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Lung-Derived Growth Factor That Stimulates the Growth of Lung-Metastasizing Tumor Cells: Identification as Transferrin

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Abstract We have previously shown that culture medium conditioned by lung fragments contains mitogenic activity for lung-metastasizing tumor cells but not for their non-metastatic counterparts. The growth-promoting component from media conditioned by rat and porcine lungs has been purified and partially characterized as a $M_r \approx$ 66,000 (unreduced) or $M_r \approx$ 72,000 (reduced) glycoprotein [*Cancer Res* 49:3928, 1989; *J Cell Biochem* 43:127, 1990]. Here we report that this factor is the iron transport protein transferrin. Migration distances in sodium dodecyl sulfate and native gel polyacrylamide electrophoresis systems were similar, as were the specific activities and spectrum of mitogenic activities of the lung-derived growth factor and transferrin. Electrophoretically separated holo-rat transferrin and rat lung-derived growth factor displayed similiar positive stains for iron. A polyclonal antibody generated against the lung-derived growth factor cross-reacted with human and rat transferrin in Western blots, and anti-human transferrin cross-reacted with rat lung-derived growth factor. All of the mitogenic activity contained in crude lung conditioned media could be removed by antibody-mediated transferrin depletion. The putative cell receptor molecular weights for the lung-derived growth factor and transferrin were similar as were the molecular weights of polypeptides produced by partial trypsin cleavage of the two. Finally, the amino acid sequence of certain regions of rat lung-derived growth factor demonstrated a high degree of homology to human transferrin. The physical and biochemical properties, antigenicity, and mitogenic activity of a previously unidentified lung-derived growth factor for lung-metastasizing tumor cells indicate that it is transferrin.

Key words: cell proliferation, cancer cells, metastasis, glycoprotein characterization

Various tumor cell and host characteristics are important in determining a tumor cell's ability to metastasize to certain sites [Nicolson, 1988, 1991]. Among these is the ability of metastasizing cells to respond to growth factors present in the target organ [Nicolson, 1987, 1988a]. We have found that the most metastatic sublines of murine B16 melanoma [Nicolson and Dulski, 1986], murine RAW117 large cell lymphoma [Nicolson, 1987] and rat 13762NF mammary carcinoma [Nicolson, 1988a] exhibited high growth rates in low-serum medium conditioned by the organ tissue that is a target for metastatic colonization. Others have reported that lung-conditioned medium [Horak, et al.,1986; Naito et al., 1987] or lung extracts [Yamori et al., 1988] preferentially enhance the growth of lung-metastasizing cell lines. Although few of these paracrine growth factors have been isolated, there are some reports on the purification and characterization of tumor cell growth factors from tissues or their conditioned media. Ogasawara et al., [1989] found that an acid stable tumor cell mitogen from porcine uterus was a truncated form of insulin-like growth factor I. Riss and Sirbasku [1987] reported that the major neutral pH extracted rat mammary adenocarcinoma mitogen from porcine pituitaries was transferrin.

One property of rapidly proliferating cells is their high number of transferrin receptors [Trowbridge and Omary, 1981; Sutherland et al., 1981; Neckers and Trepel, 1986]. Neoplastic cells often proliferate more rapidly than their normal cell counterparts, so they too usually possess increased numbers of transferrin recep-

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tors [Neckers and Trepel, 1986; Seymour et al., 1987; Soyer et al., 1987]. When the growth of normal resting cells such as lymphocytes is stimulated by mitogens, the number of transferrin receptors increases [Pauza et al., 1984]. Blood monocyte maturation into replicative-competent alveolar macrophages is accompanied by the cellular appearance of transferrin receptors [Hirata et al., 1986]. In neoplastic cells transferrin receptor number has been correlated with tumor cell invasiveness [Seymour et al., 1987] and metastatic properties [Soyer et al., 1987]. In a previous study, we found that murine B16 melanoma variants that are highly metastatic to the lung are more responsive to transferrin and have more transferrin receptors when compared to low lung-metastasizing variants. In that study, B16 sublines highly metastatic to the brain had the greatest response to transferrin and highest numbers of transferrin receptors [Nicolson et al., 1990].

Transferrin is an iron transport protein necessary for the in vitro growth of many different cell types [Aisen and Listowsky, 1980; Barnes and Sato, 1980]. It is a required component of many serum-free tissue culture media formulations [Barnes and Sato, 1980]. A number of intracellular processes require iron, and it has been suggested that supplying iron to ribonucleotide reductase is one of the major growth-promoting roles that transferrin plays in supporting the proliferation of non-erythroid cells [Chitambar et al., 1988].

In this paper we report on the characterization of a previously isolated mitogenic activity for tumor cells from lung-conditioned medium [Cavanaugh and Nicolson, 1989, 1990]. We found that the lung-derived factor was identical to transferrin.

EXPERIMENTAL PROCEDURES

Cells and Cell Culture

The MTPa cell line was established from the low metastasizing parental 13762NF rat mammary adenocarcinoma. MTLn3 cells were cloned from a rare spontaneous lung metastasis arising from the same parental tumor [Neri et al., 1982]. Cultured MTPa cells consistently produce primary tumors in the fat pads of syngeneic F344 rats, but metastasize at a low rate. Cultured MTLn3 cells display high rates of spontaneous and experimental metastasis to the lungs in syngeneic F344 rats. Both lines were maintained in culture in alpha-modified minimal essential medium (αMEM) containing 5% fetal bovine serum (FBS) in a humidified 5% CO_2–95% air atmosphere. MCF-7 cells were grown in a 1:1 (v/v) mixture of Dulbecco's-modified essential medium and F-12 medium (DMEM:F12) containing 10% FBS. Cultures were routinely checked for *Mycoplasma* and other organisms. Stock culture cells (only those of < passage 35 were used) were harvested by treatment with 0.25% trypsin, 2 mM EDTA in phosphate-buffered saline (PBS).

