Keratinocyte Growth Factor and Hepatocyte Growth Factor/ Scatter Factor Are Heparin-binding Growth Factors for Alveolar Type II Cells in Fibroblast-conditioned Medium

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Abstract

Epithelial–mesenchymal interactions mediate aspects of normal lung growth and development and are important in the restoration of normal alveolar architecture after lung injury. To determine if fibroblasts are a source of soluble growth factors for alveolar type II cells, we investigated the effect of fibroblast-conditioned medium (CM) on alveolar type II cell DNA synthesis. Serum-free CM from confluent adult human lung fibroblasts was concentrated fivefold by lyophilization. Type II cells were isolated from adult rats by elastase dissociation and incubated with [^{3}H]thymidine and varying dilutions of concentrated CM and serum from day 1 to 3 of culture. Stimulation of type II cell DNA synthesis by fibroblast-CM was maximal after 48 h of conditioning and required the presence of serum. The activity of the CM was eliminated by boiling and by treatment with trypsin, pepsin, or dithiothreitol and was additive with saturating concentrations of acidic fibroblast growth factor, epidermal growth factor, and insulin. The growth factor activity bound to heparin–Sepharose and was eluted with 0.6 and 1.0 M NaCl. Neutralizing antibody studies demonstrated that the primary mitogens isolated in the 0.6 and 1.0 M NaCl fractions were keratinocyte growth factor (KGF, fibroblast growth factor 7) and hepatocyte growth factor/scatter factor (HGF/SF), respectively. HGF/SF was demonstrated in the crude CM and KGF was detected in the 0.6 M NaCl eluent by immunoblotting. Northern blot analysis confirmed that the lung fibroblasts expressed both KGF and HGF/SF transcripts. Human recombinant KGF and HGF/SF induced a concentration- and serum-dependent increase in rat alveolar type II cell DNA synthesis. We conclude that adult human lung fibroblasts produce at least two soluble heparin-binding growth factors, KGF and HGF/SF, which promote DNA synthesis and proliferation of rat alveolar type II cells in primary culture. KGF and HGF/SF may be important stimuli for alveolar type II cell proliferation during lung growth and after lung injury. (*J. Clin. Invest.* 1993. 92:969–977.) Key words: alveolar type II cell • keratinocyte growth factor • hepatocyte growth factor • scatter factor

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Received for publication 16 July 1992 and in revised form 17 March 1993.

The Journal of Clinical Investigation, Inc.
Volume 92, August 1993, 969–977

Introduction

In most forms of diffuse lung injury, alveolar type I cells are destroyed and the alveolar epithelial lining is disrupted. Repair occurs when alveolar type II cells proliferate and differentiate into type I cells, restoring the integrity of the alveolar epithelium (1–3). If type II cell proliferation and differentiation are impaired, mesenchymal cells may migrate into the alveolar space or alveolar collapse may occur. Fibrosis ensues and alveolar gas exchange function is diminished (4, 5). Very little is known about the mechanisms that regulate the processes of cellular proliferation, differentiation, and repair in the alveolar epithelium after lung injury.

Leslie et al. (6–8) have demonstrated that factors such as insulin, cholera toxin, EGF, acidic fibroblast growth factor (aFGF),[1] and medium conditioned by alveolar macrophages stimulate DNA synthesis in alveolar type II cells in primary culture. Brandes and Finkelstein (9) have also shown that stimulated macrophages secrete a factor(s) that induces proliferation of rabbit alveolar type II cells in vitro. In addition, Leslie et al. (10) described a factor(s) in bronchoalveolar lavage fluid (BALF) from normal rats that stimulates DNA synthesis in alveolar type II cells. No defined growth factor(s) has been identified in these conditioned media or in BALF, and the role of macrophages and BALF in alveolar type II cell proliferation after lung injury or during lung growth is not known. In a previous study, we did not find an increase in the normal BALF stimulatory activity after silica-induced lung injury, a condition characterized by proliferation of type II cells (11).

In addition to inflammatory cell–derived factors, epithelial–mesenchymal interactions are believed to mediate aspects of normal lung growth and development and may be important in alveolar epithelial repair (12–15). Everett et al. (16) have demonstrated that lung fibroblasts from hyperoxia-treated hamsters produce growth factor activity that stimulates DNA synthesis and cell division in rat alveolar type II cells in primary culture. Fetal lung fibroblast CM contains mitogenic activity for fetal alveolar type II cells (17). Although these fibroblast-derived growth factor activities for alveolar epithelial cells have been partially characterized, specific growth factor(s) have not heretofore been identified. In this report we demonstrate that adult human lung fibroblasts produce two recently described heparin-binding growth factors, keratinocyte growth

1. *Abbreviations used in this paper:* aFGF, acidic fibroblast growth factor; BALF, bronchoalveolar lavage fluid; CM, conditioned medium; HGF/SF, hepatocyte growth factor/scatter factor; KGF, keratinocyte growth factor; r, recombinant.

factor (KGF) and hepatocyte growth factor/scatter factor (HGF/SF), that stimulate DNA synthesis and proliferation of rat alveolar type II cells in primary culture.

Methods

Isolation of alveolar type II cells and preparation of human lung fibroblast CM. Alveolar type II cells were isolated from specific pathogen-free Sprague-Dawley rats weighing 150–250 g (Bantin and Kingman, Inc., Fremont, CA) by elastase dissociation and purification on discontinuous metrizamide density gradients as described previously (18). Cell viability was determined by erythrocin B exclusion and the purity of alveolar type II cells was assessed by modified Papanicolaou staining (19). On the day of isolation, alveolar type II cell purity was > 75% and was > 90% on day 1 at the start of the thymidine incorporation assay. Cell viability was > 90% immediately after isolation. Adult human lung fibroblasts (no. AG02262) were obtained from the National Institute of Aging, Cell and Culture Repository, Coriell Institute for Medical Research (Camden, NJ). The cells were obtained at population doubling 4. The human lung fibroblasts were cultured in DME (GIBCO-Bethesda Research Laboratories, Gaithersburg, MD) containing 10% FCS (Sigma Chemical Co., St. Louis, MO), 2 mM glutamine, 10 μg/ml gentamicin, 2.5 μg/ml amphotericin B, 100 U/ml penicillin G, and 100 μg/ml streptomycin. The medium was changed every 2–3 d until the fibroblasts reached a dense confluency. The cells were then washed with DME and the medium was changed to DME without serum. The fibroblasts were maintained at 37°C in a humidified incubator containing 90% air/10% CO_2. The lung fibroblast culture supernatant-CM was filtered, lyophilized, and stored at −20°C. Before use, the lyophilized CM was reconstituted in one-fifth the original volume with distilled water and then dialyzed against DME in dialysis tubing with a molecular weight cutoff of 3,500 (Spectra/Por; Spectrum Medical Industries, Inc., Los Angeles, CA). Henceforth this fivefold concentrated CM will be referred to as concentrated CM.

