行政院國家科學委員會專題研究計畫　成果報告

微脂體包埋之肝臟生長因子表達質體對Bleomycin誘發肺纖維化之影響

計畫類別：個別型計畫
計畫編號：NSC91-2314-B-002-287-
執行期間：91年08月01日至92年07月31日
執行單位：國立臺灣大學醫學院內科

計畫主持人：王鶴健

報告類型：精簡報告

處理方式：本計畫可公開查詢

中　華　民　國 93 年 2 月 17 日

Introduction

Interstitial lung diseases comprise a diverse group of diseases that cause disruption of alveolar structures, often resulting in pulmonary fibrosis with typical clinical, radiographic, and physiological consequences. Unfortunately, these disorders are progressive and refractory to the limited number of therapeutic options available to clinician. Recently bleomycin-induced murine model of pulmonary fibrosis, has been developed to better understand the underlying pathophysiology and test new therapeutic approach to this disorder (1).

Hepatocyte growth factor (HGF) has been found to be a multifunctional growth factor, produced by mesenchymal cells such as endothelial cells, fibroblasts, and macrophages, and acts on a variety of epithelial cells and organs as mitogen, motogen, and morphogen (2-6). HGF acts upon epithelial tissues via its receptor, c-met (7). Thus, HGF is now recognized as humoral mediator of epithelial-mesenchymal interactions. Recently, there has been interest in the role of HGF in the lung repair process. HGF is a potent mitogen for alveolar and bronchial epithelial cells and stimulates migration and morphogenesis (8). There is no species specificity among human, rats and mouse with regard to biologic activity (9). HGF levels are increased in the bronchoalveolar lavage fluid of patients with idiopathic pulmonary fibrosis, sarcoidosis, and the interstitial disease associated with rheumatoid arthritis (10).

Yaekashiwa and coworkers has demonstrated that continuous delivery of HGF to the systemic circulation suppressed pulmonary fibrosis induced by bleomycin in murine model. HGF had a significant effect whether it was administered at the time of the injury or during the fibrotic phase (2 wk after bleomycin) (11). Makoto et al also showed that recombinant HGF given via intratracheal route on day 3 and day 6 can attenuate collagen accumulation induced by bleomycin (12) These results suggested that HGF may have therapeutic potential for pulmonary fibrosis.

We have set up a rat model of bleomycin-induced pulmonary fibrosis (Figure 1, 2). HGF DNA has been cloned into a CMV promoter driven pCDNA-3 plasmid (Invitrogen) as pCMV-HGF (Fig. 3). Quantification of secreted HGF in pCMV-HGF transfected cell lines (hepatocyte cell lines, ML-3 and HeLa cell) via ELISA has been shown in Table 1. In the current study, we plan to investigate whether rats with bleomycin-induced lung fibrosis can be prevented or treated by HGF secreted via liposome encapsulated HGF-expressing plasmid administered intravenously. Simultaneous or delayed intravenous injection of liposome encapsulated HGF-expressing plasmid in bleomycin treated rats will be performed to investigate the extent of lung injury and fibrosis on 3,7 and 14 days later and compared the severity of pulmonary fibrosis both in control and study groups. From the results of this study we can evaluate the therapeutic potential of liposome encapsulated HGF-expressing plasmid for pulmonary fibrosis.

Materials & Methods

Animal experiment

Sprague-Dawley rats weighing 180 to 200 g were injected intratracheally, while under mild ketamine induced anesthesia, with 1 mg/Kg bleomycin (Bristol-Myers Squibb Co.) in 0.5 ml sterile water. On day 0 and day 3 after bleomycin treatment, liposome encapsulated HGF-expressing plasmid (10μg/animal) (13)will be administered via injection through subclavian vein in study group (N=5). In control groups (N=5 in each group), plasmid or liposome only will be given through same route and time interval as study group. On day 7 and day 14 rats will be killed to obtain the bronchoalveolar fluid and lung tissue.

