

Heparin Potentiates Endothelial Cell Growth Factor Stimulation of Plasminogen Activator Synthesis by Diploid Human Lung Fibroblasts

Ruth S. Rappaport, Marlene Ronchetti-Blume, Robert L. Vogel, and Paul P. Hung

From the Cell Biology Unit, Microbiology Division, Wyeth Laboratories, Philadelphia, PA, USA

Key words

Heparin – Endothelial growth factor – Tissue plasminogen activator – Single chain urokinase – Diploid lung fibroblast

Summary

Endothelial cell growth factor (ECGF) stimulates the synthesis of t-PA and u-PA by confluent, diploid human lung fibroblasts, and this activity is potentiated considerably by heparin. In contrast, the malignant cell lines, Bowes melanoma and CALU-3, producers of t-PA and u-PA, respectively, are insensitive to ECGF. Studies with metabolic inhibitors and direct measurements of PA-specific mRNAs show that ECGF-mediated production of PA by human lung fibroblasts is dependent on de novo protein and RNA synthesis. The mechanism by which heparin potentiates this effect is thought to reside in its ability to prolong or strengthen the interaction of ECGF with cell surface receptors. The results raise the possibility that endogenous ECGF or related polypeptides (and heparin) may act to regulate PA synthesis by lung fibroblasts and possibly other responsive target cells in vivo.

Introduction

With the advent of genetic engineering there has been, in recent years, a surge of activity in the area of cloning human plasminogen activators (PAs) for development as specific thrombolytic agents. Native or recombinant tissue-type and urokinase-type plasminogen activators (t-PA and u-PA, respectively) are now available and at various stages of clinical investigation (1–8). Another approach to thrombolytic therapy is the search for pharmacological or hormonal agents capable of elevating endogenous levels of t-PA or u-PA. We report here the finding that bovine brain endothelial cell growth factor (ECGF) (9) stimulates the synthesis of t-PA and u-PA by diploid human lung fibroblasts and that the stimulation is potentiated by the glycosaminoglycan, heparin. Potentiation by heparin of the mitogenic activity of ECGF for endothelial cells has been reported previously (10–12) as has evidence for a structural interaction between them (11, 13, 14).

Recent studies have pointed to a role for mitogenic factors in the control of PA synthesis. One such factor, isolated from human placenta and thought to resemble basic fibroblast growth factor (bFGF) (15), was demonstrated to stimulate PA production by bovine capillary endothelial cells at concentrations that were mitogenic (16). Another such factor, isolated from sheep pituitary was shown to be responsible for enhancement of PA activity in breast tumor cells (17). This factor did not promote breast cell proliferation in vitro, but was found to be identical or closely related to bFGF in size, cationic nature, and affinity for heparin; and its activity was blocked by antibodies to the human placental

factor (16, 17). In addition, highly purified bFGF from bovine pituitary has been shown to concomitantly stimulate angiogenesis and the production of u-PA by bovine capillary endothelial cells (18).

Here we present evidence that ECGF, which is related to, but distinct from bFGF (19–21), and heparin stimulate the synthesis of t-PA and u-PA by confluent human lung fibroblasts. Under the conditions of our experiments, cell numbers did not increase substantially, although evidence for DNA synthetic activity was obtained. Thus, it appears that there are two distinct families of polypeptide mitogens (i.e., ECGF and bFGF) with affinity for heparin that are capable of augmenting target cell PA synthesis without necessarily promoting cell proliferation in vitro. These findings strengthen the view that such mitogens act to regulate PA synthesis by a variety of cell types in vitro, and possibly, in vivo; and they point to a significant auxiliary role for heparin as well.

A brief account of this work was presented at the Eighth International Congress on Fibrinolysis, Vienna, Austria, August 22–28, 1986.

Materials and Methods

Growth Factors and Reagents

Endothelial Cell Growth Supplement (ECGS), an enriched source of ECGF, was obtained from Collaborative Research, Inc., Bedford, MA. Pure α -ECGF (20), the active ingredient of ECGS, and 125 I- α -ECGF (5.4×10^4 cpm/ng to 2×10^5 cpm/ng) were gifts from T. Maciag and R. Friesel (American Red Cross, Rockville, MD). Heparin (sodium salt, grade I, from porcine intestinal mucosa), with a specific activity of 170 U/mg, was obtained from Sigma Chemical Co., St. Louis, MO.

