EPIDERMAL GROWTH FACTOR AUGMENTS POSTPNEUMONECTOMY LUNG GROWTH

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Objective: Epidermal growth factor has been shown to play an important role in prenatal and postnatal lung development, but little is known about its effects on adult lung growth. We hypothesized that postpneumonectomy compensatory lung growth can be augmented by the administration of epidermal growth factor.

Methods: Adult Sprague-Dawley rats were divided into 3 groups. Sham left thoracotomy was performed in the first group (group C), left pneumonectomy in the second group (group P), and left pneumonectomy with administration of epidermal growth factor (0.2 µg/g body weight intraperitoneally, at 72-hour intervals) in the third group (group E). The right lung growth was studied in each group 1, 3, 5, 10, and 21 days after the operation. Lung weights (in grams) and volumes (in milliliters) were expressed as a ratio to the total body weight (in kilograms) (lung weight and volume indices). Epidermal growth factor receptor was quantitated by using Western blotting.

Results: Using analysis of variance and contrast analysis, we noted a significant increase in lung weight index in group E versus group P rats at 3 days (3.08 vs 2.75; $P = .034$) and 21 days (4.62 vs 3.61; $P = .006$). Lung volume index was significantly increased in group E versus group P rats at 5 (16.98 vs 15.09), 10 (24.48 vs 18.81), and 21 (28.54 vs 21.01) days ($P < .001$). Epidermal growth factor receptor was noted to be up-regulated in the lungs of animals that received exogenous epidermal growth factor.

Conclusions: This study demonstrates that administration of exogenous epidermal growth factor has a significant effect on postpneumonectomy lung growth. This process may be mediated by an up-regulation of growth factor receptor expression in the contralateral lung. (J Thorac Cardiovasc Surg 2000;120:916-22)

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One of the problems in lung transplantation is the lack of potential growth in the transplanted organs. This problem may be more apparent in pediatric patients and in patients receiving reduced-size allografts. Traditionally, it was thought that adult lungs had reached a growth plateau, believed to be irreversible. Our laboratory recently showed that adult lungs exhibit growth when they are transplanted into immature recipients.¹ This finding led to the hypothesis that there may be specific growth factors that could induce growth in adult lung tissue similar to that seen in the perinatal state. After pneumonectomy, the remaining lung exhibits rapid and restorative growth.^{2,3} However, the stimuli, mechanisms, and regulation of this growth process remain unclear. Various growth factors have been implicated in the regulation of the different com-

ponents of lung growth and maturation. However, the role of growth factors in regulating compensatory growth after pneumonectomy or lobectomy is unknown. We chose to use a postpneumonectomy lung growth model in the rat to study lung growth because of the rapid, consistent, and well-characterized lung growth in this animal.

Epidermal growth factor (EGF) has been shown to play an important role in prenatal and postnatal lung development. EGF is a bioactive peptide that modulates epithelial maturation and regeneration, and the lung has been shown to harbor receptors for EGF on various cell types such as ciliated cells and alveolar cells. Raaberg and associates⁴ demonstrated EGF immunoreactivity in type II pneumocytes and in the cells of the bronchi and bronchioles from a few days before birth and throughout life. In addition, Ruocco and colleagues⁵ postulated that EGF, in addition to being mitogenic, functions in a paracrine fashion to help in epithelial maturation of the lung tissue. Most experimental data correlating EGF and lung growth have been generated from the study of fetal specimens. For example, Catterton and coworkers⁶ showed that EGF injected into a rabbit fetus caused accelerated maturation of the lung.

In this study, we investigated the role of exogenously administered EGF on postpneumonectomy lung growth. The lung growth was then analyzed by using lung weight and volume to determine whether EGF plays a significant role in the compensatory growth of a mature lung. Western blot analysis and morphometric techniques were used in an attempt to understand this response at the cellular level.

Material and methods

Adult male Sprague-Dawley rats were used for all experiments. Animal acquisition was under the supervision of the Department of Comparative Medicine and a licensed veterinarian. The facilities used for animal care are accredited by the American Association for Accreditation of Laboratory Animal Care. The facility is approved by the Institutional Animal Care and Use Committee. All animals received humane care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources, National Research Council, and published by the National Academy Press, revised 1996.