Lung Growth Factor Isolation

Purification of lung-derived growth factor from rat and porcine lung-conditioned medium was performed as previously described [Cavanaugh and Nicolson, 1989, 1990]. The factor was stored at a concentration of 100 μg/ml at −70°C in 25 mM Tris, pH 8.5, containing 0.02% NaN_3. For addition to cell cultures, the material was dialyzed against αMEM. Protein equilibrated in media was filter sterilized prior to use.

Transferrin Isolation

Rat and human transferrin were purchased from Sigma Chemical (St. Louis, MO). Porcine transferrin was isolated from porcine serum (Sigma). Serum (100 ml) was dialyzed against 25 mM potassium phosphate at pH 7.0 and passed through a 2.5 × 50 cm Affi-gel blue (Bio-Rad; Richmond, CA) column equilibrated with the same buffer. The void volume was collected and dialyzed against 25 mM ethanolamine, pH 9.5. The dialysate was applied to a 2.5 × 40 cm QAE-sepharose column equilibrated with the same buffer. Bound material was eluted with a 500 ml continuous, linear (0–300 mM) NaCl gradient. Fractions of 10 ml were collected at a flow rate of 0.5 ml/min. Fractions exhibiting a salmon-pink color [Riss and Sirbasku, 1987] were collected and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to verify the presence of a major $M_r \approx$ 75,000 protein. A 10 ml aliquot of the QAE-sepharose pool was concentrated to 1.5 ml using an Amicon YM30 membrane and separated by preparative native gel electrophoresis as previously described [Cavanaugh and Nicolson, 1989]. A major salmon-pink colored band of $R_f \approx 0.2$ was seen. This was cut out, and the gel slice homogenized in 25 mM Tris, pH 8.5, and stirred for 24 h. The gel pieces were removed by centrifugation (3,000*g* for 20 min), and the supernatant was collected and analyzed by native and

SDS-PAGE. A major Coomasie blue (approximately 95% of total stain) stained band of $M_r \approx$ 72,000-74,000 was seen by SDS-PAGE. Native gel electrophoresis revealed Coomasie blue stained bands of $R_f \approx 0.2$, all of which tested positive when stained for iron [Chung, 1985]. The preparative gel pool was dialyzed against water, lyophilized, and stored at −70°C. All transferrins were reconstituted in αMEM and filter sterilized prior to addition to tissue culture assays.

Cell Growth Assays

Cells were plated at 2,000 cells/well in 96-well plates in 100 μl αMEM containing 1% (v/v) FBS for 24 h. Medium was then replaced with 100 μl αMEM containing 0.3% FBS and lung growth factor, transferrin, or a negative control solution was added in a 20 μl volume and incubated for 4 days at 37°C in a 5% CO_2–95% air humidified atmosphere. Cell numbers in all wells were then quantitated by crystal violet staining [Cavanaugh and Nicolson, 1989; Gillies et al., 1986]. Briefly, well contents were aspirated and wells were rinsed 3 times with PBS. Cells were fixed with 50 μl 5% v/v glutaraldehyde for 30 min and stained with 50 μl 0.1% w/v crystal violet for 30 min. Wells were washed 5 times with 200 μl H_2O and dried. Fixed, stained cells were solubilized with 50 μl 1% w/v SDS, and absorbance at 590 nm of the resulting solution was determined using a Titertek plate reader. Absorbances correlated linearly with cell number up to ≈40,000 cells/well (data not shown).

Anti-Porcine Lung-Derived Growth Factor

Antibody production was performed by Bethyl laboratories (Montgomery, TX). Antigen (50 μg in 0.5 ml H_2O) was mixed 1:1 (v/v) with complete Freund's adjuvant and injected subcutaneously into multiple sites on the back of a New Zealand rabbit. Two and four weeks later, the animal was given similar boost injections of 50 μg growth factor in incomplete Freund's adjuvant. Six weeks after the initial injection, the animal was bled and immune serum was obtained.

Western Blotting and Immunodetection

Protein samples to be tested for antibody cross-reactivity were separated in duplicate by SDS-PAGE performed according to Laemmli [Laemmli, 1970]. Proteins were electroblotted onto polyvinylidene difluoride (PVDF; Millipore, Bedford, MA) (Matsudaira, 1987). The membranes were washed with Tris-buffered saline (TBS; 25 mM Tris, 0.15 M NaCl, pH 8.0) and were blocked by a 2 h incubation in TBS containing 10 mg/ml bovine serum albumin (BSA). Membranes were incubated in antisera or pre-immune sera (diluted in TBS-BSA) for 2 h at 25°C, washed 4 times with TBS containing 0.1% Tween-20, and incubated in TBS-BSA diluted horseradish peroxidase (HRP) conjugated anti-rabbit IgG (Sigma) or anti-goat IgG (Sigma) (the choice depending on the initial antiserum) for 2 h at 25°C. Membranes were washed 4 times in TBS–Tween-20 and were placed in peroxidase substrate (50 ml TBS containing 40 μl hydrogen peroxide and 10 ml of a 3 mg/ml 4-chloro-1-napthol solution [in methanol]) for 1 h at 25°C. The reaction was stopped by a water wash.

Antibody Treatment of Lung Conditioned Media

Fractionated goat anti-human transferrin (Sigma) or normal goat IgG (Sigma) were biotinylated using sulfo-N-hydroxysuccinimido-biotin [Updyke and Nicolson, 1984]. To 2 ml anti-human transferrin (3.5 mg/ml total protein in 0.1 M HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], pH 8.0) or 2 ml goat IgG (5 mg/ml in the same buffer) was added 220 μl of a 9 mg/ml solution of sulfo-NHS biotin. The reaction was allowed to proceed for 3 h at 25°C and the samples were dialyzed against 25 mM HEPES, pH 7.5, overnight. One-tenth volume of 1.5 M glycine (pH 8.6) was added to neutralize residual sulfo-NHS biotin and the samples were re-dialyzed. Lung-conditioned media (1 ml) was incubated with 200 μl of either antibody preparation overnight at 4°C, then with 300 μl of a 50% suspension of streptavidin-agarose for 3 h at 25°C. Agarose was removed by centrifugation at 2,000*g* for 5 min; treated media was dialyzed against 25 mM HEPES (pH 7.5) overnight, and prepared for and tested for mitogenic activity as described above.

