Amelioration of Pulmonary Emphysema by In Vivo Gene Transfection With Hepatocyte Growth Factor in Rats

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Background—Hepatocyte growth factor (HGF) is an important mitogen and morphogen that contributes to the repair process after lung injury. The goal of the present study was to characterize its role in pulmonary emphysema, which may lead to the development of new treatment strategies with HGF.

Methods and Results—HGF mRNA and protein levels in lung tissue and plasma from elastase-induced emphysema rats transiently increased, then declined significantly to below the basal level in a time-dependent manner (P<0.01). Furthermore, changes in HGF were correlated with histologically progressive emphysematous changes and deterioration in pulmonary physiology. Use of the HVJ (hemagglutinating virus of Japan) envelope method resulted in successful transfection of cDNA encoding human HGF, as demonstrated by an efficient expression of HGF in alveolar endothelial and epithelial cells. Transfection of HGF resulted in a more extensive pulmonary vasculature and inhibition of alveolar wall cell apoptosis, and those effects led to improved exercise tolerance and gas exchange (P<0.05), which persisted for more than 1 month.

Conclusions—Decreased HGF expression due to a failure in sustained endogenous production after injury was associated with emphysema-related histopathologic and physiological changes in the present rat model. In addition, induction of HGF expression by a gene-transfection method resulted in improved pulmonary function via inhibition of alveolar cell apoptosis, enhancement of alveolar regeneration, and promotion of angiogenesis. (Circulation. 2005;111:1407-1414.)

Key Words: lung ■ growth substances ■ gene therapy ■ hypoxia ■ angiogenesis

The widespread use of tobacco products has resulted in a relatively high worldwide prevalence of pulmonary emphysema. In fact, conservative predictions state that chronic obstructive pulmonary disease, which includes emphysema, will become the third-leading cause of death by the year 2020.1 Although lung volume reduction surgery emerged as a promising treatment strategy in the 1990s,2 the procedure still yields a significant number of operative deaths, and the benefits may not be long-lasting.3 Thus, development of effective therapies for patients with emphysema is critical.

Emphysema is characterized pathologically by the disappearance of alveolar septa, an overall loss of alveoli, and abnormal permanent enlargement of the airspaces; widespread destruction of the pulmonary vascular beds has also been observed. Chronic and progressive destruction of the alveolar walls and vascular beds, which may result from a protease-antiprotease imbalance,4 leads to deterioration of pulmonary function and gas exchange. Thus, the pathological processes that underlie alveolar wall destruction and pulmonary vasculature disruption may represent attractive therapeutic targets for treatment of the disease. Although several studies have characterized molecular events leading to emphysema progression,5,6 new practical therapies for effective treatment have not been elucidated.

Hepatocyte growth factor (HGF), originally purified and cloned as a potent mitogen for mature hepatocytes,7,8 functions as an essential regenerative factor in the liver, kidney, intestines, and skin, as well as other tissues,9,10 and may also act as a potent multifunctional pulmotrophic factor,11–15 inducing the formation of alveolar networks from destroyed alveolar cells in injured lung tissue. Furthermore, HGF enhances vascular endothelial cell proliferation, thereby promoting tissue remodeling via an improvement in blood perfusion.16–19 We recently reported that HGF enhanced angiogenesis in the pulmonary vasculature.20 On the basis of these findings, we hypothesized that HGF could exert beneficial effects on the pulmonary physiology of patients with emphysema.

The elastase-induced model of rat emphysema has been well characterized,21 and the pathological findings of destroyed alveolar walls, airspace enlargement, and loss of vascular beds are similar to those seen in human emphysema. In the present study, we investigated the role of HGF and the effects of its supplementation in a rat model of pulmonary emphysema.
Methods

Emphysema Induction
Emphysema was induced in anesthetized 3-month-old Sprague-Dawley rats (Japan Lab Animals Co, Ltd, Osaka, Japan) by means of a single intratracheal instillation of porcine pancreatic elastase (Roche Diagnostics, 25 U/100 g body weight, diluted in 0.8 mL of normal saline solution or an equal volume of saline alone as control. After the instillation, rats were extubated and returned to the animal care facility and managed routinely until 1 week after induction. Animal care was performed under the supervision of the Animal Research Committee in accordance with the Guidelines on Animal Experiments of our institute.