Cell Culture

Early passage human fetal lung WI-38 fibroblasts were obtained from L. Hayflick (University of Florida, Gainesville, FL); Bowes melanoma cells were a gift from D. B. Rifkin (New York University Medical Center, New York); and CALU-3 cells were obtained from the American Type Culture Collection (ATCC catalogue no. HTB-55). Cultures were grown to confluency in 24 well Linbro tissue culture plates (Flow Laboratories, Inc., Hamden, CT), employing RPMI-1640 with 25 mM Hepes (Whittaker M.A. Bioproducts, Inc., Walkersville MD) or MEM (Whittaker M.A. Bioproducts) supplemented with 10% FBS and 25 μ g/ml gentamycin. MEM was supplemented with sodium pyruvate and non-essential amino acids for cultivation of CALU-3 cells. WI-38 cells were used at tissue culture passage levels well below senescence (i.e., not above passage level 30). For all cultures, growth medium was removed at confluency and replaced with serum-free RPMI after washing the cells two or three times with phosphate-buffered saline (PBS), pH 7.4. Unless indicated otherwise, the serum-free medium was supplemented with ECGS (50 μ g/ml), heparin (90 μ g/ml), or a combination of both agents. In experiments with pure growth factor, the serum-free medium was supplemented with α -ECGF (10 ng/ml to 200 ng/ml), heparin (90 μ g/ml) or combinations thereof. Cultures were incubated at 37° C in an atmosphere of 5% CO₂, 95% air. All treatments were performed in triplicate or quadruplicate. At various times, samples (50 μ l or 100 μ l) of conditioned medium (CM) were removed from replicate wells, pooled, and stored in the presence of Trasylol (20 KIU/ml) (Mobay Corporation, New York,

Correspondence to: Ruth Rappaport, Ph. D., Wyeth Laboratories, Inc., P.O. Box 8299, Philadelphia, PA 19101, USA

NY) and Tween 80 (0.01%) at 4° C until assayed. PA activity and antigen levels remained stable for at least 1 week under these conditions.

Detection of Plasminogen Activators

Fibrinolytic Activity

Fibrinolytic activity was assessed by the plasminogen-dependent fibrin agar plate assay (22, 23). Indicator plates were prepared using SeaKem® ME agarose (FMC Bioproducts, Rockville, ME), bovine fibrinogen (95% clottable), plasminogen-free bovine thrombin (Miles Scientific, Napersville, IL), and human plasminogen (Sigma Chemical Co., St. Louis, MO). Thirty ml of warmed solution containing final concentrations of 0.4% agarose, 2 mg/ml fibrinogen, 0.195 U/ml thrombin, and 58.5 µg/ml plasminogen were poured into Dynatech Single Reservoir Innoculum Trays (13.5 cm × 8.5 cm) (Dynatech Corp., Alexandria, VA). After solidification, 3.5 mm wells were punched into the agar and 10 µl samples were applied in duplicate or triplicate to the wells. For assay of t-PA, 200 µg/ml of goat anti-urokinase (anti-UK) IgG was incorporated in the agar. This level of antibody neutralized 2 µg/ml of high molecular weight urokinase (HMW-UK). For assay of u-PA, 200 µg/ml of goat anti-tPA IgG was included in the agar. This level of antibody neutralized 2.5 µg/ml of t-PA. Bowes melanoma t-PA (500,000–693,000 IU/mg) and HMW-UK (80,000 IU/mg) were used as standards on both types of indicator plate. The polyclonal immunoglobulins and standards were obtained from American Diagnostica, Inc., Greenwich, CT. The plates were incubated at room temperature for 16 to 18 h and zones of clearing measured in two dimensions with the aid of a calibrating viewer. The results were expressed in ng/ml to permit comparisons with data generated by ELISA.