Operative model. Rats were divided into 3 major groups: C, P, and E. Each group was then subdivided into 5 groups on the basis of postoperative survival time (1, 3, 5, 10, and 21 days). We began with 8 animals in each subgroup to allow for an adequate number of tissue samples for molecular and mor-

phometric analysis. All animals were anesthetized with a combination of ketamine and xylazine injected intraperitoneally followed by endotracheal intubation with a 16F catheter. Their lungs were then ventilated with room air by using a pressure regulated rodent ventilator (Kent Scientific, Litchfield, Conn). The rats were then placed in the right lateral decubitus position and shaved and prepped in a sterile fashion.

Animals in group C underwent a sham thoracotomy on the left side. Animals in group P underwent a left pneumonectomy. Animals in group E underwent a left pneumonectomy with the administration of exogenous EGF (0.2 µg/g, dosed intraperitoneally every 72 hours, R&D Systems, Minneapolis, Minn). After the sham left thoracotomy (group C), the chest was closed after an expiratory sigh, with use of 3-0 silk sutures, and the skin was closed with surgical staples. Animals in groups P and E underwent a posterolateral thoracotomy, and the left lung was freed from the inferior pulmonary ligament. The lung was then delivered into the surgical wound, the hilum was tied with a 4-0 silk ligature, and the lung was excised. The chest wall was closed as described above. The animals were allowed to recover from the operation and their lungs were extubated after spontaneous respirations began. The animals then received analgesia in the form of buprenorphine, injected intramuscularly every 12 hours for the first 24 hours. The animals were allowed to feed freely and were kept in a controlled environment. After the designated interval, the animals were anesthetized and intubated via a tracheostomy. Exposure of the thoracic organs was obtained by a bilateral anterior sterno thoracotomy. The animals were rapidly killed by vena caval division. The right lung in half of the animals was removed, patted dry, weighed, snap frozen in liquid nitrogen, and stored at -80°C for molecular analyses. The remaining animals received intratracheal instillation of 2% glutaraldehyde to a pressure of 25 cm H₂O. The right lung was then removed with the fixative in place and ligated at the hilum. Lung volume was obtained by the volume displacement technique as described by Scherle.⁷ The lung was then embedded in paraffin, and 5-µm sections were stained with hematoxylin and eosin for morphometric analysis.

Morphometry. Lung morphometry was carried out with the point counting technique described by Gil⁸ and the 3-level sampling technique described by Davies⁹ and Wandel, Berger, and Burri.¹⁰ The volumes of the various respiratory regions were determined by using a 42-point test reticule (lattice with 85-µm grid lines) attached to a Nikon Eclipse E400 microscope. This technique is briefly described below. The first level of analysis, usually performed under gross inspection, was not performed, because of the small size of the rat lung and the lack of accurate differentiation at the gross level; thus, all the morphometric measurements need to be interpreted as relative values. The second level of analysis was performed at 50× magnification. The number of lattice points that fell on intra-acinar air space and their intervening tissue is designated as *Pr*. Points that correspond with extra-acinar

Fig 1. Lung weight index. Lung weight index (in grams per kilogram body weight) plotted at the various postoperative time intervals. Increase in lung weight index is noted in rats that underwent pneumonectomy with the administration of exogenous EGF (*E*) compared with pneumonectomy (*P*) and sham thoracotomy animals (*C*) at 3 and 21 days ($P < .05$). * $P < .05$ vs *P*, *C*.

airways and vessels less than 0.5 mm in diameter were ignored. The volume of the respiratory region (V_{vr}) was calculated by using the following equation:

$$V_{vr} = (Pr/42) \cdot 100$$

The third level of analysis was performed at 200 $\times$ magnification. The number of lattice points that overlap the respiratory airspaces was designated as *Pra*. The volume of the respiratory airspace (V_{ra}) was calculated by using the following equation:

$$V_{ra} = (Pra/42) \cdot 100$$

The next level of analysis was also performed at 200 $\times$ magnification. The number of test lines intercepting the airspace-epithelial interface was designated as *Is*. The alveolar surface density (*Sv*), a linear measurement that expresses the number of alveoli per centimeter, was then determined by using the following equation:

$$Sv = 2/d \cdot (Is/Pp)$$

where d = length of the test grid line (85 μ m) and Pp = total number of test points on the lung parenchyma = 42.