Cell culture, DNA synthesis, and cell proliferation assays. Isolated alveolar type II cells were suspended in DME containing 10% FCS and plated onto 48-well plates (Costar Corp., Cambridge, MA) at 10^5 cells per well and maintained at 37°C in a humidified incubator containing 90% air/10% CO_2. [^{3}H]Thymidine incorporation was assayed as described previously (11). Briefly, 24 h after plating, the nonadherent alveolar type II cells were removed with the medium, and 0.5 ml of concentrated CM, 0.5 μCi [^{3}H]thymidine (adjusted to a specific activity of 1.12 μCi/nmol), and FCS were added to each well along with fresh media to bring the final volume to 1.0 ml and the cells were incubated for 48 h. Each condition was tested in duplicate or triplicate wells. To determine [^{3}H]thymidine incorporation, the DNA was precipitated with cold 10% TCA, solubilized with 1.0 M sodium hydroxide, and aliquots were counted by liquid-scintillation spectrometry. To determine cell number, cells were plated in DME containing 10% FCS at 5×10^5 cells per 35-mm dish. On day 1, the nonadherent cells were removed with medium, and the growth factor(s) and 10% FCS in DME were added. After a 2-d incubation, the cells were washed with unsupplemented DME and harvested with 0.05% trypsin in 0.053 mM EDTA (GIBCO-Bethesda Research Laboratories). Trypsin was inactivated by an equal volume of DME containing 10% FCS, and the cells were counted in a hemocytometer. Autoradiography and determination of alveolar type II cell nuclear labeling index were performed as described previously (11). The time-dependent accumulation of stimulatory activity for alveolar type II cells in human lung fibroblast-CM was determined by harvesting the fibroblast supernatant-CM after 6, 12, 24, 48, 72, and 96 h. These media were lyophilized, concentrated fivefold, and then tested in the alveolar type II cell [^{3}H]thymidine incorporation assay at a concentration of 50% in the presence of 10% FCS. (The concentration of CM is defined as the percentage of the final volume that is concentrated CM.) To determine the serum dependence of the growth factor activity in fibroblast CM, the concentrated CM was tested at a concentration of 50% in the presence of 0, 1, 3, 5, 10, and 20% FCS.

Characterization of growth stimulatory activity. To characterize the mitogenic activity in the human lung fibroblast-CM, various biophysical properties of the stimulatory activity in the concentrated fibroblast-CM were determined. Heat stability was determined by heating the fibroblast-CM to 65°C for 30 min or to boiling for 10 min. To assess the effect of enzymatic protease treatment, the fibroblast-CM was treated with trypsin or pepsin. The CM was incubated with 1 mg/ml trypsin at 37°C for 2 h and the trypsin was inactivated with 2 mg/ml soybean trypsin inhibitor. As a control, the CM was incubated with 2 mg/ml soybean trypsin inhibitor at 37°C for 2 h. For the pepsin enzymatic digestion, the pH of the CM was adjusted to 2.5 with 1 N HCl, and the CM was incubated with 0.2 mg/ml pepsin at 37°C for 2 h and the pH was then readjusted to 7.4 with 1 N NaOH. Reduction by DTT was determined by maintaining the CM in 2 mM DTT at 37°C for 2 h. Sensitivity to acid was determined by adjusting the pH of the fibroblast CM to 2.5 with 1 N HCl, incubating the CM at 37°C for 2 h, and readjusting the pH to 7.4 with 1 M NaOH. After each treatment, the CM was dialyzed against DME, diluted twofold with DME, and incubated with alveolar type II cells in the presence of [^{3}H]thymidine and 10% FCS from day 1 to 3 in culture. To determine if fibroblast-CM activity was additive with known growth factors for alveolar type II cells, saturating concentrations of insulin (10 μg/ml), EGF (20 ng/ml), and aFGF (200 ng/ml) alone or in combination were tested in the presence or absence of 50% concentrated fibroblast-CM.

Identification of KGF and HGF/SF in the CM. The stimulatory activity in the fibroblast-CM supernatant was partially purified by heparin-Sepharose affinity chromatography. The fibroblast-CM was harvested after 48 h of conditioning, filtered through a 0.45-μm filter, and frozen at −20°C before use. Approximately 150–200 ml of the fibroblast-CM was loaded onto a heparin-Sepharose column (Pharmacia Inc., Piscataway, NJ) with a bed volume of 4 ml. The column was washed with 0.2 M NaCl in 20 mM Tris (pH 7.5) until the A_{280} was 0 and eluted at a flow rate of 20 ml/h with a step gradient of increasing NaCl concentration in 20 mM Tris (pH 7.5). 6-ml fractions were collected and dialyzed against DME, diluted 2-fold with DME, and incubated with alveolar type II cells in the presence of 10% FCS in the DNA synthesis assay.