On each animal, the chest will be opened and the lungs allowed to collapse. A tracheostomy will be performed and lung was lavaged twice with 5 ml of Tris-based saline solution containing 10 μl per ml of protease inhibitor cocktail (Sigma, St Louis, MO). After lavage, the samples per animal will be pooled. An aliquot will be used later to measure protein content and the HGF level by enzyme-linked immunosorbent assay (ELISA) (R & D Co.).

Lung tissue

After lavage, the left lobes of lung will be tied off, removed, and frozen for later measurement of total hydroxyproline. The right lobes will be re-inflated with buffered formaldehyde overnight, then 6 random tissue blocks will be cut for processing and embedded in paraffin. The sections will be prepared and stained with hematoxylin and eosin (for evaluation of the severity of inflammation), and immunohistochemical stain for collagen fiber (for evaluation of severity of fibrosis).

Determination of lung HGF content

HGF content in the lung will be measured by an ELISA kit. After the rats were sacrificed, the lung will be homogenized in four volumes of Tween 80, 1 mM phenymethylsulfonyl fuloride (PMSF), and 1 mM EDTA. The supernatant was used for quantification of HGF, which was expressed as nanogram per gram wet lung tissue.

Hydroxyproline assay

For an evaluation of lung collagen content, hydroxyproline will be measured by modifications of colorimetric method. After perfusion the lungs with PBS, the lung will be cut and hydrolyzed in 2 ml HCl at 110^0 C for 14 h. The pH of the samples will be adjusted to between 6 and 7. A 2-ml aliquot from the total samples volume of 20 ml will be added to 1 ml of 1.4% chloramine T and incubated at room temperature for 20 min, then 1 ml of 3.15 M perchloric acid will be added and incubated for 5 min, followed by the addition of 1 ml of Erlich solution (1 M p-dimethylaminobenzaldehyde in ethylene glycol monomethyl ether). After incubation at 65^0 C for 20 min, absorbance will be measured at 562 nm

with Molecular Devices UV MAX plate reader. The assay will be carried out in quadruplicate for each lung sample. The amount of hydroxyproline will be determined by comparison with a standard cure prepared from known concentration of reagent hydroxyproline.

Results

We have set up a rat model of bleomycin-induced pulmonary fibrosis (Figure 1, 2). HGF DNA has been cloned into a CMV promoter driven pCDNA-3 plasmid (Invitrogen) as pCMV-HGF (Fig. 3). Quantification of secreted HGF in pCMV-HGF transfected cell lines (hepatocyte cell lines, ML-3 and HeLa cell) via ELISA has been shown in Table 1.

Severe pulmonary embolism was ensued when liposmon encapsulated HGF-expressing plasmid was injected via subclavian to the animal. Pulmonary hemorrhage developed soon after the injection & animal died within few minutes So we cant not evaluate the therapeutic effect of liposmon encapsulated HGF-expressing plasmid.