Radioiodination of Lung-Derived Growth Factor or Transferrin

To 100 μg of either protein in 0.5 ml PBS were added 0.5 mCi Na[^{125}I] and 10 μl 1% (w/v) chloramine T. This mixture was incubated at 25°C for 1 h, and 10 μl 2% (w/v) sodium bisulfite was added. Free Na[^{125}I] was removed by passage of the sample through a 1 × 10 cm Bio-Gel P-10

column eluted with PBS. Specific activity was 400,000–500,000 cpm/μg protein.

Binding and Cross-Linking Studies

MTLn3 cells were grown to confluency on 12-well cluster plates. One day prior to assay, medium was replaced with αMEM only. When the assays were initiated, cell monolayers were washed 3 times with binding buffer (25 mM HEPES, pH 7.5, 0.15 M NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mg/ml BSA), 1 ml binding buffer was added to all wells, and the plate was incubated at 4°C for 20 min. ^{125}I-labeled factor was added to test wells (10–20 μl; 3.5×10^5–1×10^6 cpm), and the plate was incubated at 4°C for 2 h. Wells were then washed 5 times with BSA-free binding buffer. For cross-linking studies, cells were incubated at 4°C in 1 ml BSA-free binding buffer containing 0.25 mM Bis (sulfosuccinimidyl) suberate. Cell monolayers were washed 5 times with 10 mM Tris, 0.25 M sucrose, 1 mM EDTA (pH 7.5) and solubilized in the same buffer containing 1.0% (v/v) Nonidet P-40. Solubilized cells were centrifuged at 10,000*g* for 10 min. Supernatants were concentrated with 10,000 M_r cut-off filters to 5,000–10,000 cpm/100 μl, treated with Laemmli SDS-PAGE treatment solution, and separated by SDS-PAGE performed according to Laemmli [Laemmli, 1970]. Radioiodinated bands were detected by autoradiography.

Trypsin Treatment of Porcine Transferrin and Lung-Derived Growth Factor

To 100,000 cpm of either radiolabeled factor (in 100 μl of 2 mM Tris, pH 8.0) was added 0.1 unit bovine trypsin. This mixture was incubated at 37°C for 2 h, made 100 μM in phenylmethyl sulfonyl fluoride, lyophilized, taken up in SDS-PAGE treatment solution, and separated on a 15% acrylamide gel run according to Laemmli [Laemmli, 1970]. Products were detected by autoradiography.

Protease Digestion, HPLC Separation, and Microsequencing of Rat Lung-Derived Growth Factor

Digestion and sequencing were performed at the Harvard Microchemistry Facility (Cambridge, MA). In situ V8 protease digestion of rat lung-derived growth factor blotted onto nitrocellulose was performed essentially as described by Aebersold et al., [1987], with the exception that the NaOH wash was eliminated. After digestion, the preparation was stored at −20°C. V8 protease generated peptides were separated by narrow-bore reverse phase HPLC on a Hewlett-Packard model 1090 HPLC equipped with a 1040 diode array detector, and a Vydac 2.1 mm × 150 mm C18 column. Buffer A was 0.06% trifluoroacetic acid/H_2O and buffer B was 0.055% trifluoroacetic acid/acetonitrile. The gradient used was 5% B at 0 min, 33% B at 63 min, 60% B at 95 min, and 80% B at 105 min at a flow rate of 150 μl/min. Fractions collected were stored at −20°C. Fractions to be sequenced were applied directly to a polybrene pre-cycled glass fiber filter and placed in the reaction cartridge of an Applied Biosystems model 477A protein sequencer. Samples were subjected to automated Edman degradation using the program NORMAL-1, which was modified for faster cycle time. Resultant penylthiohydantoin derivitized amino acids were identified using an on line Applied Biosystems model 120A HPLC and a Shimadzu CR4A integrator.

RESULTS

When analyzed by SDS-PAGE performed under reducing conditions, rat and porcine lung-derived growth factors migrate with an $M_r \approx$ 72,000 [Cavanaugh and Nicolson, 1989, 1990]. Published molecular weights for transferrin tend to be somewhat higher and range from 76,000–81,000 [Aisen and Listowsky, 1980]. In our hands, rat, porcine, and human transferrin and rat and porcine lung-derived growth factor migrated to similar positions in SDS-PAGE gels (Fig. 1), indicating similar molecular weights. Like transferrin [Aisen and Listowsky, 1980], the lung-derived growth factors lack disulfide linked subunits because their molecular weights (as determined by SDS-PAGE) rose slightly upon reduction with mercaptoethanol [Cavanaugh and Nicolson, 1989, 1990].

The migration pattern of rat lung-derived growth factor and rat transferrin in a native gel electrophoresis system were identical, as determined by Coomasie blue staining of the electrophoresed proteins (Fig. 2). The presence of iron in duplicate runs of the two was verified by a positive stain response to ferene-S [Chung, 1985]. The iron positive bands were identical in running position to those seen with Coomasie blue.

When assayed against MTLn3 cells, both porcine and rat lung-derived growth factor dis-