To determine if the stimulatory activity eluting from the heparin-Sepharose column was due to known heparin-binding growth factors, active fractions were incubated with inhibitory antibodies to KGF or hepatocyte growth factor/scatter factor (HGF/SF) in the DNA synthesis assay. The anti-KGF antibody was a murine monoclonal antibody that, at a final concentration of 1 μg/ml, inhibited ≥ 85% of the activity of a near maximal dose and ≥ 98% of half-maximal recombinant (r)KGF-induced stimulation of BALB/MK keratinocytes (J. S. Rubin, manuscript in preparation). The anti-HGF/SF antibody was the purified IgG fraction of serum from rabbits immunized with purified HGF/SF and inhibited ≥ 90% of the activity of half-maximal rHGF/SF-induced stimulation of B5/589 human mammary epithelial cells at a dilution of 1:250 (~ 8 μg/ml) (20). The anti-KGF antibody was used at a final concentration of 5.0 μg/ml and the anti-HGF/SF antisera was tested at a dilution of 1:200 in the [^{3}H]thymidine incorporation assay with the fractions eluting from heparin-Sepharose in 0.6 and 1.0 M NaCl as well as crude CM (see Fig. 6). All samples were tested in the presence of 10% FCS. Bacterially expressed human rKGF and baculovirus-expressed rHGF/SF (J. S. Rubin, unpublished data) were diluted with DME alone or DME containing 10% FCS and tested at various concentrations in the [^{3}H]thymidine incorporation assay (see Fig. 8).

Total cellular RNA was isolated by lysis of human lung fibroblasts in guanidine isothiocyanate and centrifugation through a CsCl cushion (21). RNA was fractionated on 1% agarose gels containing formaldehyde and transferred to nylon membranes. The KGF probe was a 712-bp fragment corresponding to nucleotides 17–729 (22) and the HGF/SF probe was a 2.2-kb fragment corresponding to nucleotides −24 to +2187 (23). Probes were labeled with [^{32}P]dCTP using nick translation. For HGF/SF, the blots were hybridized overnight at 42°C in 50% formamide, 5× SSC, 10 mM sodium phosphate, pH 6.6, 0.5% SDS

containing 50 μg/ml denatured salmon sperm DNA. They were then washed with 0.2× SSC, 0.1% SDS for 30 min once at room temperature and twice at 60°C. For KGF, the blots were hybridized overnight at 42°C in 50% formamide, 5× SSC/25 mM sodium phosphate, pH 6.6, 2.5× Denhardt's solution containing 50 μg/ml denatured salmon sperm DNA and then washed with 0.2× SSC, 0.1% SDS for 30 min once at room temperature and twice at 60°C.

Approximately 100 ng of heparin-Sepharose-purified KGF from the culture supernatant of M426 fibroblasts (24) was resolved by 12.5% SDS-PAGE (25) alongside a concentrate of the 0.6 M NaCl heparin-Sepharose fraction obtained from 50 ml of adult lung fibroblast-CM. Proteins were transferred to Immobilon (Millipore Corp., Bedford, MA) according to the method of Towbin et al. (26). The Immobilon was blocked with 5% BSA in 20 mM Tris-HCl, pH 7.4, for 2 h at 42°C and blotted with 0.1 μg/ml affinity-purified rabbit polyclonal antiserum raised against a synthetic peptide corresponding to the carboxy-terminal 14 amino acids of the KGF sequence (22). After a 1-h incubation at room temperature, the blot was washed six times with PBS, 0.05% Tween, 0.02% azide, incubated with [^{125}I]protein A for 30 min at room temperature, washed six times with PBS, Tween, azide, and exposed to x-ray film at −70°C. To detect HGF/SF by immunoblotting, ~ 40 ml of fibroblast-CM was incubated with 200 μl of a 50% heparin-Sepharose slurry in 20 mM Tris, 0.3 M NaCl, pH 7.4 for 4–5 h at 4°C. The resin was precipitated by centrifugation, washed with equilibration buffer, and eluted with 60 μl of Laemmli buffer, which was loaded directly onto a 12.5% polyacrylamide gel. The purified IgG fraction of anti-HGF/SF rabbit polyclonal serum (~ 8 μg/ml) was used to detect HGF/SF using the same protocol as for KGF.

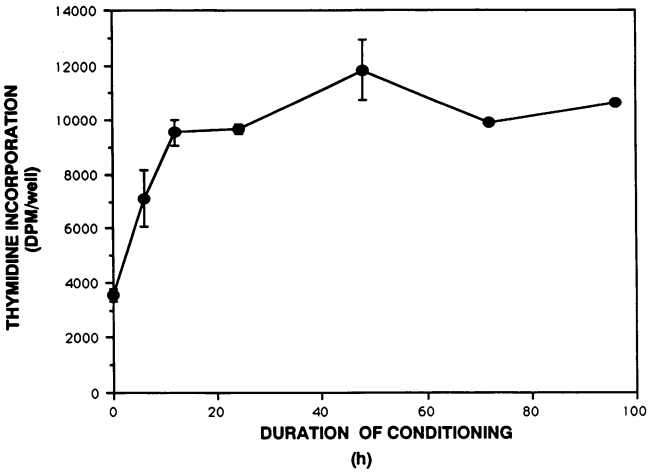

Figure 1. Time-dependent accumulation of human lung fibroblast-CM growth factor activity. Human lung fibroblasts were grown to confluence in DME supplemented with 10% FCS. The cells were washed with DME and then incubated with unsupplemented medium. The CM was concentrated fivefold by lyophilization. Alveolar type II cells were isolated from adult male rats by elastase dissociation and purification on discontinuous metrizamide gradients. The type II cells were incubated with [^{3}H]thymidine and 50% concentrated CM supplemented with 10% serum from day 1 to 3 in culture. Values are the mean±SEM of four experiments.

Results

Time-dependent accumulation and serum dependence of fibroblast-CM stimulatory activity. Initial studies were performed with fibroblasts isolated from embryonic and adult rat lungs by mechanical disruption and explant culture. Medium conditioned by these fibroblasts stimulated alveolar type II cell DNA synthesis in the [^{3}H]thymidine incorporation assay but this activity was dependent upon the fibroblast isolation and the passage number. Whereas there was no apparent difference in stimulatory activity between medium conditioned by fetal and adult fibroblasts, CM derived from fibroblasts maintained in culture for > 3 wk had decreased stimulatory activity compared with freshly isolated fibroblasts (data not shown). In contrast, medium conditioned by the human lung fibroblast cell line AG02262 maintained constant stimulatory activity for several months while being maintained in DME and 10% FCS and passaged every 5–7 d. CM was obtained throughout this period. In addition, fibroblasts were maintained in roller bottles and cycled (periods of DME supplemented with 10% FCS alternating with DME alone). Cells were maintained and media conditioned under these conditions for ~ 2 mo.