Antigen Content

Antigen concentrations of t-PA and u-PA were each determined by ELISA. T-PA levels were measured using the IMUBIND t-PA kit (American Diagnostica, Inc., Greenwich, CT). The kit employs purified Bowes melanoma t-PA as the standard and in this laboratory, the assay detected as little as 0.4 ng/ml t-PA. U-PA levels were determined by a sandwich assay employing murine monoclonal anti-UK IgG (MoAb No. 390, American Diagnostica) as the capture antibody and alkaline phosphatase (Boehringer Mannheim Biochemicals, Indianapolis IN) conjugated goat anti-UK IgG (American Diagnostica) as the detecting antibody. The conjugate was prepared according to instructions from Boehringer Mannheim Biochemicals. HMW-UK (American Diagnostica) was employed as the standard and p-nitrophenylphosphate (Boehringer Mannheim Biochemicals) as the substrate. The sensitivity of the assay was 5 ng/ml of HMW-UK. In some instances, a double sandwich was employed using rabbit anti-UK IgG as capture antibody and goat anti-UK IgG plus alkaline phosphatase conjugated rabbit anti-goat IgG (Cappel Laboratories, Cochranville, PA) as detecting antibodies. The rabbit and goat anti-UK IgGs were purified by ammonium sulfate precipitation and DEAE cellulose chromatography from serum obtained from Biotech Research Laboratories (Rockville, MD) and American Diagnostica, respectively. This assay detected as little as 0.2 ng/ml of HMW-UK.

Zymography

Selected samples were analyzed by SDS-PAGE using 10% or gradient (10–20%) polyacrylamide gels and a tris-glycine buffer system. The gradient gels were obtained from Integrated Separation Systems, Newton, MA. Following electrophoresis, SDS was removed by soaking the gels in 2.5% Triton X-100 for 1.5 h (23). After rinsing with water, the gels were placed on fibrin agar or fibrin agar containing goat anti-t-PA or anti-UK IgG, prepared as described above. Bowes melanoma t-PA (American Diagnostica) and HMW-UK (Biotech Research Laboratories) were used as standards. The indicator gels were allowed to develop at 4° C, 22° C, or 37° C, depending on the samples under evaluation.

Metabolic Labeling and Immunoprecipitation

Confluent Bowes melanoma ($2-3 \times 10^5$ cells per well) or WI-38 ($1-1.5 \times 10^5$ cells per well) cultures were washed twice with PBS and then incubated at 37° C in 0.3 ml (per well) of methionine-free MEM (Flow Laboratories McLean, VA) with or without ECGS (50 µg/ml) and heparin

(90 µg/ml) and supplemented with 50 µCi of 35 S-methionine (1000–1500 Ci/mmol) (Amersham Corp., Arlington Heights, IL). After 8 h, conditioned medium from duplicate wells was harvested, pooled, and centrifuged at 13,000 × g for 2 min to remove cell debris. To each 500 µl sample, 100 µg of goat anti-t-PA IgG or goat anti-UK IgG was added and immunoprecipitation allowed to proceed for 16 h at 4° C in the presence of final concentrations of 1% NP-40, 1% bovine serum albumin (BSA), 0.2 M NaCl, 50 mM tris, pH 8.0, 1000 KIU/ml Trasylol, and 0.3 mM phenylmethylsulfonyl fluoride (Sigma Chemical Co.) (24).

Antigen/antibody complexes were adsorbed onto a 10% suspension of Sepharose CL-4B beads (Pharmacia, Inc. Piscataway, NJ) by gentle end to end agitation for 2.5 h at 4° C. After centrifugation, the beads were washed five times with 0.1% NP-40 in 50 mM tris, 1 M NaCl, pH 8.4, two times with 1% NP-40 and 0.3% SDS in 50 mM tris, 0.1 M NaCl, pH 6.8, and lastly, one time with distilled water (24). Protein was eluted from the beads by heating them at 100° C for 5 min in 63 mM tris, pH 6.8, containing 1% SDS, 10% glycerol, 5% mercaptoethanol and 0.005% bromophenol blue.