The relative values of the various respiratory compartments in the lung were then calculated as follows:

The total volume of respiratory region (TV_{vr}) = $V_{vr} \cdot VL$, is a measure of the total volume of alveoli and the intervening gas exchange tissue in the lung.

The total volume of the respiratory airspace (TV_{ra}) = $V_{ra} \cdot V_{vr} \cdot VL$, is a measure of the volume of alveoli in the lung, where VL = total volume of the lung.

Western analysis. Total lung protein (120 μ g) was fractionated on a 7.5% (weight per volume) sodium dodecyl sulfate polyacrylamide gel and transferred to nitrocellulose with use of an electrophoretic transfer cell (BioRad, Hercules, Calif). The blot was blocked and incubated with 1- μ g/mL primary rabbit-polyclonal anti-EGF-receptor (EGFR) anti-

body (Santa Cruz Biotechnology, Santa Cruz, Calif) for 1 to 3 hours at room temperature, followed by washing with 50 mmol/L Tris HCl pH 7.4, 150 mmol/L NaCl, 0.1% Tween. The blot was then incubated for 1 hour with mouse-monoclonal anti-rabbit IgG secondary antibody coupled to horseradish peroxidase (1:3000, Biorad) and washed as before. Protein bands were visualized by chemiluminescence (ECL; Amersham, Piscataway, NJ) and quantitated by means of computerized densitometry (Alpha Innotech Corp, San Leandro, Calif).

Statistical methods. Measurements are reported as the mean $\pm$ SD. One-way analysis of variance (ANOVA) and contrast analysis were used to determine whether a difference existed between study groups. The Bonferroni adjustment (multiple comparison test) was used when appropriate.

Results

The right lung weights and volumes were analyzed among the 3 groups (*C*, *P*, *E*) at the various time intervals (1, 3, 5, 10, and 21 days). The lung weight (in grams) and volume (in cubic centimeters) were expressed as a ratio to the total body weight of the animal (in kilograms), which are designated as the lung weight and volume index. Comparison of lung weight index is shown in Fig 1. At 1 day there was no difference between the various groups. At 3 days there were significant differences for *C* vs *P* and *E* ($P = .004$). At 5 days there were significant differences for *C* vs *P* and *E* ($P = .020$). At 10 days we noted significant differences between *C* vs *P* and *E* ($P = .001$). At 21 days there were significant differences for *C* and *P* vs *E* ($P = .006$). Both the EGF treatment group and the pneumonectomy group had significantly higher lung weight index at 3, 5, 10, and 21 days when compared with the

Fig 2. Lung volume index. Lung volume index (in milliliters per kilogram of body weight) plotted at the various postoperative time intervals. Increase in lung volume index is noted in rats that underwent pneumonectomy with the administration of exogenous EGF (*E*) compared with pneumonectomy (*P*) and sham thoracotomy animals (*C*) at 5, 10, and 21 day intervals ($P < .001$). * $P < .001$ vs *P*, *C*.

Table I. Results of morphometric analysis

Group	<i>Sv</i> (cm^{-1})	<i>TVvr</i> (mL)	<i>TVvra</i> (mL)
C10	286.3 $\pm$ 62.6	3.87 $\pm$ 0.3	2.39 $\pm$ 0.8
P10	288.2 $\pm$ 26.9	5.32 $\pm$ 1.0	3.41 $\pm$ 0.9
E10	280.1 $\pm$ 47.5*	6.78 $\pm$ 1.3†	4.59 $\pm$ 1.7‡
C21	324.9 $\pm$ 75.2	5.56 $\pm$ 0.4	3.39 $\pm$ 1.1
P21	298.8 $\pm$ 46.6	7.19 $\pm$ 0.3	4.40 $\pm$ 1.4
E21	265.8 $\pm$ 30.1§	9.72 $\pm$ 1.5	6.74 $\pm$ 2.7¶

Sv, Alveolar surface density; *TVvr*, total volume of respiratory regions; *TVvra*, total volume of respiratory airspaces.

* $P = .932$ vs C10, P10.

† $P < .001$ vs C10, P10.

‡ $P = .003$ vs C10.

§ $P = .087$ vs C21.

|| $P < .001$ vs C21, P21.

¶ $P = .003$ vs C21, P21.

sham group. Using contrast analysis, we tested our hypothesis for any difference between groups *P* and *E* at the various time points. We noted significant increase in lung weight index at 3 days ($P = .034$) and 21 days ($P = .014$) in group *E* compared with group *P*.