(**A)** **(B)**

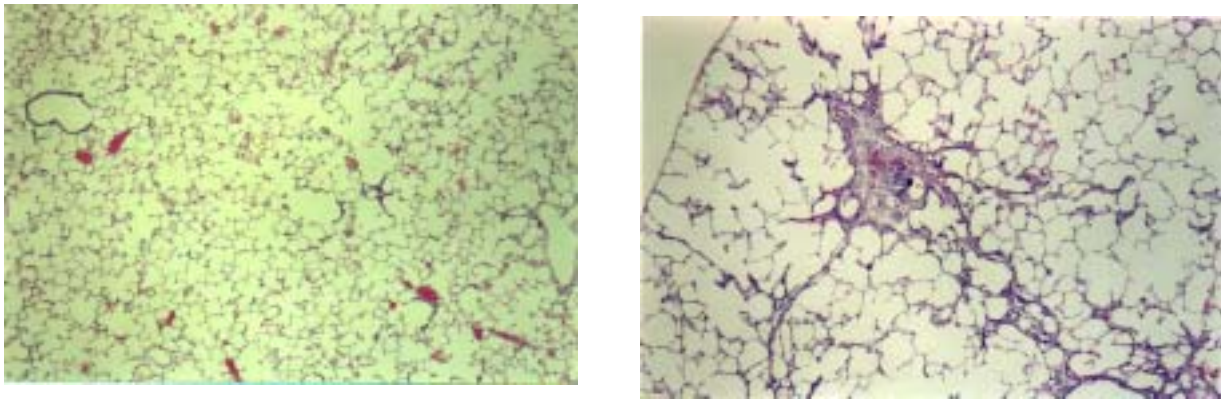
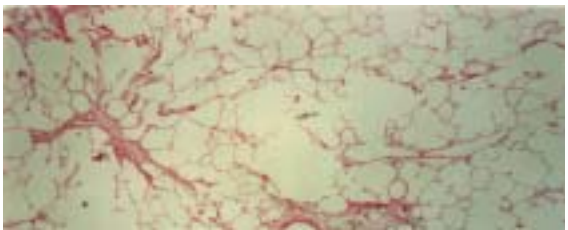


Figure 1. Photomicrographs of lung specimen of control rat (A) and 7 days after bleomycin intratracheally instillated rat (B) (HE stain) X 100

(A) **(B)**



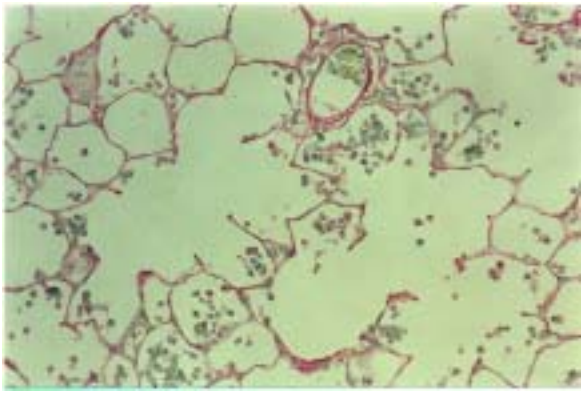


Figure 2. Sirius staining for collagen fiber in control rat (A) and bleomycin intratracheally instillated rat (B).

(A) **(B)**

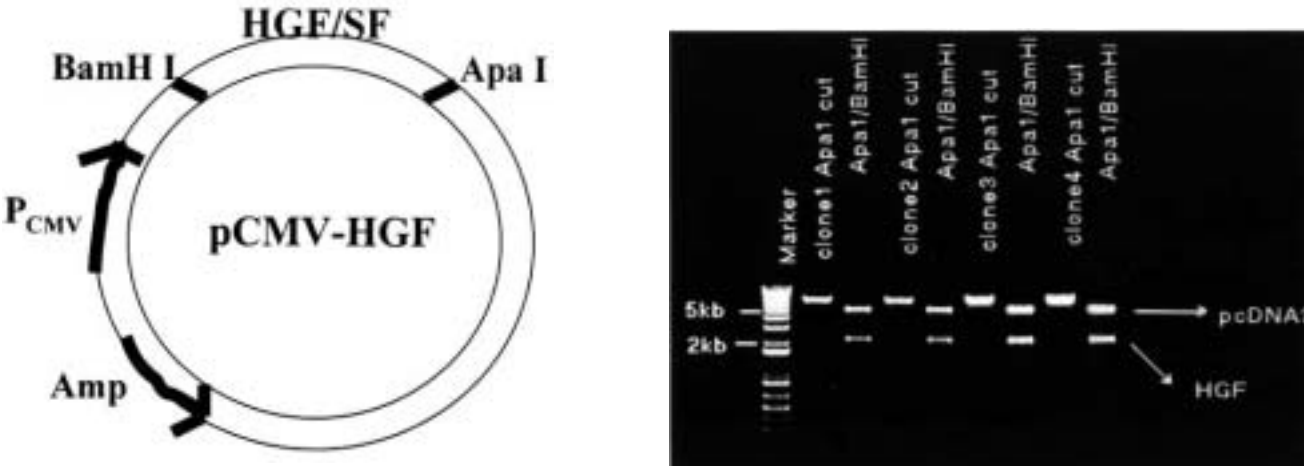


Figure 3. Construct map of plasmid pCMV-HGF (A), and agarose gel eletrophoresis results of different restriction enzymes digestion (B).