[3 H]-Thymidine and [3 H]-Uridine Incorporation

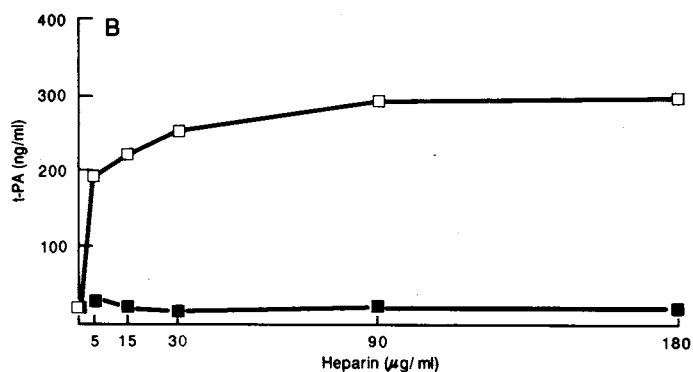
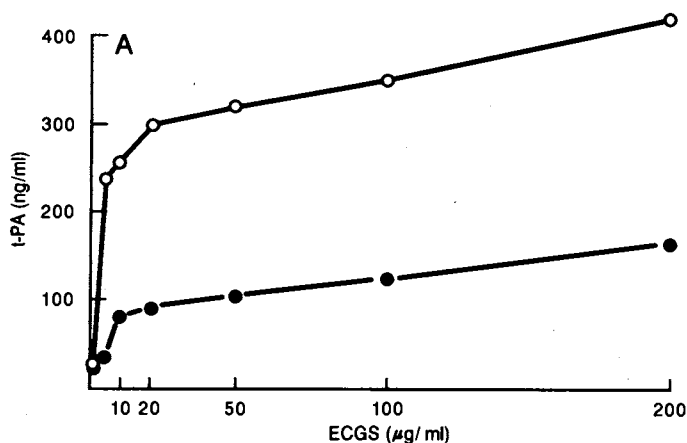
Quiescent cultures (approximately 1×10^5 cells per well) were washed twice with PBS and then incubated in serum-free RPMI with or without ECGS (50 µg/ml) and heparin (90 µg/ml) under the same conditions described above. At various times during a 36 h period, triplicate cultures were pulsed for 4 h with 1 µCi/well of [3 H]-thymidine (22 Ci/mM) or 1 µCi/well of 5,6-[3 H]-uridine (45 Ci/mM) (Amersham Corp., Arlington Heights, IL). At the end of the incubation period, the cultures were rinsed three times with 2 ml cold PBS and precipitated for 10 min with 1 ml of 10% ice cold trichloroacetic acid. The precipitated cells were washed three times with 2 ml ice cold distilled water and then removed from the wells with two consecutive 500 µl aliquots of 0.3 M NaOH and 1% SDS (25). The acid precipitable radioactivity was added to liquid scintillation cocktail (Hydrofluor, National Diagnostics, Somerville, NJ) and counted in a Packard liquid scintillation counter. The results are presented in terms of the % radioactivity incorporated in ECGS and heparin treated cultures versus control cultures.

Cellular RNA Extraction and Dot Blot Hybridization

WI-38 cells were grown in multiple 150 cm² tissue culture flasks. At confluency, the cultures were washed with PBS and incubated in serum-free RPMI with and without ECGS (50 µg/ml) and heparin (90 µg/ml). After incubation at 37° C for 48 h, the cultures were washed twice with PBS and the cells were scraped from the flasks. Total RNA was extracted from approximately 40×10^6 cells for each condition by sarkosyl guanidinium isothiocyanate treatment and cesium chloride centrifugation (26, 27). The RNA pellets were extracted twice with phenol and chloroform and precipitated with ethanol. The precipitates were dissolved in water and quantitated spectrophotometrically. Graded and equal amounts of RNA from control and treated cultures (2, 10 and 50 µg, respectively) were applied to duplicate nitrocellulose membranes using a vacuum manifold device (Schleicher & Schuell, Inc., Keene, NH). The filters were dried for 1 h at room temperature and then baked for 2 h at 80° C under vacuum. Recombinant plasmid DNAs containing the genes for t-PA and for u-PA, respectively, were used as standards with one standard each applied to one of the nitrocellulose membranes in 2, 10, and 50 ng amounts. The two membranes were hybridized separately to the appropriate 32P nick-translated plasmid DNA probe (about 10^6 cpm/ml) under high stringency conditions of hybridization and washing (28). The probes used were 1.3 kb (bp 517–1805) and 0.34 kb (bp 283–623) DNA fragments obtained from t-PA and u-PA cDNA clones, respectively (29, 30), and they were nick-translated to 10^7 cpm/pg. Both probes allowed detection of 2 ng or less of the appropriate cDNA.

ECGF Receptor Binding Studies

Confluent cell monolayers were washed three times with ice cold DMEM (Whittaker M.A. Bioproducts) containing BSA at 1 mg/ml and 50 mM Hepes, pH 7.4 (DMEM/BSA) (11). Replicate well cultures were incubated at 4° C for 90 min in DMEM/BSA containing 5.0×10^4 cpm/ml of 125 I- α -ECGF and various concentrations of heparin (0, 0.5, 5.0, and 50 µg/ml). Duplicate cultures were incubated under the same conditions