Tissue Processing and Morphological Assessment
After completion of the treatment period, rats were anesthetized and intubated again. After a midline thoracotomy, the heart and lungs were excised en bloc, and lungs were inflated and fixed with intratracheal instillation of ethanol at a constant pressure of 25 cm H2O. Both lungs were then embedded in paraffin, sectioned sagitally, and stained with hematoxylin-eosin for standard histological analysis. To estimate morphological changes in alveolar epithelial cells, the radial alveolar count (RAC) index was used as described previously.14 Briefly, RACs were performed to determine the number of septa that intersected a perpendicular line drawn from the center of a respiratory bronchiole to the distal acinus (connective tissue septum or pleura). At least 40 counts were performed per rat, and measurements were performed at each time point.

Measurement of HGF in Tissues and Plasma and Real-Time Quantitative Reverse Transcriptase–Polymerase Chain Reaction
Measurement of tissue and plasma HGF concentrations with an ELISA kit for rodent HGF (Institute of Immunology) was performed from 5 rats at each time point as described previously.25 HGF mRNA levels in lung tissue were also determined by real-time quantitative reverse transcriptase–polymerase chain reaction (RT-PCR) as described previously.25 Briefly, RACs were performed to determine the number of septa that intersected a perpendicular line drawn from the center of a respiratory bronchiole to the distal acinus (connective tissue septum or pleura). At least 40 counts were performed per rat, and measurements were performed at each time point.

Plasmid DNA
An HGF expression vector was prepared by insertion of human HGF cDNA into the Not VIII site of the pUC-SR expression vector plasmid as reported previously.24 A control expression vector without the HGF gene was also constructed.

Preparation of Hemagglutinating Virus of Japan Envelope Vector
Hemagglutinating virus of Japan (HVI; also known as Sendai virus) was amplified as described previously.25

In Vivo Gene Transfer via the Vein Dorsalis Penis Superficialis
Elastase-treated rats were randomly divided into 2 groups and anesthetized. A 26-gauge Angiocath (Becton Dickinson) was inserted into the vein dorsalis penis superficialis. Then, the HVJ envelope-plasmid complex (0.5 mL, including 100 µg of cDNA) was infused via the catheter. The animal was allowed to recover in a warm, oxygenated environment. The expression vector with HGF cDNA was transfected into 20 rats [PPE(+) plus-HGF group], and the vector without HGF was transfected into another 20 rats, which served as controls [PPE(+) plus-vector group]. Additionally, we set normal untreated rats [PPE(−) group] and rats initially treated with elastase [PPE(+) group] as negative and positive controls, respectively. The transfection was performed on day 7 after introduction of elastase. Five rats in each group were euthanized for evaluation of histopathologic analysis and pulmonary function at 7, 14, and 28 days after transfection, respectively. The remaining 5 rats in each group were euthanized 7 days after transfection to assess pulmonary blood perfusion analysis with laser Doppler image (LDI). Furthermore, luciferase gene–loaded HVJ envelope vector was injected to examine tissue-specific expression of the target HGF, Luciferase activity was measured at 1 day after transfection in lung and each other organ as described previously.25

Immunohistochemical Analysis (c-Met/HGF Receptor, PCNA, TUNEL, Factor VIII, Human HGF)
Immunohistochemical staining was performed with antibodies against factor VIII (1:3; DAKO), proliferating cell nuclear antigen (PCNA; 1:50; Santa Cruz), and terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL; Takara Biomedical). After transfection, we used a rabbit polyclonal antibody against human HGF (1:200) as described previously.20 This antibody specifically detects human HGF but not rat HGF.20 For the quantification of proliferating alveolar cells and apoptotic cells and the factor VIII analysis, we used the same parameters described in our previous report.20 Furthermore, to specify localization of cells that express the c-Met/HGF receptor in the lung tissue, tissue sections were also stained with rabbit polyclonal anti-mouse c-Met (1:200; SP 260; Santa Cruz).