The results of the lung volume index are shown in Fig 2. At 1 day there were no differences between the groups ($P = .491$, NS). At 3 days there was a significant increase in *C* compared with *P* ($P = .04$). At 5 days there were significant increases in *E* vs *P* and *C* and in *P* vs *C* ($P < .001$). At 10 days there were significant increases noted in group *E* vs *P* and *C* and in *P* vs *C* ($P < .001$). Similarly, at 21 days there were significant increases noted in group *E* vs *P* and *C* and *P* vs *C* ($P < .001$). We noted that the EGF treatment group and the pneumonectomy group had a significantly higher lung volume index at 3, 5, 10, and 21 days when compared with the sham group. Contrast analysis done to test the validity of our hypothesis

revealed a significant increase in lung volume index in group *E* compared with group *P* at 5, 10, and 21 days ($P < .001$).

Morphometric analysis was performed on the lung tissue from the 3 groups at 10 and 21 days because maximal variability in lung volume was noted at these time points (Table I). The surface density of alveoli (*Sv*) was found to be similar among all groups at both time intervals. The volume of the respiratory region (*TVvr*) was increased in group *E* at the 10-day (6.78 ± 1.3 mL; $P < .001$ vs P10 and C10) and 21-day (9.72 ± 1.5 mL; $P < .001$ vs P21 and C21) intervals. The volume of the respiratory airspace (*TVvra*) in the lung was elevated in group *E* at the 21-day interval (6.74 ± 2.7 mL; $P = .003$ vs P21 and C21).

Western analysis was performed for the detection of EGFR in the lung tissue from the 3 groups at 1-, 3-, and 5-day intervals. We also used lung tissue from unoperated age-matched animals (*N*) for internal control. The

Fig 3. Western blot analysis for epidermal growth factor receptor (EGFR). The Western blot is shown on **top** and the computerized densitometry shown at the **bottom**. The EGFR expression in the EGF treatment group at 1 day (E1) was significantly higher when compared with unoperated normals (N), sham thoracotomy (C1), and pneumonectomy (P1) animals ($P < .001$). Similarly, EGFR expression in E5 was significantly higher than N, C5, and P5 ($P < .001$).

results are shown in Fig 3. The Western blotting was performed on tissue from 1, 3, and 5 days because we thought that molecular events would be more evident at these earlier time points. These earlier time points were also thought to represent the periods of rapid growth. At 1 day we noted an up-regulation in EGFR expression in group E vs N, C1, and P1 (3.47 ± 0.49 ; $P < .001$). Similarly, at 5 days we noted a significant upregulation of EGFR in E5 vs N, C5, and P5 (2.22 ± 0.35 ; $P < .001$).

Discussion

Growth of a mature lung has been observed after both lung injury and pneumonectomy. After lung epithelial injury, type II cells proliferate and eventually line the alveolar septa as rows of columnar cells. During the course of healing, these type II cells differentiate into type I cells to allow the reconstitution of normal alveolar parenchymal architecture. Type II cell proliferation is the critical step in the structural and functional restoration after lung epithelial injury.¹¹ Pneumonectomy and lobectomy initiate compensatory growth that results in restoration of lung volume, compliance, and mass to the remaining lung. Restoration of whole lung levels of protein, RNA, DNA, collagen, and elastin, along with essentially normal lung cell populations, also occurs.² Lung expansion, or stretch, has a definite role in the initiation of compensatory growth. Although there is no dispute that the remaining tissue undergoes growth after pneumonectomy, the nature and extent of the growth are not fully understood.

Whether compensatory growth of the lung involves formation of new alveoli is controversial, and several biochemical studies indicate that lung mass is restored by hyperplastic growth of the remaining tissue rather than by hypertrophy of individual cells.² This is exemplified in the rat within the first day after pneumonectomy by pleural, mesothelial, and peripheral alveolar cell division, and 6 to 7 days later mitoses are found throughout the lung.¹² There is evidence that type II cells play a role in compensatory growth since type II cells isolated from the lungs of pneumonectomized rats exhibited metabolic changes typical of accelerated cell growth.¹³

Utilizing the rodent model of postpneumonectomy lung growth, we were able to demonstrate that the administration of exogenous EGF had a significant effect on the growth of the contralateral lung. The effect of EGF in augmenting lung growth is illustrated by an increase in the lung weight index noted at 3 and 21 days. Wet/dry ratios were not determined because it has been shown in the literature that compensatory growth response of the lung after pneumonectomy is not caused by an accumulation of blood or fluid but by an increase in lung tissue.¹⁴ This is further supported by the fact that the lung volume index was significantly elevated at 5, 10, and 21 days in animals that received exogenous EGF.