Figs. 1A, B Effect of ECGS and heparin on the production of t-PA by diploid human lung fibroblasts. WI-38 cells were cultivated in 24-well Linbro tissue culture plates. At confluency, the wells were washed twice with PBS and then incubated, in triplicate, for 72 h in serum-free medium containing (A) various concentrations of ECGS in the presence (open circles) and absence (closed circles) of heparin (90 $\mu\text{g/ml}$) or (B) various concentrations of heparin in the presence (open squares) or absence (closed squares) of ECGS (50 $\mu\text{g/ml}$). The media were collected and t-PA antigen levels determined by ELISA

except that a 100-fold excess of unlabeled purified α -ECGF was added (approx. 100 ng/well). At the end of the incubation period, triplicate wells for each condition were washed three times with DMEM/BSA and the cells were lysed with 0.3 M NaOH and 1% SDS. The samples were counted in a Packard gamma counter and the amount of cell associated radioactivity was corrected for non-specific binding. The remaining cultures were washed in a similar manner and then incubated at 37° for up to 5 h in DMEM/BSA. At appropriate times, cultures were cooled to 4° C, rinsed three times with DMEM/BSA and the cell-associated radioactivity was recovered by solubilization in 1 ml of 0.3 M NaOH, 1% SDS. The amount of specifically bound radioactivity remaining after various times of incubation at 37° C was determined as a function of heparin concentration.

Results

Heparin Potentiates ECGS Stimulation of PA Synthesis

Well cultures of WI-38 fibroblasts were grown to confluency and exposed for 72 h to various concentrations of ECGS (see

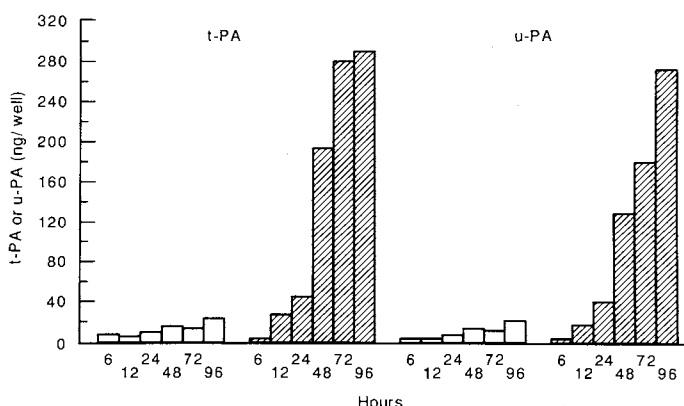


Fig. 2 Time course of production of t-PA and u-PA by diploid human lung fibroblasts cultivated in the presence or absence of ECGS and heparin. WI-38 cells were grown to confluency in 24-well Linbro tissue culture plates, washed twice with PBS, and then incubated in serum-free RPMI medium alone (open bars) or serum-free medium supplemented with ECGS (50 $\mu\text{g/ml}$) and heparin (90 $\mu\text{g/ml}$) (lined bars). At various intervals, aliquots of conditioned media were removed from replicate wells, pooled, and stored at 4° C as described in Materials and Methods. Upon completion of the experiment, t-PA and u-PA antigen levels were determined by ELISA

Materials and Methods) in the presence and absence of heparin. Under these conditions, t-PA antigen levels in conditioned medium increased as a function of ECGS concentration both in the presence and absence of heparin (Fig. 1A). The addition of heparin consistently resulted in a three-fold or greater increase in t-PA levels above those elicited by ECGS alone. In contrast, heparin alone, at various concentrations, did not stimulate t-PA production, exerting its effect only in the presence of ECGS (Fig. 1B). Similar results were obtained when the same conditioned media were analyzed for u-PA (data not shown). Optimal stimulation of t-PA and u-PA production occurred at an ECGS concentration of about 50 $\mu\text{g/ml}$, whereas optimal potentiation of ECGS by heparin occurred at a heparin concentration of about 90 $\mu\text{g/ml}$. These concentrations of ECGS and heparin (factors) were therefore employed in subsequent experiments unless noted otherwise.

Elevated synthesis of t-PA and u-PA by WI-38 cells incubated in the presence of factors became most evident after 24 h with maximal production occurring between 72 and 96 h (Fig. 2A and B). The increment of increased antigen production was generally on the order of at least tenfold in the presence of ECGS and heparin versus serum-free medium alone. In contrast, Bowes melanoma and CALU-3 cultures, which produce t-PA and u-PA, respectively, did not increase their level of production in the presence of ECGS or ECGS and heparin (data not shown).