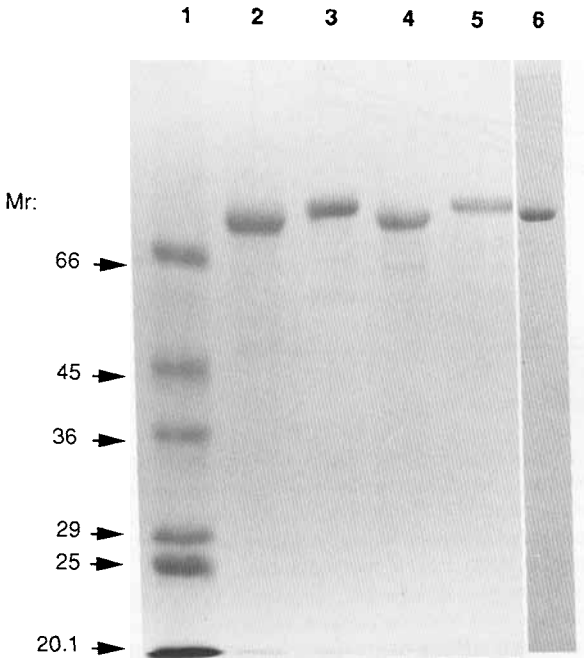

Fig. 1. Migration of lung-derived growth factor and transferrin in SDS-PAGE gels. Results obtained when 5 μg each of human transferrin **(lane 2)**, rat transferrin **(lane 3)**, porcine transferrin **(lane 4)**, rat lung-derived growth factor **(lane 5)**, and porcine lung-derived growth factor **(lane 6)** were separated on a 10% w/v acrylamide SDS-PAGE gel run according to Laemmli (Laemmli, 1970), and stained with Coomasie blue. Migration positions of molecular weight standards are shown in **lane 1.** Standards and molecular weights were bovine serum albumin ($M_r \approx 66{,}000$), ovalbumin ($M_r \approx 45{,}000$), glyceraldehyde-3-phosphate dehydrogenase ($M_r \approx 36{,}000$), carbonic anhydrase ($M_r \approx 29{,}000$), trypsinogen ($M_r \approx 25{,}000$), and soybean trypsin inhibitor ($M_r \approx 20{,}100$).

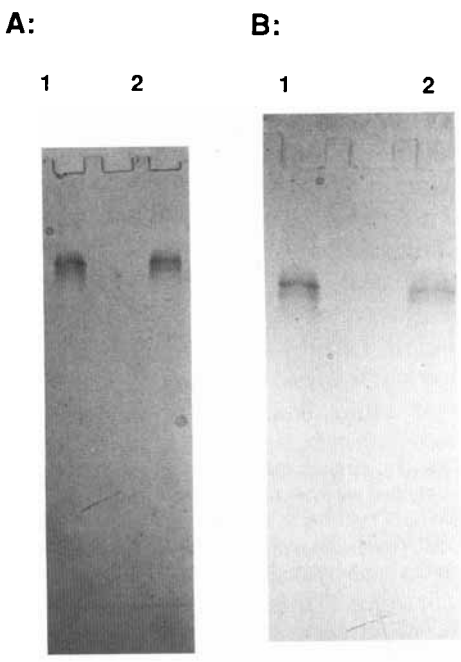

Fig. 2. Comparison of Coomasie blue and ferene-S stained rat lung-derived growth factor and rat transferrin separated by native gel electrophoresis. **A:** 10 μg each of rat transferrin **(lane 1)** or rat lung-derived growth factor **(lane 2)** were electrophoresed and stained for protein with Coomasie blue. **B:** 20 μg each of rat transferrin **(lane 1)** or rat lung-derived growth factor **(lane 2)** were electrophoresed and stained for iron using ferene-S.

played equal potency (Fig. 3). When 20–80 ng of the factors was introduced into cell cultures, linear increases in cell numbers were seen. This response was mimicked precisely by human transferrin (Fig. 3). The specific activities of all three proteins were similar. In contrast, epidermal growth factor produced a mitogenic effect on MTLn3 cells at much lower concentrations [Lichtner et al., 1988].

As we had previously reported, the low metastatic rat MTPa mammary adenocarcinoma cell line responded poorly to lung-derived growth factor, whereas the highly metastatic MTLn3 line and human MCF-7 mammary adenocarcinoma lines demonstrated a pronounced proliferative response [Cavanaugh and Nicolson, 1989]. The effect of porcine lung-derived growth factor and human transferrin on these cell lines was measured. The mitogenic behavior of transferrin was identical to that of the lung factor (Fig. 4) in that it stimulated MTLn3 and MCF-7 cells but not MTPa cells. As determined with these three lines, the spectrum of mitogenic activity of all proteins was similar.

The specificity of a polyclonal antiserum to porcine lung-derived growth factor was determined by electrophoresing both crude porcine lung-conditioned medium and purified lung-derived growth factor, blotting onto PVDF membranes, and testing for antibody cross-reactivity (see Experimental Procedures). Only one band in the crude conditioned medium was HRP positive, similar to the purified preparation (Fig. 5). When rat and human transferrin and rat and porcine lung-derived growth factors were electrophoresed, blotted, and tested for reactivity with anti-porcine lung-derived growth factor, a strong positive reaction was seen with the porcine factor. Positive but lower intensity binding was observed with the other three proteins (Fig. 6), indicating antigenic similarity among all four. When the same four proteins were examined for reactivity towards goat anti-human transferrin (Sigma), positive binding to all was seen; however, cross-reactivity of the porcine lung-derived

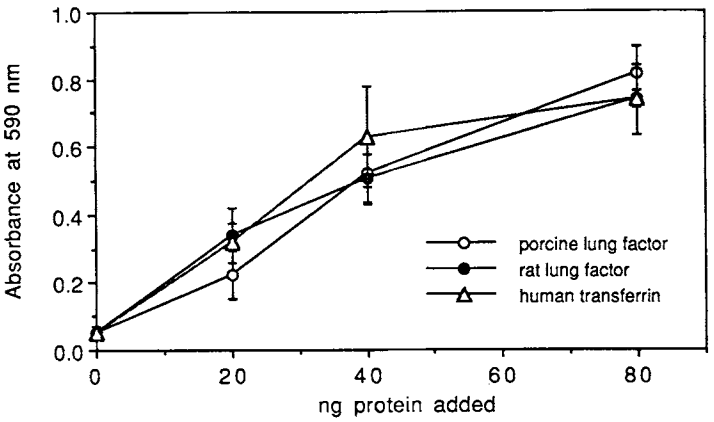

Fig. 3. Growth response of lung-metastasizing tumor cells to lung-derived growth factor and transferrin. Results obtained when increasing amounts of rat or porcine lung-derived growth factor or human transferrin were added into MTLn3 cultures in medium containing 0.3% FBS. Cells were plated in 96-well plates at an initial density of 2,000 cells/well in a volume of 100 μL. Four days after the addition of transferrin or factor, cell numbers were quantitated using the crystal violet cell quantitation assay. Data are expressed as mean ± SD of four replicates.

factor was very weak (Fig. 6), indicating antigenic similarity between rat and human transferrin and rat lung-derived growth factor.

