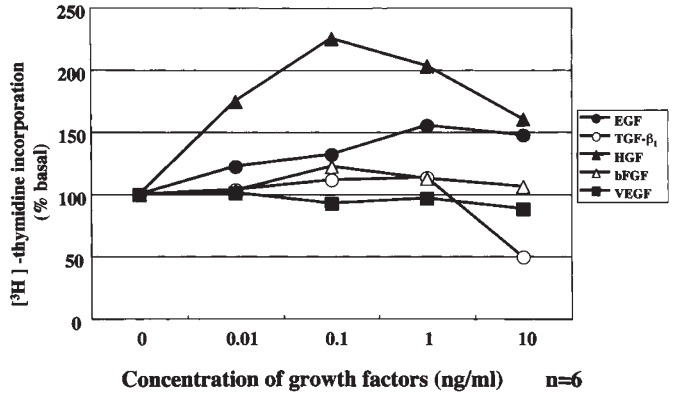

Figure 6. Incorporation of [^{3}H]-thymidine into cultured human lung epithelial cells incubated with recombinant human EGF, TGF-β_1, HGF, bFGF, or VEGF.

A549 cells are a highly transformed and aggressive lung adenocarcinoma cell line that includes several subclones. Results from studies that use these cell lines should be extrapolated with caution to true primary alveolar cells. However,

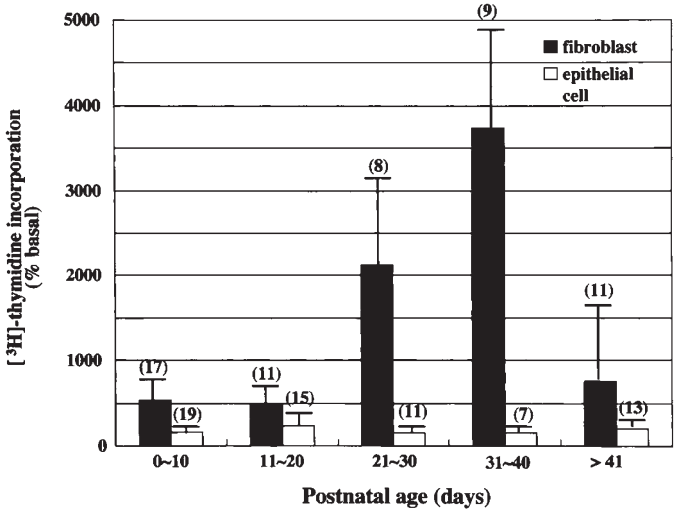

Figure 7. Postnatal trends of the mitogenic activity of tracheal effluent on lung fibroblasts and lung epithelial cells. Samples were obtained from 16 patients between 0 and 62 d of age. Bars show mean ± SD. Sample numbers are in parenthesis.

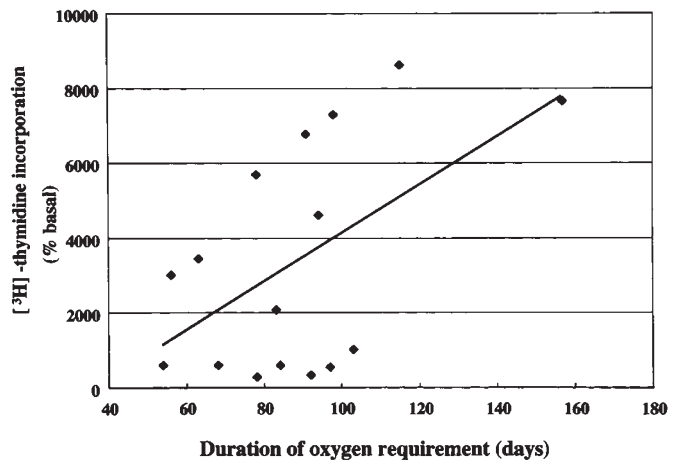

Figure 8. Positive correlation between duration of oxygen requirement and maximum fibroblastic stimulation of tracheal effluent in very low birth weight infants; $p = 0.033$, $R = 0.54$.

A549 cells have been shown to retain their type II alveolar epithelial features and hence have been used as models of neonatal alveolar epithelial cells (16, 17).