The human lung fibroblast-CM stimulatory activity accumulated in a time-dependent manner (Fig. 1). Activity increased rapidly over the first 12 h and was maximal after 48 h. To determine if the conditioning and processing protocol altered the DME and produced stimulatory activity, DME was incubated for 48 h in the absence of fibroblasts and processed in the same manner as the fibroblast-CM. This medium did not stimulate DNA synthesis in alveolar type II cells (data not shown).

The fibroblast-CM stimulatory activity for alveolar type II cells demonstrated an absolute requirement for serum (Fig. 2). At FCS concentrations < 3%, 50% concentrated CM did not stimulate an increase in DNA synthesis (data not shown). Half-maximal stimulatory activity occurred at 5% FCS and the stimulatory activity was maximal at 10% FCS. In the presence of 10% FCS, the stimulatory activity of fibroblast-CM was concentration dependent (Fig. 2). Half-maximal activity occurred at 10% concentrated CM and maximal activity was produced by 50% concentrated CM.

To demonstrate that the thymidine incorporation was occurring in type II cells and to evaluate cell proliferation, alveolar type II cell-labeling indexes and changes in cell number were measured. When type II cells were incubated with 10% FCS from day 1 to 3 of culture, the cell number increased from 18.1±1.4 × 10^4 at day 1 to 25.7±2.2 × 10^4 at day 3

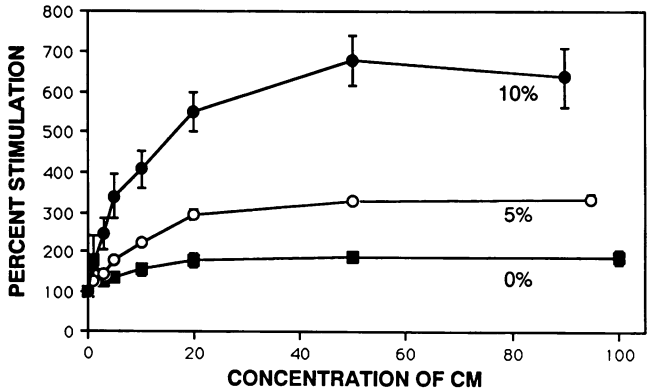

Figure 2. Growth factor activity in fibroblast-CM is concentration and serum dependent. The cells were incubated with [^{3}H]thymidine and varying concentrations of fivefold concentrated CM and serum (*filled circles*, 10%; *open circles*, 5%; *filled squares*, 0%) from day 1 to 3 in culture. [^{3}H]Thymidine incorporation is expressed as percent stimulation; 100% is the level of thymidine incorporation in the absence of CM. Values are the mean±SEM of four experiments.

(mean±SEM, n = 4). In the presence of 10% FCS supplemented with 10 ng/ml rHGF/SF, 10 ng/ml rKGF, or both factors together, the cell number increased to 31.9±2.5, 34.7±3.0, and 46.8±5.8 × 10^4 at day 3 ($P = 0.05$, $P < 0.02$, $P < 0.01$, respectively, compared with cells cultured in 10% FCS alone). rHGF/SF and rKGF increased the alveolar type II cell-labeling index (Table I). Thus, rKGF and rHGF/SF induced not only alveolar type II cell DNA synthesis but also stimulated cellular division and an increase in type II cell number.

Partial characterization of the fibroblast-CM stimulatory activity. The fibroblast-CM was subjected to the treatments outlined in Fig. 3. These studies demonstrated that the stimulatory activity was trypsin and pepsin sensitive, heat labile, and partially diminished by acid or reduction with DTT. The fibroblast-derived stimulatory activity was additive with all growth factors for alveolar type II cells tested individually or in combination (Fig. 4). EGF, insulin, and aFGF were tested at maximal stimulatory concentrations, suggesting that the stimulatory activity in the fibroblast-CM was different from these previously identified growth factors for alveolar type II cells.

Identification of KGF and HGF/SF. The stimulatory activity of the fibroblast-CM was partially purified by heparin-Sepharose affinity chromatography. Crude CM was applied to a heparin-Sepharose column and eluted with a step gradient of increasing salt concentration. Stimulatory activity eluted from heparin-Sepharose in 0.6 and 1.0 M NaCl (Fig. 5). Because the flowthrough from the heparin-Sepharose column did not stimulate type II cell DNA synthesis (data not shown), the stimulatory activity in the fibroblast-CM was due to at least two distinct heparin-binding fractions.

To determine if the stimulatory activity of the fibroblast-CM was attributable to KGF and HGF/SF, two heparin-binding growth factors that elute from heparin-Sepharose in 0.6 and 0.8–1.0 M NaCl, respectively, the crude CM, and the fractions eluting from heparin-Sepharose in 0.6 and 1.0 M NaCl were incubated with neutralizing antibodies to KGF and HGF/SF in the alveolar type II cell DNA synthesis assay (Fig. 6). The

Table I. rHGF/SF and rKGF Increased the Alveolar Type II Cell-labeling Index

		Alveolar type II cells			
		Mononuclear		Binuclear	
Condition	Purity	Labeled	Unlabeled	Labeled	Unlabeled
			%		
FCS	90.9±0.9	2.9±0.8	96.2±0.7	0.2±0.2	0.8±0.3
rHGF/SF	89.7±0.7	5.3±1.4	92.7±1.5	1.2±0.3	0.9±0.2
rKGF	90.4±0.8	6.1±1.5	91.7±1.2	1.4±0.2	1.2±0.3
rHGF/SF + rKGF	88.0±0.9	15.1±3.3	79.5±4.0	4.7±1.3	0.6±0.3

Alveolar type II cells were incubated with 10 ng/ml rHGF/SF, 10 ng/ml rKGF, or both factors in the presence of 10% FCS from day 1 to 3 of culture. Autoradiography was performed and type II cells were identified by their characteristic inclusions by staining with osmium tetroxide and tannic acid. The alveolar type II cell-labeling index was calculated as previously described (11). Data are expressed as mean±SEM, n = 4.