Laser Doppler Blood Flow Analysis
Lung surface blood perfusion was evaluated with an LDI analyzer (Moor Instruments). Blood flow measured by LDI correlated well with capillary density, and we previously demonstrated the utility of LDI for assessment of rat lung angiogenesis.20

Pulmonary Function Test (Arterial Blood Gas, Treadmill Test)
To assess pulmonary function at rest, arterial blood gas analysis was performed with blood samples from ascending aorta with an ABL 505 system (Radiometer). These data were obtained with a ventilator with oxygen supplementation (FIO2 = 0.3) at 60 breaths/min. To determine the adequacy of pulmonary capacity under exercise stress, a treadmill testing was performed with a small-animal treadmill system that consisted of an acrylic plastic chamber with a small-rat treadmill (Shizume Medical) as described previously.27 Cardiopulmonary functional capacities were determined with the values of maximum running speed and O2 uptake (VO2max) during the treadmill test.

Statistical Analysis
Data are expressed as mean ± SEM. The means of different groups were compared by 1-way ANOVA. An unpaired Student t test was used for statistical analysis, and a probability value of <0.05 was considered statistically significant.

Results

Characterization of Morphological and Physiological Phenotypes in Rats
We initially characterized morphological and physiological phenotypes in our rodent model during the experimental periods. In the elastase-treated rats, pathological findings (such as airspace enlargement and progressive destruction of alveolar wall structures) became evident in a time-dependent manner, whereas alveoli of rats in the saline-treated control group remained normal in size and appearance. To quantify the alveolar injuries, we measured RACs in emphysematous lung tissue at each time point. The RAC values decreased rapidly as early as day 3 (from 11 ± 0.8 to 7.1 ± 1.2; P < 0.05)
and then gradually decreased thereafter (Figure 1A). Consistent with the histological changes, the elastase-treated rats manifested a progressive loss in pulmonary function: PaO\textsubscript{2} decreased rapidly as early as day 1 (from 160±110 to 112±15 mm Hg; \(P<0.05\)) and then decreased gradually until day 28 (Figure 1B). Likewise, maximum running speed and the value of V\textsubscript{O\textsubscript{2}}\text{max} on the treadmill test decreased in a time-dependent manner (Figure 1B).

**Reciprocal Changes in Endogenous HGF and c-Met Expressions and Relationship Between HGF Levels and the Development of Emphysema**

Using the successful model, we addressed whether expressions of HGF and c-Met/HGF receptor levels may be modulated under the pathological conditions. The intrinsic HGF levels in lungs of the elastase-induced emphysema rats increased as early as day 1 and reached a peak on day 5. Thereafter, they decreased to a level below preoperative values (Figure 2A). Of note, local HGF levels on day 7 were significantly lower than that of the control rats (21±5 versus 46±3 ng/g tissue, \(P<0.01\)). Likewise, circulating HGF levels in the elastase-induced emphysema rats increased to a maximum on day 3 (3.53±0.45 ng/mL) and then decreased rapidly below normal levels between 7 and 28 days after elastase induction (Figure 2A). Furthermore, lung HGF mRNA levels increased on day 1 (162±12% of the level in normal lung, \(P<0.05\)) but returned to pretreatment levels from day 5 after elastase injection (Figure 2A). For example, the lung HGF mRNA level was significantly lower on day 7 than on day 0 (82±9% versus 102±7%, \(P<0.01\)). In contrast to lung tissues, there were significant increases in renal or hepatic HGF mRNA and protein levels. Both the renal and hepatic HGF levels reached a peak on day 3, which was 3- to 5-fold higher than pretreatment levels. This extrapulmonary support system may reflect the transient increase in plasma...
HGF. We next determined whether endogenous HGF levels are linked to the development of emphysema in our rat model. Because HGF is a potent regenerative factor, we hypothesized that enhancement of alveolar repair by HGF might retard the progression of the emphysema. To test our hypothesis, we counted the number of PCNA-positive alveolar cells at each time point in emphysematous lung tissue. The PCNA index increased, reaching a peak on day 5. Thereafter, it decreased rapidly to a level below basal values on day 7 (Figure 2B, left). In the natural course of the emphysema, endogenous HGF levels correlated well with alveolar regeneration, as evidenced by PCNA index (Figure 2B, right). In other words, the loss in endogenous HGF production may participate in destruction of the lung parenchymal structure. Finally, we examined c-Met/HGF receptor expression to determine HGF-targeting cells in the lung tissues; we found that c-Met was localized mainly to the alveolar cells and vascular endothelial cells. Expression was more prominent in cells consistent with type II pneumocytes, which were located primarily in corners of alveoli (Figure 2C).