An indication that transferrin was the major mitogen for MTLn3 cells in crude rat lung-conditioned media was made evident by the fact that media treated with biotinylated anti-transferrin followed by streptavidin-agarose was largely devoid of growth-promoting activity whereas that treated likewise with an excess of biotinylated normal goat IgG produced a proliferative effect very nearly equal to that of untreated media (Fig. 7).

Trypsin digestion of porcine transferrin and lung-derived growth factor also indicated a similarity between the two. Porcine transferrin was purified from porcine serum, since a commercial source of it could not be found. Analyses of the final product are shown in Figure 8. Upon short-time exposure to trypsin, both ^{125}I-labeled porcine lung-derived growth factor and ^{125}I-labeled porcine transferrin were cleaved into five major

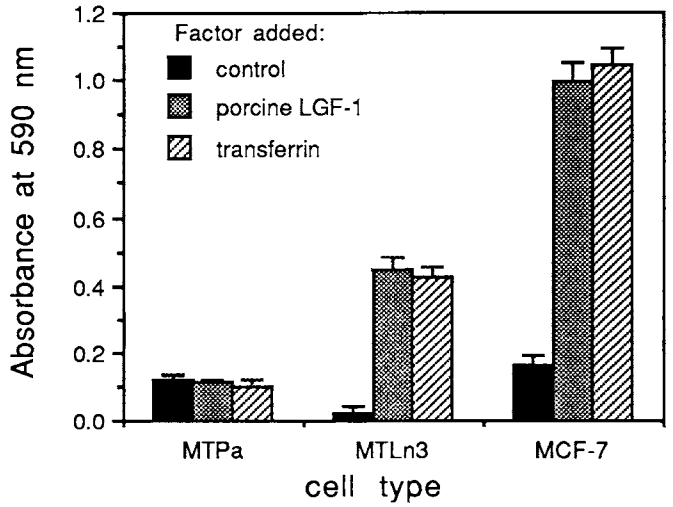

Fig. 4. Spectrum of activity of lung-derived growth factor and transferrin. Results obtained when 100 ng of porcine lung-derived growth factor or human transferrin were added into cultures of various cell lines in medium containing 0.3% FBS. Control wells received factor solvent only. Cells were plated in 96-well plates at an initial density of 2,000 cells/well in a volume of 100 μL. Four days after the addition of transferrin or factor, cell numbers were quantitated using the crystal violet assay. Bars represent the mean ± SD of four replicates. MTPa cells displayed a threefold increase in A_{590} in response to 10% FBS (data not shown).

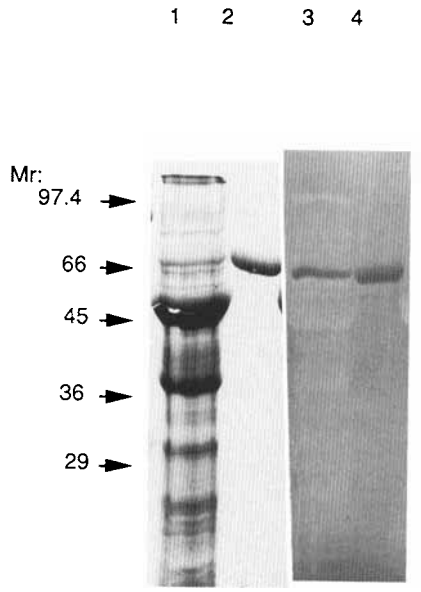

Fig. 5. Specificity of polyclonal anti-porcine lung-derived growth factor. **Left:** Appearance of a Coomasie blue stained 12% Laemmli SDS-PAGE gel which received 80 μg porcine lung-conditioned medium protein **(lane 1)** or 5 μg pure porcine lung-derived growth factor **(lane 2). Right:** Appearance of the same samples of crude lung-conditioned medium **(lane 3)** and pure lung-derived growth factor **(lane 4)** when blotted onto PVDF and examined for reactivity towards anti-porcine lung-derived growth factor (performed as in Experimental Procedures). Bands seen are sites of horseradish peroxidase activity. Running positions of molecular weight standards are shown to the left. Standards and molecular weights are phosphorylase b ($M_r \approx 97{,}400$), bovine serum albumin ($M_r \approx 66{,}000$), ovalbumin ($M_r \approx 45{,}000$), glyceraldehyde-3-phosphate dehydrogenase ($M_r \approx 36{,}000$), and carbonic anhydrase ($M_r \approx 29{,}000$).

polypeptides of identical molecular weight ($M_r \approx 67{,}000$, 55,000, 38,500, 31,000, and 15,500) as determined by SDS-PAGE (Fig. 9). Trypsin cleavage was apparently incomplete, since fragment molecular weights did not add up to the intact protein molecular weight.

The major radiolabeled complex obtained when MTLn3-bound ^{125}I-porcine transferrin was cross-linked to its cellular receptor possessed an $M_r \approx 320{,}000$ (Fig. 10). This most likely consisted of a complete $M_r \approx 180{,}000$ transferrin receptor homodimer with a transferrin cross-linked to each monomer [Trowbridge and Omary, 1981; Sutherland et al., 1981; Schneider et al., 1982]. A minor cross-linked band of $M_r \approx 250{,}000$ was also seen, which probably consisted of a complete dimeric transferrin receptor cross-linked to one transferrin molecule (Fig. 10). Dissociation of transferrin cross-linked dimeric receptors into transferrin cross-linked receptor monomers under reducing conditions was not observed, presumably because the cross-linked agent had linked these also. The molecular weights of complexes obtained when ^{125}I-porcine lung-derived growth factor was cross-linked to its MTLn3 cell receptors were similar (Fig. 10), indicating that the two radiolabeled proteins had target cell receptors of the same molecular weight and behavior under reducing conditions. No high molecular weight radiolabeled bands were detected when bis(sulfosuccinimidyl) suberate was not included (data not shown).