To determine whether the antigen levels established by ELISA reflected enzymatically active t-PA and u-PA, conditioned media from WI-38 cultures incubated in the presence and absence of factors were analyzed for fibrinolytic activity. The individual contribution of each PA to total fibrinolytic activity was assessed by incorporating appropriate levels of the irrelevant neutralizing antibody in the fibrin agar plate assay for the respective PAs. Levels of fibrinolytic activity computed on a per cell per day basis were generally consistent with the same computation from ELISA values, corroborating the significant increment of increased antigen production observed when cultures were incubated in the presence of factors (Table 1).

Heparin Potentiates ECGF Stimulation of PA Synthesis

Limited quantities of highly purified α -ECGF (20) became available subsequent to completion of studies with ECGS. To confirm that results obtained with ECGS could be duplicated with purified growth factor, experiments similar to those

Table 1 Analysis of WI-38 cell synthesis of t-PA and u-PA in the presence and absence of ECGS and heparin: Comparison of plasminogen-dependent fibrinolysis in agar (FA) and enzyme-linked immunosorbent (ELISA) assays

Experiment number	Cell number per well ($\times 10^5$)	t-PA				t-PA				u-PA				u-PA			
		FA		Fold Δ		ELISA		Fold Δ		FA		Fold Δ		ELISA		Fold Δ	
		-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+
1	1.2	1.3	0.09	1.32	14.7	0.12	1.45	12.1		0.12	0.98	8.1		0.13	1.07	8.2	
2	1.0	1.1	0.06	0.92	15.3	0.05	0.82	16.4		0.25	2.05	8.0		0.16	1.28	8.0	

FA and ELISA in $\text{pg cell}^{-1} \text{day}^{-1}$.

In two independent experiments, confluent well cultures of WI-38 cells were washed three times with PBS and then incubated in serum-free RPMI medium alone (–) or supplemented with ECGS (50 $\mu\text{g/ml}$) and heparin (90 $\mu\text{g/ml}$) (+). Conditioned medium from triplicate wells was harvested at an appropriate time, pooled, and were analyzed independently for t-PA and u-PA by plasminogen-dependent fibrinolysis. Specific antibodies were incorporated in the agar to neutralize one or the other PA as described in Materials and Methods. Identical samples were evaluated for t-PA and for u-PA by ELISA. The cultures were trypsinized and the cells were counted in a hemocytometer

described above were performed. In the absence of heparin, α -ECGF only weakly stimulated the production of t-PA, whereas in the presence of heparin (90 $\mu\text{g/ml}$), α -ECGF promoted a substantial rise in t-PA levels in a dose dependent manner (Fig. 3A). At concentrations between 100 ng/ml and 200 ng/ml, α -ECGF (and heparin) drove t-PA production to levels equivalent to those produced by 50 $\mu\text{g/ml}$ of ECGS (and heparin) in numerous independent experiments (data not shown), thus providing an estimate of the effective amount of ECGF present in ECGS. A direct comparison of ECGS and ECGF corroborated these results (Table 2). Potentiation of α -ECGF by heparin was less pronounced in the case of u-PA, but the rate of induction was more rapid than for t-PA (Fig. 3B). It is noteworthy that the concentration of α -ECGF required to produce half-maximal t-PA stimulation (Fig. 3A) was at least an order of magnitude greater than required for half-maximal stimulation of mitogenesis (12).

Zymography and Immunoprecipitation

Conditioned media obtained from WI-38 cultures at various times after addition of ECGS and heparin were examined by zymography. To establish that the PAs represented t-PA and u-PA, replicate gels were overlaid with fibrin agar or fibrin agar containing anti-t-PA IgG or anti-UK IgG. In the absence of antibody, two zones of clearing were observed which comigrated with Bowes melanoma t-PA (63–65 K) and high molecular weight urokinase (55 K), respectively (Fig. 4A). In the presence of anti-t-PA IgG, only the 63–65 K species disappeared (Fig. 4C); whereas in the presence of anti-UK IgG, only the 55 K species disappeared (Fig. 4B). It is noteworthy that the zymograms showed no evidence of light chain t-PA or low molecular weight urokinase. The appearance of a minor band of activity at about 100 K represents an inhibitor complex (Fig. 4C, lane 6) and it most likely reflects activation of a small quantity of single chain urokinase which occurred during prolonged incubation (72–96 h).