**Successful Expression of Exogenous HGF Introduced by Gene Transfection in Emphysematous Lungs**

At 7 days after transfection of plasmid, human (ie, exogenous) HGF was extensively identified in the cytoplasm of the alveolar epithelium and the endothelium, as evidenced by immunohistochemistry with antibody reactive for human (but not rat) HGF (Figure 3A). These findings were found predominantly in the more emphysematous areas than the less injured areas. To verify expression levels, the concentrations of human exogenous and rat endogenous HGF in lung tissue were measured by ELISA at 1, 2, 3, and 4 weeks after transfection. At 1 week, human HGF could be detected at levels as high as 13.0±1.8 ng/g tissue in lung tissue of rats transfected with human HGF vector, whereas human HGF protein could not be detected in control rats. Interestingly, an increase in rat endogenous HGF was also observed in rats transfected with human HGF vector at levels almost 5-fold higher than with control vector (P<0.01). Furthermore, rat HGF expression levels remained at considerably high levels up to 3 weeks after transfection (Figure 3B). Importantly, no HGF gene expression could be detected in other organs, including brain, liver, kidney, and spleen (Table), thus suggesting that the present method with an HVJ envelope leads to selective and persistent expression of exogenous HGF at local sites in emphysematous lung tissues.

**Regenerative and Protective Effects of HGF on Emphysematous Lungs**

HGF gene transfection was performed in elastase-treated rats on day 7 when the HGF levels in lung tissue and plasma were low, as demonstrated previously. Histological examination demonstrated that the lungs from rats that underwent HGF transfection had a decreased area of airspace enlargement and showed increased vascularity. The number of RACs in the HGF group was significantly higher than that in the PPE(-) plus-vector group and PPE(-) group (7.1±0.6 versus 4.6±1.7 and 2.6±1.9, P<0.01; Figure 4A). We next addressed whether HGF, as a potent regenerative factor, alters alveolar regeneration in vivo. PCNA-positive cells were detected frequently in intra-alveolar, septal, and endothelial cells in both HGF-treated and control rat lungs (Figure 4B). The number of PCNA-positive alveolar cells in the HGF group was more than that in the PPE(+) plus-vector group and PPE(+) group (4.3±1.4/mm² versus 3.0±1.1/mm² and 2.0±0.4/mm², respectively). The TUNEL technique was also
used to assess for apoptosis in the lung tissues. Apoptotic cells were frequently detected within the thin alveolar septal walls and at intra-alveolar sites in rats after elastase treatment, whereas apoptotic cells were significantly decreased in HGF-treated rats compared with control rats (0.8 ± 0.7/mm² versus 4.5 ± 1.6/mm² and 6.2 ± 0.6/mm²; Figure 4C).

Improvement of Gas Exchanges Concomitant With Enhanced Angiogenesis

**Angiogenesis**

To evaluate lung angiogenesis in vivo, an immunohistochemical examination with anti-factor VIII was performed. A marked increase in the number of factor VIII–positive pulmonary capillaries was observed in the HGF-treated group compared with the PPE(+)–plus-vector group and PPE(+) group (Figure 5A), and the difference was significant (13.0 ± 1.9/mm² versus 6.0 ± 1.4/mm² and 4.8 ± 0.4/mm², respectively; P<0.01).

**Laser Doppler Analysis for Lung Blood Perfusion**

To determine whether blood perfusion increased in HGF gene–transfected lung, blood perfusion of the lung surface was measured with LDI. Representative images, obtained 1 week after gene transfection, are shown in Figure 5B. Blood perfusion in the lower lung in the HGF group was remarkably increased.

**Effects of HGF Gene Transfection on Pulmonary Function**

The value of PaO₂ in the HGF group 1 week after transfection was higher than that in the control group and was significantly higher at 2 weeks after treatment (87 ± 9 versus 61 ± 7 mm Hg, P<0.05; Figure 5B). Maximum running speed and VO₂max (Figure 5C) were also significantly higher in the HGF group than in the control group as early as 1 week after treatment (30 ± 2.7 versus 20 ± 5.6 m/min and 55 ± 2.4 versus 36 ± 7 mL/kg⁻¹·min⁻¹, P<0.05). At 4 weeks after transfection, significantly higher levels of PAO₂ and exercise tolerance parameters continued. Overall, HGF gene therapy was found to improve the impairment in pulmonary function via alveolar and vascular regeneration.