Morphometric analysis of lung tissue revealed an increase in the volume of the respiratory regions and respiratory airspace in animals that received exogenous EGF. However, the alveolar surface density remained

unchanged, which indicates that the microscopic architecture of the alveoli was preserved. This evidence of increased volume of respiratory region and respiratory airspace when combined with the increased total lung volume noted by volumetry indicates that EGF has a substantial role in regulating the replication and growth of the various components of the lung parenchyma. These data, combined with the constant nature of the surface density of alveoli, indicate that EGF acts in a bimodal fashion in the lung. Initially, there must be a hyperplastic phase, mediated by the growth factor receptor, and a secondary process during which the replicated alveolar units are restored to their original size by a hypertrophic response.

Western blot analysis of the lung tissue for EGFR revealed that its expression was up-regulated in rats that received exogenous EGF at 1 and 5 days. This could represent an autoregulatory process, whereby the exogenous growth factor induces the expression of its receptor. This up-regulation of EGFR could account for the effect of exogenously administered EGF on lung growth. EGFR has been shown to be vital to the development of the lung; EGFR-deficient mice were shown to have evidence of lung immaturity as a result of impaired branching and deficient alveolization and septation.¹⁵ The regulation of EGFR has been shown to be important for the timing of mesenchymal-epithelial cell communication, which is necessary for surfactant synthesis.¹⁶

We believe this is the first evidence that administration of exogenous growth factor can influence the growth of adult lung tissue. This study illustrates the role of a single growth factor on lung growth. Further investigations are needed to determine the various signals and growth factors that can initiate growth in adult lung tissue. The next step is to understand the role of EGF in experimental transplantation and to determine whether it has a role in augmenting the growth of transplanted lungs. The clinical applications of understanding lung growth are manifold. It could help us modulate the growth of reduced-size lung transplants and transplants in the pediatric population.

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Discussion

Dr Eric A. Rose (*New York, NY*). Will the growth factors stimulate growth of malignant cells? Is this something that, if it were to be used clinically in the setting of a pneumonectomy for lung cancer, would potentially cause recurrence of disease?

Dr Kaza. There have been various papers that have indicated that EGF does indeed cause the transformation, but as far as our animals were concerned, these were healthy animals. We did not see a transformation. However, we feel the clinical application of this research is for the nonmalignant patients, if any, for the patients with insufficient pulmonary reserves, and for the modulation of growth in the lung transplant patients, specifically, the pediatric patients.

Dr Edward D. Verrier (*Seattle, Wash*). Could you notice any functional differences in any of the animals?

Dr Kaza. We did not measure any functional parameters. However, there have been classical studies that have shown that oxygenation in compensatory lung growth equals that of a bilateral lung ventilation.

Dr Verrier. I am still concerned about the evidence that you actually have cellular replication. There has got to be

another way to get at showing that there is actually increased alveolar replication. I recognize that you have tried to get at that issue. It seems to me to be a critical issue that you actually are inducing—because that is a little unusual in adult tissue, in the heart and other tissues, in order to actually induce new cells to replicate. It is very difficult to do. Does anybody else have that concern?

Dr Kaza. Yes, we are actually looking at that. We are doing immunostaining with BRDU to detect actual cell mitoses in the pneumocytes, and we are actually using the technique in the experiments that we are conducting right now, and we are going to go back and look at this tissue as well.

Dr Verrier. I think it is a fundamentally important question and one that is not the easiest to get at experimentally.

Dr Glen S. Van Arsdell (Toronto, Ontario, Canada). You did extensive morphometric analysis of alveolar changes. Did you also look at the vasculature that supplied it?

Dr Kaza. No, we did not. This morphometric analysis that is described by Dr Paul Davies is specifically used to quantitate the various respiratory compartments in the lung and the surface density of alveoli.

Dr Van Arsdell. Have you looked at the effects of EGF without removal of the lung?

Dr Kaza. No, we have not.

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