Tracheal effluent samples obtained from human neonatal lung exhibit sample variability, and the volume of fluid recovered is one important variable. In this study, there is a slight possibility that the effluents recovered earlier in the disease process consisted largely of saline, whereas during a later secretory phase the effluents included a higher proportion of lung fluid. This could influence changes in fibroblastic stimulation as a function of time. However, inasmuch as effluent stimulation of epithelial cells was constant as a function of time, this seems unlikely. A number of attempts have been made to normalize tracheal effluent samples based on modulation of various factors, including concentration of urea, secretory IgA, and albumin (18). However, it remains unclear whether tracheal aspirates should be corrected for dilution using these techniques. We followed the recommendation of

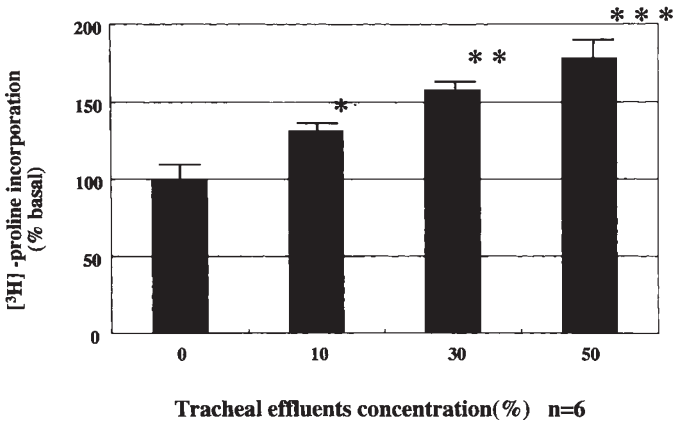

Figure 9. Incorporation of [^{3}H]-proline into cultured fetal lung fibroblasts incubated with tracheal effluent from infants with chronic lung disease. Samples were obtained from six patients between 14 and 28 d of age. Bars show mean ± SD. *$p = 0.0004$, **$p = 0.0001$, ***$p < 0.0001$ compared with the concentration of 0%, respectively.

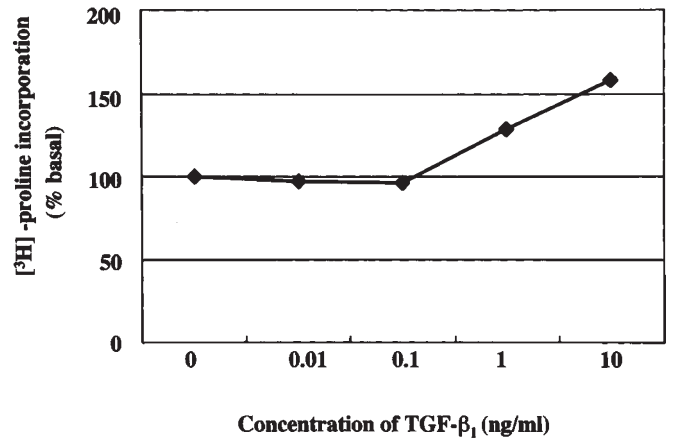

Figure 10. Incorporation of [^{3}H]-proline into cultured fetal lung fibroblasts incubated with TGF-β_1.

the European Respiratory Task Force on Bronchoalveolar Lavage in children (19) and did not correct our results for dilution. D'Angio *et al.* (20) reported that tracheal effluent might be a suitable substitute for bronchoalveolar lavage samples because they are readily obtained, often several times a day, as part of the routine care of an intubated infant.

We used thymidine incorporation to evaluate cell proliferation because this technique is simple and can deal with more samples with less variation than the cell count technique. Incubation of cultured lung fibroblasts with tracheal effluents from infants with CLD produced a dose-dependent increase in [^{3}H]-thymidine incorporation, and stimulation was partially abolished by incubation with the tyrosine kinase inhibitor genistein. Additionally, a blocking study with antibodies and a stimulation study with recombinant growth factors suggest that TGF-β_1, bFGF, and VEGF contribute to mitogenic activity in cultured human fetal fibroblasts.