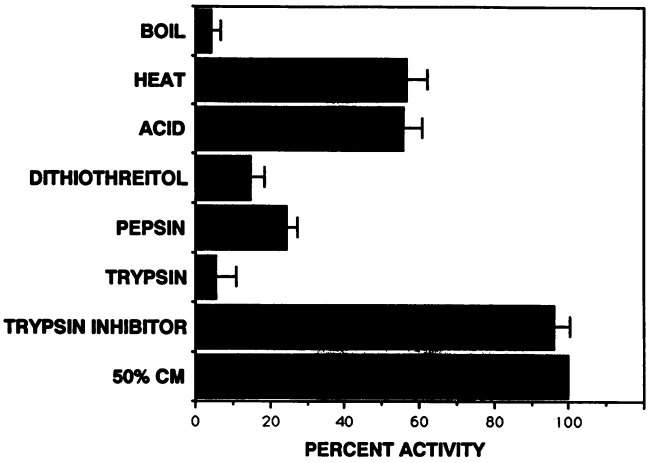

Figure 3. Partial characterization of the growth factor activity in fibroblast-CM. CM and alveolar type II cells were prepared as described in Fig. 1. The CM was subjected to the following treatments: *BOIL,* CM was boiled for 10 min; *HEAT,* CM was heated to 65°C for 30 min; *ACID,* pH of CM was adjusted to 2.5 with 1 N HCl and the CM was incubated at 37°C for 2 h and then the pH was readjusted to 7.4 with 1 M NaOH; *DITHIOTHREITOL,* CM was incubated with 2 mM dithiothreitol at 37°C for 2 h; *PEPSIN,* pH of CM was adjusted to 2.5 with 1 N HCl and the CM was incubated with 0.2 mg/ml pepsin at 37°C for 2 h and the pH was readjusted to 7.4 with 1 M NaOH; *TRYPSIN,* CM was incubated with 1 mg/ml trypsin at 37°C for 2 h and the trypsin was inactivated with 2 mg/ml soybean trypsin inhibitor; *TRYPSIN INHIBITOR,* CM was incubated with 2 mg/ml soybean trypsin inhibitor at 37°C for 2 h. *50% CM,* untreated concentrated CM. After each treatment, the CM was dialyzed against DME, diluted twofold with DME, and incubated with type II cells in the presence of [^{3}H]thymidine and 10% FCS from day 1 to 3 in culture. Percent activity was calculated from the thymidine incorporation (decay per minute per well) by this formula: 100 × [(test condition − 10% FCS alone)/(50% CM − 10% FCS alone)]. Values are the mean±SEM of four experiments.

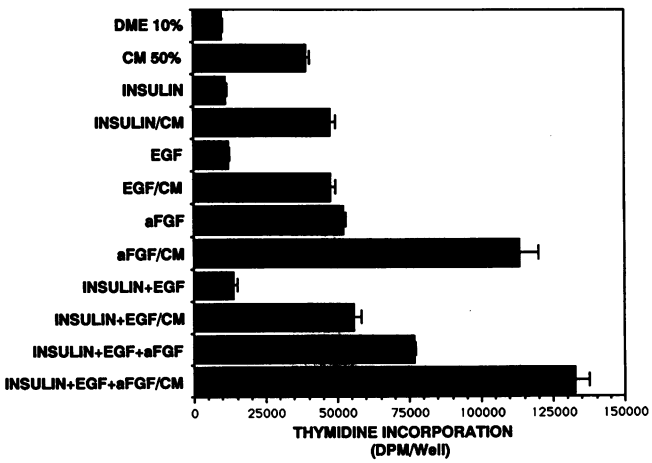

Figure 4. Activity of fibroblast-CM is additive with known growth factors for alveolar type II cells. CM and type II cells were prepared as in Fig. 1. Type II cells were incubated with [^{3}H]thymidine, 10% FCS, and saturating doses of growth factors known to stimulate type II cell DNA synthesis: insulin (10 μg/ml); *EGF,* epidermal growth factor (20 ng/ml); *aFGF,* acidic fibroblast growth factor (200 ng/ml), in the presence or absence of 50% concentrated CM from day 1 to 3 of culture. DME 10% is DME supplemented with 10% serum. Values are the mean±SEM of four experiments.

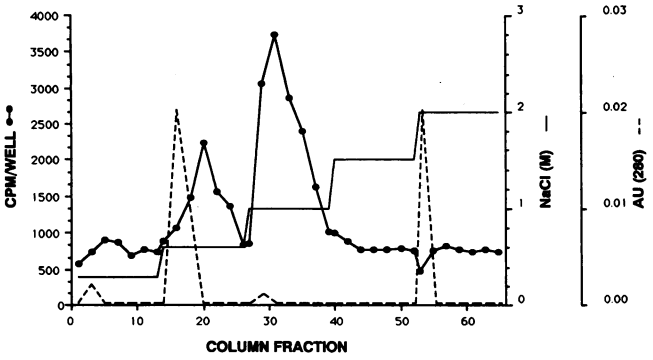

Figure 5. Heparin-Sepharose chromatography of fibroblast-CM demonstrates stimulatory activity eluting in 0.6 and 1.0 M NaCl. 120 ml of fibroblast-CM was loaded onto a heparin-Sepharose column (4.0-ml bed volume). The column was washed with 0.2 M NaCl in 20 mM Tris, pH 7.5, until the A_{280} was 0 and eluted with a step gradient of increasing NaCl concentration in 20 mM Tris, pH 7.5. 6-ml fractions were eluted at a flow rate of 20 ml/h. The fractions were dialyzed against DME, diluted twofold with DME, and incubated with type II cells in the presence of 10% FCS [^{3}H]thymidine from day 1 to 3 of culture. A representative experiment of many similar elution profiles is shown.

anti-KGF antibody inhibited 80±11% of the activity eluting in 0.6 M NaCl and the anti-HGF/SF antisera blocked 84±5% of the activity eluting in 1.0 M NaCl (n = 3). Neither antibody inhibited basal thymidine incorporation by type II cells incubated in the presence of 10% serum. On the basis of the degree of inhibition of the crude CM activity by the anti-KGF and anti-HGF/SF antibodies and the absolute level of thymidine incorporation induced by the fractions eluting in 0.6 and 1.0 M NaCl, HGF/SF appears to be the dominant stimulant of alveolar type II cell DNA synthesis in the fibroblast-CM.