Amino acid sequencing of intact lung-derived growth factors was unsuccessful, apparently due to the presence of a blocked amino terminus. Cleavage of rat lung-derived growth factor by V8 protease produced 63 major peptides which were separated by reverse phase HPLC (Fig. 11). Sequencing of three of the peptides indicated approximately 85% similarity to internal sequences of human transferrin [Yang et al., 1984]. These were the only peptides analyzed from which there were sequences long enough to gain reliable information from the Genetics Computer Group (Madison, WI) gene bank. A full-length sequence of rat transferrin was not contained in the amino acid sequence databases. These data, along with those shown above, indicate that the lung-derived growth factor is indeed transferrin.

Although rats were perfused prior to obtaining lungs and lung-conditioned medium, the identification of the lung-derived growth factor as transferrin suggested that the source of the factor was from residual serum contamination of the lung-conditioned medium and was not from a lung-specific release or secretion phenomenon. Supporting this was the fact that the addition of cylcohexamide (15 μg/ml) to the lung fragment culture had no apparent effect on the production of lung-derived growth factor (data not shown), indicating that its presence was not dependent on lung tissue protein synthesis. However, if residual serum was the sole source of the transferrin in lung-conditioned media, then other serum proteins should have been present in the medium at normal serum ratios to transferrin. SDS-PAGE analysis of Coomasie blue–stained equivilant protein loads of rat serum and rat lung-conditioned media indicated that certain serum proteins were absent in lung-conditioned media. Further exploration of this by Western blotting and immunodetection of specific serum proteins revealed that lung-conditioned media contained virtually no

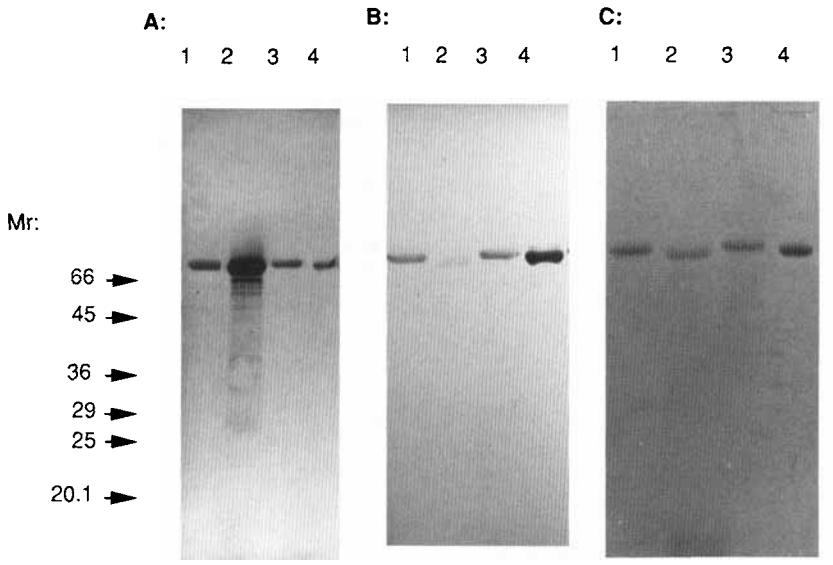

Fig. 6. Reactivity of lung-derived growth factor and transferrin to anti-lung-derived growth factor and anti-transferrin. **A:** Reactivity of rabbit anti-porcine lung-derived growth factor antisera (used at 1:1,000 dilution) towards 5 μg of SDS-PAGE separated (12% Laemmli gel) and PVDF blotted rat lung-derived growth factor **(lane 1);** porcine lung-derived growth factor **(lane 2);** rat transferrin **(lane 3);** and human transferrin **(lane 4). B:** Reactivity of fractionated goat anti-human transferrin (Sigma; used at a 1:1,000 dilution) towards a duplicate blot of the same proteins as listed in A. **C:** A Coomasie blue stain of a duplicate blot demonstrating identical protein load. After application of the antibodies, blots were washed and incubated with a 1:1,000 dilution of HRP-conjugated goat anti-rabbit IgG (A) or HRP-conjugated rabbit anti-goat IgG (B). Blots were then rewashed and incubated in HRP substrate. Duplicate blots of these same proteins which were treated with rabbit pre-immune sera or normal goat gamma globulin instead of the first antibody displayed no bands (data not shown). Running positions of molecular weight standards (identical to those in Fig. 1) are shown to the left.

IgG or fibronectin (both were present in normal amounts in serum; data not shown). However, both contained the same amount of transferrin on a per protein basis (≈ 7 μg/100 μg protein; data not shown). Therefore, the isolated material could have originated from lung-bound pools of transferrin that may have originated from a serum source, but was not from residual serum contamination of the lung-conditioned medium. The perfused adult murine lung has been shown to be a major localization site for transferrin [Meek and Adamson, 1985]. Another indication that low residual serum levels were present in lung conditioned media was that the erythrocyte or hemoglobin contamination of rat lung-conditioned media was negligible (data not shown).

DISCUSSION

A previously purified glycoprotein growth factor found in lung-conditioned medium that is more mitogenic for lung-metastasizing tumor cells than for non-metastatic cells [Cavanaugh and Nicolson, 1989, 1990] has been identified here as transferrin. This raises the possibility that highly lung-metastasizing tumor cells re-

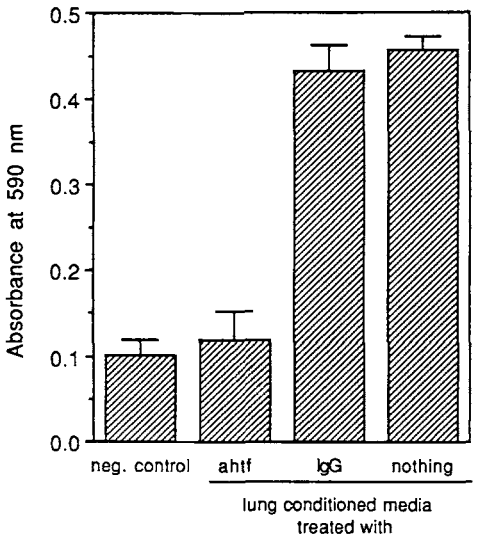

Fig. 7. Elimination of mitogenic activity from rat lung-conditioned media by treatment with anti-transferrin. Lung-conditioned media was treated with biotinylated goat anti-human transferrin, biotinylated normal goat IgG, or nothing. Biotinylated components were removed by treatment with streptavidin-agarose and centrifugation. Treated media was added into MTLn3 cultures (2,000 cells/well in 96-well plates in αMEM + 0.3% FBS) at 10% (v/v). Four days after the addition, cell numbers were quantitated using a crystal violet stain assay. Bars represent the mean ± SD of four replicates.