Table 1. Determination of secreted HGF protein in PCMV-HGF transfected cell lines with different Liposome formula

HGF (pg / ml)	ML-3			He La		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
PCMV-HGF	-123.676	-101.1	-123.676	-139.802	-146.253	-123.676
PCMV-HGF+DOC	60.16	237.546	505.237	1585.68	3627.231	3540.151
PCMV-HGF+LPD-1	1727.588	1347.015	2669.347	1353.465	1630.832	1359.916

DOC: Cationic liposome + cholesterol

LPD-1: Liposome from Dr. Leaf Huang

Reference

1.Crouch E. Pathobiology of pulmonary fibrosis. Am J physiol 1990;259:L159-L148.

1. Ynagita K, Nagaike M, Ishibashi H, Niho Y et al. Lung may have an endocrine function producing hepatocyte growth factor in response to injury of distal organ. Biolchem. Biophys. Res Commun. 1992;182:802-809.
2. Nakamura T, Nishizawa T, Hagiya M, Seiki T, et al. Molecular cloning and expression of human hepatocyte growth factor. Nature 1989; 342:440-443.
3. Matsumoto K, HashimotoK, Yoshikawa K, Nakamura T. Marked stimulation of growth and motility of human keratinocytes by hepatocyte growth factor. Exp. Cell Res.1991;196:114-120.
4. Weidner KM, Arakaki N, Hartmen G et al. Evidence for the identity of human scatter factor and human hepatocyte growth factor. Proc. Natl. Acad. Sci. U.S.A. 1991;88:7001-7005.
5. Montesano R, Matsumoto K, Nakamura T, et al. Identification of fibroblast-derived epithelial morphogen as hepatocyte growth factor. Cell 1991;67:901-908.
6. Rosen EM, Nigam SK, Goldberg ID et al. Scatter factor and the c-met receptor: a paradigm for mesenchymal/epithelial interaction. J cell Biol 1994;127:1783-1787
7. Singh-Kaw P, Zarnegar R, Sigfried JM, et al. Stimulated effects of hepatocyte growth factor on normal and neoplastic human bronchial epithelial cells. AM J Physiol 1995;268:L1012-L1020.
8. Ishiki Y, Ohnishi H, Muto Y, et al. Direct evidence that hepatocyte growth factor is a hepatotrophic factor for liver regeneration and has a potent antihepatitis effect in viva. Hepatol. 1992;16:1227-1235.
9. Sakai T, Satoh K, Matsushima K, et al. Hepatocyte growth factor in brochoalveolar lavage fluids an cells in patients with inflammatory chest diseases of the lower respiratory tract: detection by RIA and in situ hybridization. Am J Respir Cell Mol Biol 1997;16:388-397.
10. Yaekashiwa M, Nakayama S, Ohnuma K, et al. Simultaneous or delayed administration of hepatocyte growth factor equally represses the fibrotic changes in murine lung injury induced by bleomycin a morphologic study. Am J Respir Crit Care Med 1997;156:1937-1944.

12. Dohi M Hasegawa T, Yamamoto K, et al. Hepatocyte growth factor attenuates collagen accumulation in a murine model of pulmonary fibrosis. Am J Respir Crit Care Med 2000;162:2302-2307.

13. Lee CH, Ni YH, Chen CC, et al: Synergistic effect of polyethylnimine and cationic liposomes in nucleic acid delivery to human cancer cells. Biochimica et Biophysica Acta 2003, 1611: 55-62.