In a parallel series of experiments, confluent cultures of Bowes melanoma and WI-38 cells were radiolabeled with ^{35}S -methionine and the conditioned media immunoprecipitated with goat anti-t-PA IgG, goat anti-UK IgG, or goat non-immune IgG. A major band of 63–65 K and another band of about 100 K, representing an inhibitor complex, appeared in the WI-38 sample precipitated with anti-t-PA IgG, whereas only the 63–65 K band appeared after immunoprecipitation of the Bowes melanoma conditioned medium (Fig. 5, lanes 2 and 3). Increased amounts of both the 63–65 K and 100 K components were obtained when ECGS and heparin were included in the serum-free culture medium (Fig. 5, lane 4). Thus, the combination of ECGS and heparin acted to augment the synthesis not only of t-PA, but the endothelial cell

Table 2 Comparison of the effect of endothelial cell growth supplement (ECGS) and endothelial cell growth factor (ECGF) on the synthesis of t-PA by WI-38 fibroblasts

Treatment ¹	Concentration (ng/ml)	t-PA – Heparin (ng/ml)	t-PA + Heparin (ng/ml)	Statistical ² significance
None	—	<10	10	—
ECGS	50,000	78.2 ± 4.5^3	255 ± 38.7	$p < 0.01$
α -ECGF	10	<10.0	10.0	—
	50	26.0 ± 4.0	117.4 ± 7.1	$p < 0.01$
	100	60.0 ± 4.5	153.1 ± 6.1	$p < 0.01$

¹ Confluent well cultures of WI-38 were washed and incubated in serum-free RPMI medium alone or supplemented with ECGS or α -ECGF in the presence or absence of heparin (90 $\mu\text{g/ml}$), as indicated. Conditioned medium from triplicate well cultures was harvested 72 h after treatment, pooled, and assayed for t-PA by ELISA.

² Data from two independent experiments were analyzed by two-way analysis of variance (48) to determine statistical significance.

³ Mean \pm standard error.

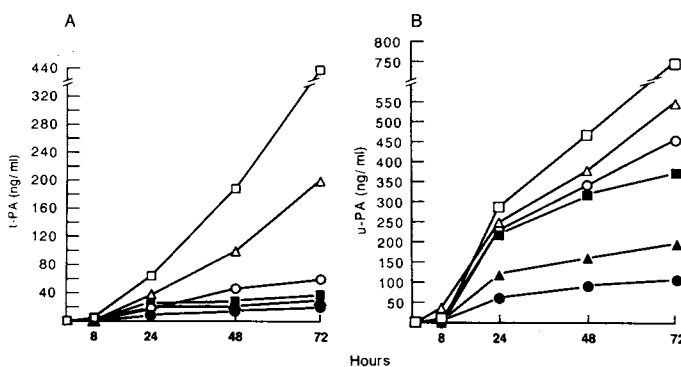


Fig. 3 Effect of α -ECGF and heparin on the production of t-PA and u-PA by human lung diploid fibroblasts. WI-38 cells were grown to confluency in 24-well Linbro tissue culture plates, washed twice with PBS and incubated in serum-free RPMI medium supplemented with α -ECGF at 50 ng/ml (closed circles), 100 ng/ml (closed triangles), and 200 ng/ml (closed squares), respectively, or with the same concentrations of α -ECGF and heparin (90 $\mu\text{g/ml}$) (corresponding open symbols). Controls consisted of cultures incubated in the presence of serum-free RPMI medium alone or supplemented with heparin (90 $\mu\text{g/ml}$). At various time intervals, aliquots were removed from replicate wells, pooled, and stored at 4°C as described in Materials and Methods. Upon completion of the experiment, t-PA (A) and u-PA (B) antigen levels were determined by ELISA. The control cultures produced very little t-PA or u-PA, less than or equal to 20 ng/ml (t-PA) and 40 ng/ml (u-PA), respectively (data not shown)