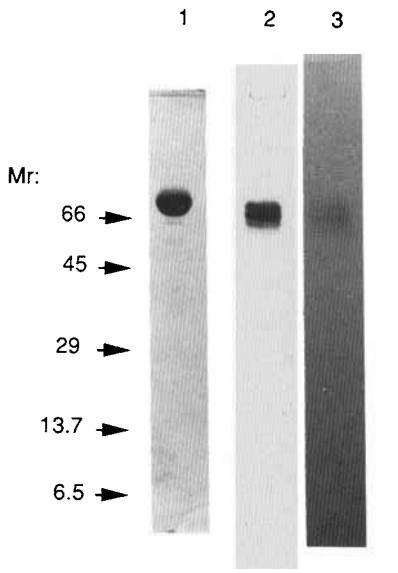

Fig. 8. Appearance of various electrophoretic analyses of purified porcine transferrin. **1:** SDS-PAGE separated and Coomasie blue stained (12% polyacrylamide Laemmli gel). Molecular weight standard running positions are indicated to the left. Standards and molecular weights were bovine serum albumin ($M_r \approx 66{,}000$), ovalbumin ($M_r \approx 45{,}000$), carbonic anhydrase ($M_r \approx 29{,}000$), ribonuclease ($M_r \approx 13{,}700$), and aprotinin ($M_r \approx 6{,}500$). **2:** Native gel separated and Coomasie blue stained. **3.** Native gel separated and stained for iron using ferene-S. Ten micrograms of protein were loaded onto each lane.

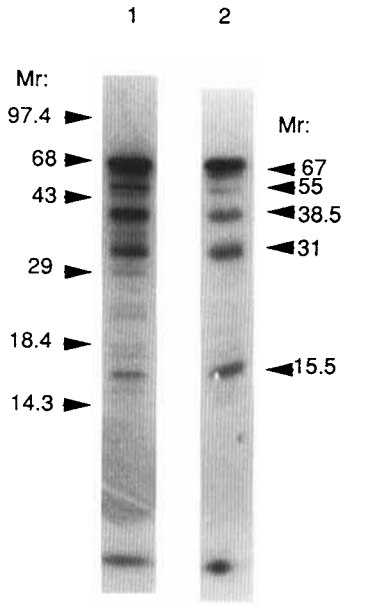

Fig. 9. Comparison of products resulting from trypsin treatment of radioiodinated lung-derived growth factor and transferrin. Results obtained from the SDS-PAGE separation (15% polyacrylamide Laemmli gel) of cleavage fragments of porcine lung-derived growth factor (**lane 1**) or porcine transferrin (**lane 2**). Fragments were detected by autoradiography. The positions of major cleavage products are indicated to the right. Running positions of molecular weight standards are shown to the left. Standards and molecular weights are phosphorylase b ($M_r \approx 97{,}400$), bovine serum albumin ($M_r \approx 68{,}000$), ovalbumin ($M_r \approx 43{,}000$), carbonic anhydrase ($M_r \approx 29{,}000$), β-lactoglobulin ($M_r \approx 18{,}400$), and lysozyme ($M_r \approx 14{,}300$).

spond to transferrin at lower concentrations than their non-metastatic counterparts. In agreement with this hypothesis, we have found that highly metastatic murine B16 melanoma cells possess higher numbers of transferrin receptors and respond mitogenically to lower levels of transferrin than do their low metastatic counterparts [Nicolson et al., 1990].

One remarkable quality of the lung-derived growth factor purified previously was its relatively low specific activity: half maximal stimulation of cell growth was observed at 0.5–1.0 μg/ml [Cavanaugh and Nicolson, 1989]. In contrast, the published ED_{50} of most other growth factors is markedly lower: that is, epidermal growth factor at 2 ng/ml [Carpenter and Cohen, 1976], platelet-derived growth factor at 0.2 ng/ml [Deuel et al., 1981], fibroblast growth factor at 40 pg/ml [Thomas et al., 1984]. The low specific activity of our pure lung-derived growth factor suggested that a major contaminant might have existed in our preparations. However, we were unable to detect any mitogenic activity in the pure material not associated with the major band detected by native or SDS-PAGE [Cavanaugh and Nicolson, 1989, 1990].

In an effort to identify the purified lung-derived growth factor, attempts at comparing it to known growth factors of similar molecular weight and potency were made. This review process drew our attention to nutrient transport molecules, such as transferrin and insulin, which tend to act at higher concentrations than non-nutrient growth factors [Barnes and Sato, 1980; Riss and Sirbasku, 1987]. The identification of the lung-derived growth factor as transferrin was not initially made because the lung-derived mitogen was active in assays that were performed in 0.3% FBS. Fetal bovine serum contains transferrin at concentrations of ≈ 2,000 μg/ml [Heubers et al., 1987], making basal levels of transferrin in our system ≈ 6 μg/ml. It seemed unlikely that the addition of 0.5–1.0 μg/ml of transferrin or a transferrin-like material to the cultures would have had any mitogenic effect. However, since a mitogenic result was obtained with similar levels of pure human transferrin in these 0.3% FBS containing cell cultures