KGF was demonstrated in the 0.6 M NaCl heparin-Sepharose fraction and HGF/SF was detected in the crude CM by immunoblotting (Fig. 7, *A* and *B*, respectively). The human lung fibroblasts expressed transcripts for both KGF and HGF/SF (Fig. 7 *C*). Both rKGF and rHGF/SF induced type II cell

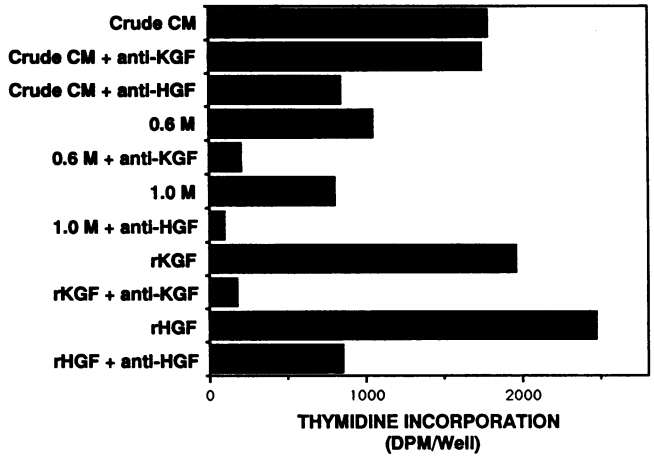

Figure 6. Stimulatory activity eluting from heparin-Sepharose in 0.6 and 1.0 M NaCl is inhibited by neutralizing antibodies to KGF and HGF/SF, respectively. Heparin-Sepharose chromatography and sample preparation were performed as described in Fig. 5. 15 min before adding the samples to the alveolar type II cells, neutralizing antibodies were added to the indicated samples. The anti-KGF murine monoclonal antibody was tested at a final concentration of 5.0 μg/ml and the rabbit anti-HGF/SF sera was tested at a dilution of 1:200. Crude CM is unprocessed fibroblast-CM tested at 1:10 dilution; 0.6 M is the fraction eluting from heparin-Sepharose in 0.6 M NaCl tested at 1:10 dilution; 1.0 M is the fraction eluting from heparin-Sepharose in 1.0 M NaCl tested at 1:5 dilution; rKGF was tested at 1 ng/ml; rHGF/SF was tested at 1 ng/ml. All samples were tested in the presence of 10% FCS. Thymidine incorporation is expressed as: measured thymidine incorporation (cells incubated with the test condition) − basal thymidine incorporation (cells incubated with DME supplemented with 10% FCS only), 1,980 dpm per well. A representative experiment is shown; these data were reproduced in two additional independent experiments.

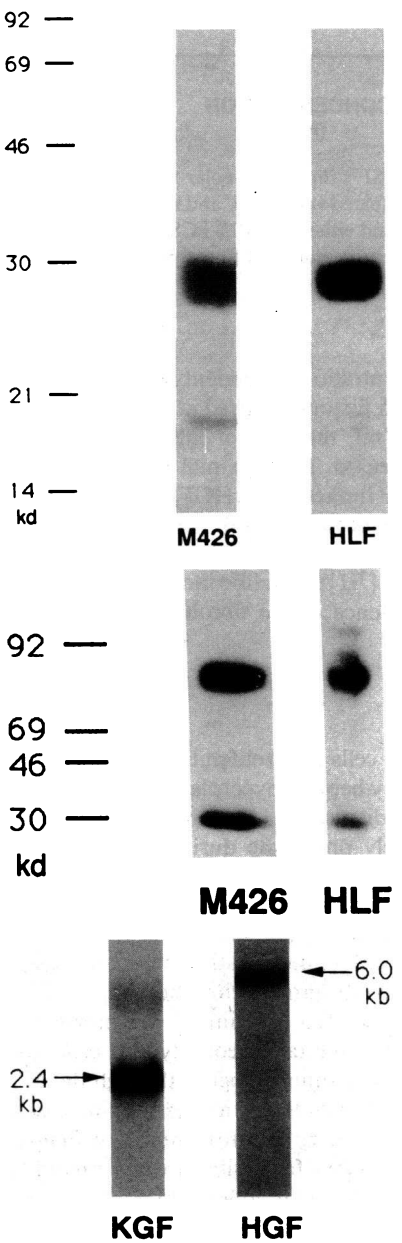

Figure 7. (*A*) Immunoblot demonstrating KGF in the 0.6 M NaCl fraction eluting from heparin-Sepharose. CM was processed and chromatography was performed as in Fig. 5. The 0.6 M NaCl heparin-Sepharose fraction was concentrated, resolved by 12.5% SDS-PAGE, transferred to Immobilon, and incubated with rabbit antisera to KGF. The M426 sample was heparin-Sepharose–purified KGF isolated from M426-CM, the original source of KGF (24). In addition to the 26–28-kD KGF band, an ~ 18-kD proteolytic fragment was observed in both M426 and adult human lung fibroblast (*HLF*) CM. (*B*) Immunoblot demonstrating HGF/SF in the crude fibroblast CM. Heparin-Sepharose was incubated with CM and eluted with Laemmli buffer, which was loaded directly onto a 12.5% polyacrylamide gel. HGF/SF was detected as described in Methods. M426 and HLF CM were processed in parallel. The ~ 75-kD band corresponded to HGF/SF, whereas the ~ 30-kD band represented the product of an alternatively spliced, truncated form of HGF/SF (74) (*C*) Northern blot analysis of KGF and HGF/SF mRNA expression in HLF. Total RNA (10 μg) from HLF was separated by electrophoresis on 1% agarose containing formaldehyde and hybridized with either KGF or HGF/SF probes. The sizes in kilobases of the major transcripts were determined by comparison with RNA standards.