**Discussion**

The limitations of such medical treatments as lung volume reduction surgery² and the shortage of donors for lung

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Figure 4. Beneficial effects of HGF gene transfection on lung regeneration and protection from emphysematous changes at 7 days after transfection. Three groups were designated as controls. A, Top, Hematoxylin-and-eosin–stained lung tissue at 7 days after transfection; Bottom, changes in number of RACs. Each value represents mean±SEM of values obtained with 5 rats. *P<0.01 vs PPE(+)–plus-vector group. B, Changes in number of PCNA-positive alveolar cells in lung after transfection. Top, Representative photomicrographs subjected to immunohistochemical staining with anti-PCNA antibody; Bottom, semiquantification of these histological findings (mean±SEM, n=5). *P<0.01 vs PPE(+)–plus-vector group. C, Apoptosis in lung tissue and changes in number of TUNEL-positive alveolar septal cells (black arrow) in lung after transfection. Top, Representative photomicrographs stained by TUNEL technique; Bottom, semiquantification of these histological findings (mean±SEM, n=5). *P<0.01 vs PPE(+)–plus-vector group.
transplantation have made the establishment of new treatment strategies for pulmonary emphysema an important global priority. The present results showed that supplementation with a growth factor may ameliorate the lowered levels of exercise tolerance and gas exchange associated with emphysema via inhibition of alveolar wall cell apoptosis, as well as by enhancement of alveolar regeneration and angiogenesis in the pulmonary vasculature.

We found that HGF was upregulated in plasma and lung tissue in the acute phase after elastase induction. We considered that these increased HGF levels were the result of a paracrine mechanism in lung macrophages and endothelial cells and an endocrine mechanism in the liver and kidney. Pulmonary emphysema is clinically characterized by local hypoxia, whereas several in vitro studies demonstrated that hypoxia downregulates HGF gene expression in several cells. Thus, the endocrine system appeared to contribute more predominantly to a transient increase in HGF compared with the paracrine system in emphysematous lungs, given the extrapulmonary HGF levels in the present study. In our previous studies, we demonstrated that HGF acts as an important mitogen in the repair process after lung injury, which was corroborated by the present data, as shown in Figure 2B. However, we also found that disease progression was accompanied by a marked decrease in HGF production, possibly in response to progressive hypoxia locally and in distant organs. In addition, a decrease in vascular beds reflects the marked loss of endothelial cells, which are sources of HGF, which leads to a lack of persistent HGF accumulation and exhausted endogenous HGF production that might be related to the progressive destruction of lung parenchyma, with an associated widespread loss of alveoli and vasculature, due to the failure of tissue regeneration.

To test our hypothesis that a failure of sustained endogenous HGF production may result in aggravation of the pulmonary pathophysiology, human HGF was transfected into elastase-treated rat models. Induction of HGF expression was achieved, which resulted in histological and functional

Figure 5. Therapeutic effects of transfection on lung blood perfusion and pulmonary function. A, Changes in number of vascular density. Vascular density was determined as number of factor VIII-positive capillaries <100 µm in diameter per square millimeter. Top, Distribution of capillary vessels in lung after transfection; Bottom, semiquantification of these histological findings (mean ± SEM, n = 5). *P < 0.01 vs PPE (+)-plus-vector group. B, Top, Representative LDI analysis of lung blood perfusion at 7 days after transfection; Bottom, inhibitory effect of HGF on progression of systemic hypoxemia compared with control vector (Con), as determined by PaO2 levels at 7, 14, and 28 days after transfection. C, Amelioration of cardiopulmonary capacity under exercise stress by HGF gene transfection compared with control vector (Con), as evaluated by maximal (Max) running speed and VO2max in treadmill test at 7, 14, and 28 days after transfection. Each value represents mean ± SEM of values obtained with 5 rats. *P < 0.01 vs control.
pulmonary recoveries that were associated with regeneration of the nearby alveolar cells. Interestingly, in addition to the increase in human HGF, rat HGF concentration in lung tissue was also significantly increased 5- to 8-fold compared with the nontreatment group. With regard to this, an in vitro study demonstrated that exogenous human HGF dramatically enhances rat endogenous HGF production in cultured fibroblast-like and endothelial cells (private communications from S.M. and T.N). Furthermore, the same effects were observed in vivo, as reported. Therefore, we speculated that the exogenous HGF augmented the secretion and/or production of endogenous HGF by the autinduction of HGF.