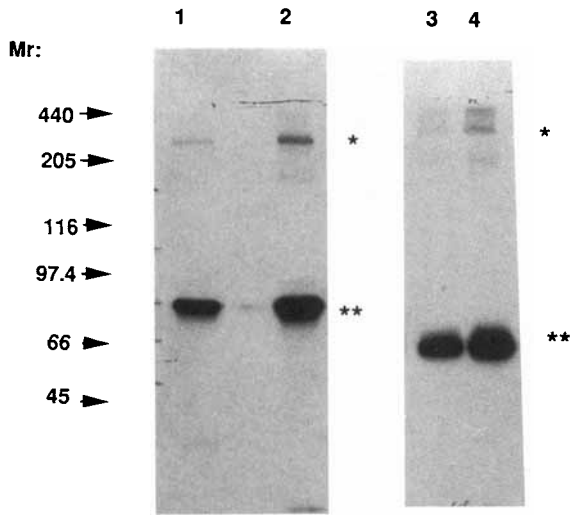

Fig. 10. Analysis of cell surface receptors for lung-derived growth factor and transferrin. Results obtained when ^{125}I-porcine transferrin or ^{125}I-porcine lung-derived growth factor were incubated with MTLn3 cell monolayers, cross-linked to their cell receptors with bis(sulfosuccinimidyl) suberate, and analyzed by SDS-PAGE (7% Laemmli gel) followed by autoradiography. Binding was performed as described in Experimental Procedures. The major radiolabeled cross-linked band is denoted by *, and noncross-linked factors are marked by **. **Lane 1:** Porcine lung-derived growth factor (reduced). **Lane 2:** Porcine transferrin (reduced). **Lane 3:** Porcine lung-derived growth factor (non-reduced). **Lane 4:** Porcine transferrin (non-reduced). Molecular weight standard running positions are indicated to the left. Standards and molecular weights are laminin subunit ($M_r \approx 440{,}000$), myosin ($M_r \approx 205{,}000$), β-galactosidase ($M_r \approx 116{,}000$), phosphorylase b ($M_r \approx 97{,}400$), bovine serum albumin ($M_r \approx 66{,}000$), and ovalbumin ($M_r \approx 45{,}000$).

(Fig. 2), there was an indication that low levels of pure transferrin had an unusual, additional growth stimulatory effect on these target cells.

Transferrin's ability to promote cell growth is thought to be due primarily to its ability to transport iron into cells, since lipophilic iron chelators can mimic transferrin's activity [Laskey et al., 1988; Tsao et al., 1987]. However, some studies have suggested that iron transport alone cannot explain transferrin's growth stimulating activity. Using a mouse hybridoma system, Kovar and Franek [1989] found that the intracellular iron transport specific activity for bovine and porcine transferrin was the same, but the specific activity for growth stimulation induced by porcine transferrin was more than 10 times greater than that of bovine transferrin. Other properties of transferrin that do not involve growth stimulation have also been reported. For example, avian transferrin has been reported to influence the adhesion of chick mesoderm cells to collagen [Sanders, 1986].

Our work indicates that in several animal tumor metastatic models, the response to transferrin is increased in cells that are more metastatic to lung. This suggests that metastasis formation in certain environments, such as lung, is aided by the increased ability of tumor cells to grow rapidly in response to available transferrin [Cavanaugh and Nicolson, 1989, 1990; Nicolson

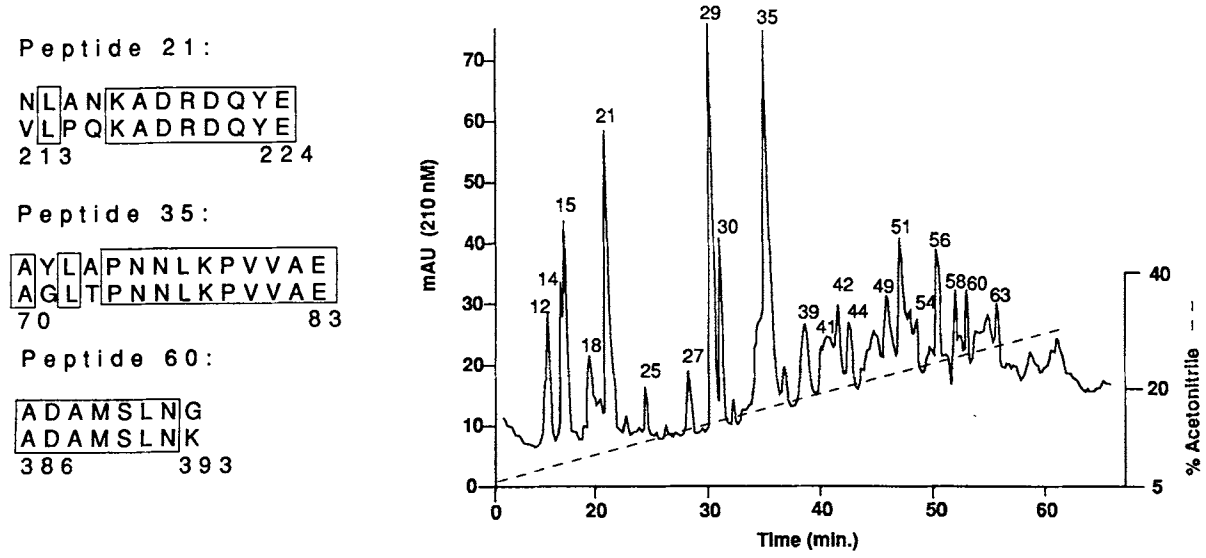

Fig. 11. Partial amino acid sequence of rat lung-derived growth factor and comparison to that of human transferrin. **Right:** The chromatogram obtained when V8 protease-digested rat lung-derived growth factor was separated by reverse phase HPLC. **Left:** The upper sequences are those obtained from the rat factor peptides. The lower sequences are those published for human transferrin, and the numbers below indicate the amino acid numbers. Identical residues are shown by the boxed regions. Procedures were as described in Experimental Procedures.

et al., 1990]. Other cellular properties affected by transferrin or increased numbers of transferrin receptors may be involved in enhancing the metastatic behavior of tumor cells and their ability to selectively colonize certain organs.

ACKNOWLEDGMENTS

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