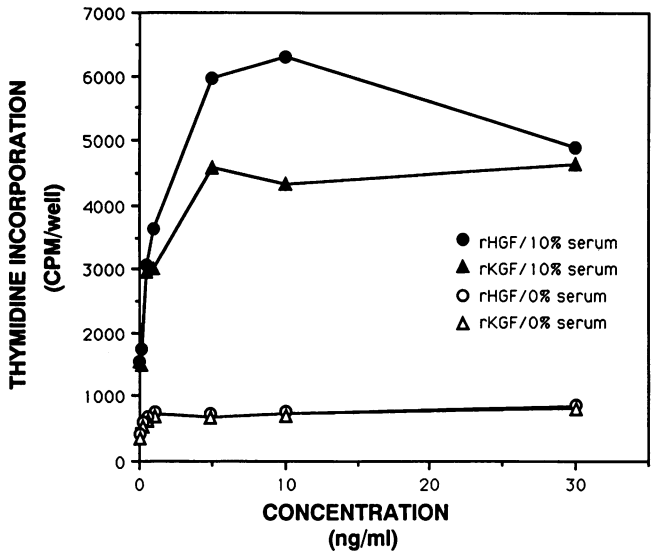

Figure 8. rKGF and rHGF/SF stimulate alveolar type II DNA synthesis. Type II cells were incubated with rKGF and rHGF/SF in the presence of [^{3}H]thymidine and either 0 or 10% FCS from day 1 to 3 in culture. An experiment representative of four similar studies is shown.

DNA synthesis in a concentration-dependent manner (Fig. 8). Maximal activity for both factors occurred at 5–10 ng/ml. At concentrations > 10 ng/ml, the level of DNA synthesis induced by rHGF/SF decreased. The maximal absolute level of thymidine incorporation induced by rHGF/SF was ~ 30–35% greater than that induced by rKGF. In addition, when tested in the absence of serum, both rKGF and rHGF/SF produced < 50% increases in [^{3}H]thymidine incorporation, similar to the serum dependence of the fibroblast-CM (Figs. 2 and 8).

Discussion

Although alveolar type II cells are proliferatively quiescent in normal adult animals, they become hyperplastic and hypertrophic in many pulmonary diseases and lung injury models (27–32). Type II cells actively proliferate during neonatal lung growth and, when they are isolated, these cells multiply in primary culture (33–36). In contrast, normal adult type II cells do not multiply in vitro in the absence of mitogens (6) and, in the presence of growth factors, their proliferation is limited (3, 6). Few studies have examined the growth characteristics of adult alveolar type II cells isolated after lung injury. We previously demonstrated that hypertrophic rat alveolar type II cells isolated after silica-induced lung injury progress through the cell cycle, are committed to DNA synthesis, and exhibit increased levels of DNA synthesis in primary culture compared with normotrophic type II cells isolated from silica-treated rats (11, 37). Although repair of the alveolar epithelium after lung injury presumably is important for the restoration of normal alveolar architecture and physiological function, the factors controlling the regulation of alveolar type II cell hyperplasia and hypertrophy are not known. Several investigators have demonstrated that medium conditioned by lung macrophages or fibroblasts can stimulate DNA synthesis in alveolar epithelial cells (7, 9, 16, 17, 38); however, no specific growth factor(s) have been identified from either macrophage or fibroblast-CM. In this report we demonstrate that human adult lung fibroblasts produce KGF and HGF/SF and that these factors stimulate DNA synthesis and proliferation of rat alveolar type II cells in primary culture.

Several properties of the adult human lung fibroblast-CM suggest that most if not all of the activity stimulating alveolar type II cell DNA synthesis is due to KGF and HGF/SF. The activity was heat and partially acid labile and sensitive to protease degradation. The additive effect of the fibroblast-CM with saturating concentrations of aFGF, EGF, and insulin indicated that the CM contained one or more agents that would act through receptors other than the ones recognized by these factors. The CM activity was separated into two peaks of activity by heparin-Sepharose affinity chromatography. The first peak eluted from heparin-Sepharose in 0.6 M NaCl, suggesting that the heparin affinity of this activity was similar to KGF. Further, this activity was inhibited by a neutralizing murine monoclonal antibody that is specific for KGF. By immunoblot analysis, the 0.6 M NaCl eluent contained an immunoreactive protein with a molecular mass of 26–28 kD, indistinguishable from KGF. The second peak of activity eluted from heparin-Sepharose with a NaCl concentration corresponding to that required for HGF/SF. This activity was completely blocked by a rabbit antiserum that specifically neutralizes the mitogenic effect of HGF/SF. Western blot analysis detected an ~ 75-kD protein in crude CM under nonreducing conditions that corresponds to HGF/SF. Also, the adult human lung fibroblasts expressed transcripts for both KGF and HGF/SF. Finally, human rKGF and rHGF/SF stimulated alveolar type II cell DNA synthesis in vitro in a manner similar to that seen with the CM. Thus, the stimulatory activity for alveolar type II cells in fibroblast-CM appears due largely, if not exclusively, to the heparin-binding growth factors KGF and HGF/SF. On the basis of the degree of inhibition of the fibroblast-CM stimulatory activity by the anti-KGF and anti-HGF/SF antibodies and the difference in mitogenic potency of the 0.6 and 1.0 M NaCl heparin-Sepharose fractions as well as rKGF and rHGF/SF, HGF/SF appears to be the dominant heparin-binding growth factor for alveolar type II cells in fibroblast-CM.

KGF is a recently described epithelial cell–specific heparin-binding growth factor that was originally isolated from the CM supernatant of the human embryonic lung fibroblast cell line M426 (24). The KGF gene appears to be transcribed as two alternative mRNAs, a predominant 2.4-kb transcript and a less abundant 5-kb transcript. KGF mRNA is translated into a monomeric polypeptide that is both acid and heat sensitive and has an apparent molecular mass of 28 kD according to SDS/PAGE. KGF (FGF-7) is a member of the fibroblast growth factor family, which includes acidic and basic FGF (FGF-1 and FGF-2, respectively), *Int*-2 (FGF-3), *HST* (FGF-4), FGF-5, and FGF-6 (22, 39–41). Unlike acidic and basic FGF, the primary KGF translation product contains a hydrophobic amino-terminal peptide sequence that is not present in the mature KGF molecule, suggesting that it is a signal sequence for secretion (22). KGF mRNA is expressed in various tissues including normal adult kidney, colon, and ileum (22). In contrast to the other known members of the FGF family, KGF has a unique target cell specificity restricted to epithelial cell types (22, 24) whereas acidic and basic FGF also exert potent effects on fibroblasts, endothelial cells, melanocytes, and neurons (39). Analysis of the interaction of KGF with BALB/MK

mouse epidermal keratinocytes demonstrated saturable specific high affinity binding as well as a high capacity/low affinity heparin-like binding site (42). The high affinity KGF receptors on BALB/MK keratinocytes bind aFGF equally well but bFGF with an order of magnitude lower affinity. Molecular cloning of the KGF receptor established that it is a membrane-spanning tyrosine kinase derived from an alternatively spliced form of the *bek* (FGFR-2) gene (43, 44). This alternatively spliced transcript has only been detected in epithelial cells and, thus, may account for the unique target cell specificity of KGF. *bek* is expressed in the epithelium of the bronchial tree and the alveoli in the embryonic mouse (45, 46).