TGF-β has been shown to induce collagen production by human embryonic fibroblasts (9). In our study, tracheal effluents increased collagen production by fibroblasts dose dependently, and this increase was inhibited by antibodies against TGF-β_1. Kotecha *et al.* (5) reported that concentration of TGF-β_1 in tracheal effluents is increased in infants with CLD and that TGF-β_1 might contribute to the fibrotic response observed in CLD. The number of fibroblasts is increased in CLD, and this proliferation has been found to be associated with the presence of TGF-β (13). In addition, TGF is considered one of the key cytokines in pulmonary fibrosis (21). Consequently, TGF-β_1 in the lungs of infants with CLD could worsen pulmonary function by inducing fibrosis secondary to increased proliferation of fibroblasts or increased collagen production. bFGF and VEGF may also promote fibrosis by increasing proliferation of lung fibroblasts.

Incubation of cultured epithelial cells with tracheal effluents from infants with CLD dose dependently increased [^{3}H]-thymidine incorporation, and stimulation was partially abolished by incubation with genistein. Additionally, activity was blocked by antibodies against growth factors, suggesting that HGF contributes to mitogenic activity in cultured human lung epithelial cells. Yaekashiwa *et al.* (22) reported that exogenous HGF has a pulmotrophic function and prevents the progression of bleomycin-induced lung injury in mice. These results suggest that HGF may contribute to alveolar epithelialization by increasing proliferation of alveolar epithelial cells. VEGF also has been shown to enhance alveolar epithelial recovery in acute lung injury (8, 11, 23, 24). However, VEGF did not stimulate epithelial cell proliferation in this study.

Jobe *et al.* (25) conceptualized CLD as a "new bronchopulmonary dysplasia (BPD)" in which there are fewer and larger alveoli with decreased pulmonary microvascular development. The lungs of infants delivered after <28 wk of gestation are characterized by reduced alveolar growth. In this study, epithelial proliferation was enhanced by anti-TGF-β_1 antibodies, and recombinant human TGF-β_1 inhibited the proliferation of lung epithelial cells. TGF-β_1 may contribute to BPD by inhibiting the proliferation of alveolar epithelial cells.

Recombinant EGF increased the proliferation of both lung fibroblasts and epithelial cells. However, antibodies against EGF did not inhibit stimulation by tracheal effluent. There are two possible explanations for this finding. First, EGF in tracheal effluent may not contribute to mitogenic activity because its concentration is below the level needed to stimulate mitogenicity. Second, EGF may contribute to differentiation rather than proliferation of lung epithelial cells (12).

Fibroblastic stimulation by tracheal effluents reached a maximum when the infants were 40 d old, a time when CLD was getting worse. On the other hand, epithelial cell stimulation was not age-dependent, but the level of activity for epithelial cells was much lower than that for fibroblasts. These results suggest that in the lungs of infants with CLD, mitogenic activity for fibroblasts may be higher when CLD is worse. Duration of oxygen therapy reflects the severity of CLD, and maximum mitogenic activity of tracheal effluents for fibroblasts correlated with the duration of oxygen therapy. Thus, the more severe CLD is, the more extensive pulmonary fibrosis might be. Lecart *et al.* (26) reported that high initial levels of bioactive-TGF-β in extremely low-birth-weight infants was predictive of the need for oxygen therapy at home. The higher the TGF-β concentration was, the more extensive the degree of fibrosis, and the longer duration of oxygen therapy required.

Currie *et al.* (27) and Dik *et al.* (28) have reported that the bronchoalveolar lavage fluid of infants with CLD possesses mitogenic activity for mouse lung fibroblasts and human fetal fibroblasts. However, they did not find any change in mitogenic activity during the neonatal period. Their study period was much shorter than ours, which might account for the difference between their findings and ours. Dik *et al.* (28) found that thrombin contributed to the mitogenicity of bronchoalveolar lavage fluid during the first 7 d of life, whereas we studied the mitogenicity from 14 to 28 d.

Growth factors that modulate fetal lung fibroblast and lung epithelial cell proliferation and differentiation are released into the tracheal effluents of newborns with lung injury. TGF-β_1 in the lungs of infants with CLD may worsen CLD by inducing fibrosis secondary to fibroblast proliferation or increased collagen production. Furthermore, TGF-β_1 may arrest alveolarization by inhibiting proliferation of epithelial cells. bFGF and VEGF may induce fibrosis by stimulating fibroblasts, whereas HGF may favor resolution of CLD by promoting epithelialization. A more complete understanding of the roles played by these growth factors in the pathogenesis of CLD might facilitate the development of new therapeutic strategies. Western blot and/or immunohistochemical analyses to detect changes in growth factor signaling or gene expression are required for future investigation.

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