This paracrine mode of HGF-mediated regeneration would produce beneficial effects that are locally restricted to the lungs, with only minimal side effects for other organs. Furthermore, in addition to the suggestion that transforming growth factor-β1 (TGF-β1) was involved in arresting the regeneration process via inhibition of local HGF production under hypoxic conditions, HGF can also suppress TGF-β1 production and inhibit TGF-β1-induced signal transduction. Taken together, these findings seem to show that HGF supplementation reverses the failure of alveolar regeneration in the context of emphysema.

It is understood that functional impairments associated with emphysema occur in parallel with the degree of loss of pulmonary vascular beds. Furthermore, the addition of secondary vasoconstriction that occurs in response to hypoxia results in irreversible progression toward pulmonary hypertension. Liebow37 introduced the “vascular concept,” which states that a reduction in the blood supply of the small precapillary blood vessels might contribute to the pathogenesis of emphysema. Interestingly, the present study revealed a marked increase in pulmonary vasculature in elastase-induced emphysema rats in response to HGF treatment, whereas previous studies have shown that HGF potently induces angiogenesis in heart, skin, and lung tissues.16–20 Although a large number of angiogenic factors have been characterized, it remains to be shown whether other angiogenic factors, such as basic fibroblast growth factor (b-FGF) and vascular endothelial growth factor (VEGF), which have a proliferative effect on alveolar epithelial cells. Additionally, in contrast to VEGF, which is induced and upregulated by hypoxia and may cause tissue edema, b-FGF may promote fibrosis, HGF does not appear to have such adverse effects.20,38

Several recently reported lines of evidence suggest that the process of programmed cell death, apoptosis, triggers and precedes the progressive destruction of lung tissue in emphysema. Furthermore, the elastase/antielastase imbalance seen in the disease may result from alveolar cell death, followed by inflammatory cell invasion and disruption of the extracellular matrix. Therefore, the present finding that alveolar sepal cell apoptosis was inhibited by HGF may provide a mechanistic explanation for the prevention of progressive tissue destruction in the present model. Many studies have demonstrated the antia apoptotic effects of HGF on various cell lines, whereas it has also been shown to act as a septation-induced factor that promotes branching morphogenesis in injured lungs. Indeed, the promotion of alveolar septation in adults remains a major challenge in the development of adequate treatments for emphysema. However, HGF can exert multiple biological effects in lung tissue after injury, including alveolar proliferation, angiogenesis, tubule formation, and the prevention of apoptosis. Taken together, we considered that HGF supplementation may benefit pulmonary physiological functions in emphysema by minimizing the extent of lung injury and stimulating alveolar septation.

In the present study, HGF supplementation was achieved via intravenous injection of an HVJ envelope vector, and the effect provided by a single injection with this method showed local persistence for several months, which may be controlled by regulating the expression together with a suicide gene. We believe that it is essential for therapeutic strategies to maintain a stable and long-lasting expression in addition to prolonging the effects of HGF, especially for chronic diseases like emphysema. The method used in the present study may be ideal in that sense.

Massaro and coworkers conducted a pioneer study of the ability of retinoic acid to induce the formation of alveoli in a rat emphysema model. Administration of HGF, which has both pulmotrophic and angiogenic activities, is different from their method in that it is ideal as a resuscitation therapy, given the molecular pathogenesis of the disease, and is a rational strategy to pursue because of the self-repairing system inherent in humans. To the best of our knowledge, this is the first report to demonstrate the potential of growth factors in ameliorating the pulmonary pathophysiology of emphysema using animal models. Future studies that utilize a combination of growth factors and gene-transfection technologies that target and are restricted to lung tissue may be useful for clarifying the molecular mechanisms for other lung diseases. Additional detailed studies are required to determine the beneficial effects of HGF on emphysema in clinical trials, as we consider that the effects may depend on dosage, as well as age and animal species.

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References

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