HGF/SF is a heparin-binding growth factor that was initially isolated from plasma (47, 48) and platelets (49, 50) and more recently purified from CM supernatant of the human embryonic lung fibroblast cell line M426 (20). HGF/SF is a disulfide-linked heterodimer consisting of a 55–60-kD alpha subunit and a 32–34-kD beta subunit (47, 48, 50) that are proteolytically derived from a single precursor polypeptide (23, 51). The cDNA of HGF/SF reveals no homology with known growth factors but is ~ 38% identical to plasminogen, including the presence of four kringles and a serine protease–like domain. Northern blot analysis has demonstrated the expression of HGF/SF mRNA in various tissues, including the liver, kidney, lung, and brain (52); in stromal fibroblasts derived from adult skin, lung, gastrointestinal tract and prostate; and in embryonic lung fibroblasts (20). Immunohistochemical studies have demonstrated HGF/SF immunoreactivity in the tracheal, bronchial, and bronchiolar epithelium and in endothelial cells and macrophages within the alveolus (53). HGF/SF is mitogenic for hepatocytes, epithelial cells, melanocytes, and endothelial cells but does not stimulate fibroblast DNA synthesis (20, 54, 55). HGF/SF is identical to scatter factor (56, 57), a fibroblast- and smooth muscle cell–derived factor that stimulates epithelial and endothelial cell motility and causes the dissociation of epithelial cell colonies (58–60). The hepatocyte growth factor receptor is the c-*met* protooncogene product (61, 62) and *met* is expressed highly in mouse lung tissue (63, 64). In addition, HGF/SF binds specifically to plasma membranes isolated from lung tissue (64).

Neither fibroblast-CM nor rKGF or rHGF/SF stimulated alveolar type II cell DNA synthesis in the absence of serum. In the presence of 50% concentrated CM, half-maximal stimulatory activity occurred at 5% FCS and maximal activity occurred at 10% FCS (Fig. 2). Similarly, aFGF does not effectively induce type II cell DNA synthesis in the absence of serum (8). Thus, serum contains components that are necessary for these heparin-binding growth factors to stimulate DNA synthesis in type II cells. Leslie et al. (8) showed that rat HDL could substitute effectively for serum in the presence of aFGF. However, the mechanism by which HDL enables aFGF to stimulate alveolar type II cell DNA synthesis is not known.

Mesenchymal cells have been shown to stimulate epithelial cell proliferation in several different systems (65–73). Fibroblast-CM stimulates mammary (65), corneal (66), tracheal (67, 68), and bronchial (69) epithelial cell as well as keratinocyte (24, 70) proliferation. In addition, clonal proliferation of mammary (71), skin (72), and bronchial (73) epithelial cells occurs when they are grown on fibroblast feeder layers. Previous studies have shown that medium conditioned by lung fibroblasts derived from hyperoxia-exposed animals (16) or fibroblasts exposed to hyperoxia in vitro (38) stimulates alveolar type II cell proliferation in primary culture. In addition, fetal lung fibroblast-CM stimulates DNA synthesis in fetal epithelial cells (17). Of all these activities only KGF and HGF/SF have been purified (20, 24). Shoji et al. (69) showed that medium conditioned by human fetal lung fibroblasts contained a protease-sensitive, acid-stable, nondialyzable, lipid-inextractable protein with an apparent molecular mass of 6 kD that stimulated the proliferation of human bronchial epithelial cells. Fetal lung fibroblast–derived mitogenic activity for fetal alveolar epithelial cells was reportedly due to a heat-labile, trypsin-sensitive factor with an apparent molecular mass of 30 kD (17), whereas the factor produced by hyperoxia-treated lung fibroblasts that stimulated DNA synthesis in pulmonary epithelial tumor (A549) cells had an apparent molecular mass of 20–26 kD (38). The physical properties of both these activities are consistent with KGF.

Ultrastructural studies of lung specimens obtained after lung injury provide further evidence of epithelial–mesenchymal cell interactions. Epithelial cytoplasmic protrusions extending through discontinuities in the basement membrane and forming intimate associations with stromal cells have been described in electron micrographs of lung biopsy specimens from patients with interstitial lung disease and from mice that have been exposed to butylated hydroxytoluene and hyperoxia (14, 15). Although these associations suggest intercellular communication between stromal and epithelial cells, their significance is not known.

In summary, we have shown that medium conditioned by human adult lung fibroblasts stimulates DNA synthesis and proliferation of rat alveolar type II cells in primary culture. This stimulatory activity is mediated by two soluble heparin-binding growth factors, KGF and HGF/SF. Human rKGF and rHGF/SF stimulate a concentration-dependent increase in alveolar type II cell DNA synthesis. Production of KGF and HGF/SF by lung fibroblasts may be important for the proliferation of alveolar type II cells after lung injury and during normal and compensatory lung growth.

Acknowledgments

We thank Xue Song Wang and Kathleen McCormick-Shannon for technical assistance, Dr. Karl Csaky for Western blot analysis of HGF/SF, Dr. Dina Ron for engineering recombinant expression of human KGF, and Drs. Andrew Chan and Roy Jensen for recombinant expression of human HGF/SF.

This work was performed in part in the Lord and Taylor Laboratory of Lung Biochemistry in the Anna Perahia Adatto Clinical Research Center of the National Jewish Center for Immunology and Respiratory Medicine and was funded by National Institutes of Health grants HL-27353 and HL-29891 (R. J. Mason) and an American Lung Association Research grant (R. J. Panos). R. J. Panos is a Parker B. Francis Fellow in Pulmonary Research.

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