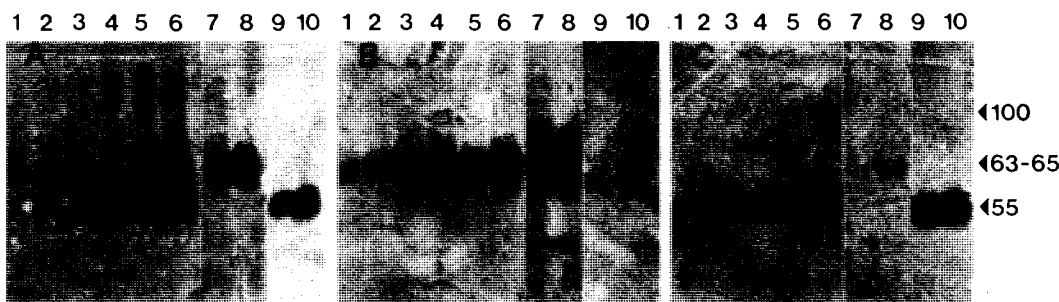


Fig. 4 Zymographic analysis of t-PA and u-PA secreted by WI-38 cells as a function of time in the presence of ECGS (50 μ g/ml) and heparin (90 μ g/ml). Culture conditions were the same as those described in the legend to Fig. 2. Serum-free conditioned media (30 μ l aliquots) were analyzed by SDS-PAGE (10% gels) followed by plasminogen-dependent fibrin agar zymography to reveal PA activity in the absence of antibody (A) and in the presence of anti-UK IgG (B) and anti-t-PA IgG (C), respectively. Lanes 1–6 represent samples harvested at 8, 12, 24, 48, 72, and 96 h, respectively. Lanes 7 and 8 represent Bowes melanoma t-PA (American Diagnostica) at 15 ng and 30 ng, respectively. Lanes 9 and 10 represent HMW urokinase (Biotech Research Laboratories) at approximately 15 ng and 30 ng, respectively. Very little or no clearing was exhibited by test samples overlaid with fibrin agar lacking plasminogen

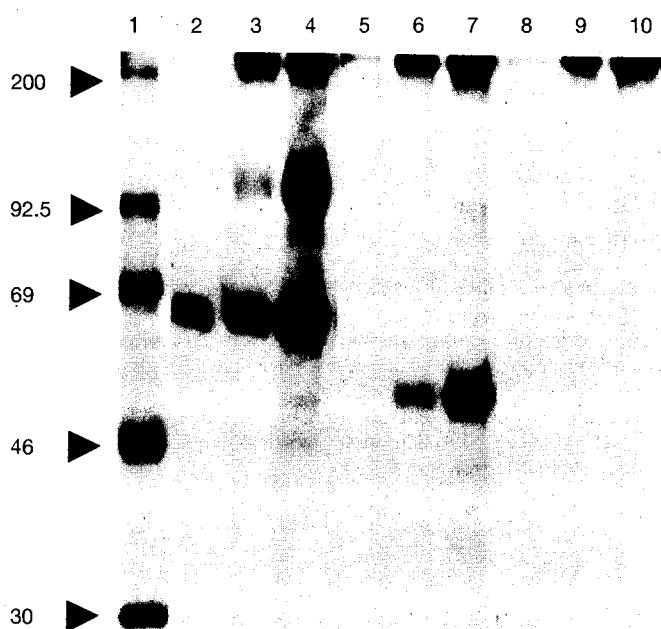


Fig. 5 Analysis by immunoprecipitation of radiolabeled PAs secreted by Bowes melanoma and WI-38 cells in the presence or absence of ECGS and heparin. Confluent well cultures were radiolabeled with 35 [S]-methionine and conditioned media, harvested at 8 h, was immunoprecipitated with anti-t-PA IgG, anti-UK IgG, or non-immune IgG as described in Materials and Methods. The respective immunoprecipitates were adsorbed to and eluted from Sepharose CL-4B beads and analyzed by SDS-PAGE (10% gels) and subsequent radioautography. Lane 1, 14 C-labeled protein standards; lanes 2, 5 and 8, Bowes melanoma conditioned medium lacking ECGS and heparin immunoprecipitated with anti-t-PA IgG, anti-UK IgG, and non-immune IgG, respectively; lanes 3, 6 and 9, WI-38 conditioned medium lacking ECGS and heparin immunoprecipitated with anti-t-PA IgG, anti-UK IgG, and non-immune IgG, respectively; and lanes 4, 7 and 10, WI-38 conditioned medium supplemented with ECGS and heparin immunoprecipitated with anti-t-PA IgG, anti-UK IgG, and non-immune IgG, respectively

type inhibitor, PAI-1 (32), as well. Similar results were observed in the case of proteins immunoprecipitated with anti-UK IgG: a 55 K band of was observed in the WI-38 sample and it increased in amount when ECGS and heparin were present in the culture medium (Fig. 5, lanes 6 and 7). Just as in the zymogram (Fig. 4C), a weak band of about 95 K also appeared, probably reflecting the presence of a minute amount of two-chain u-PA. As expected, no

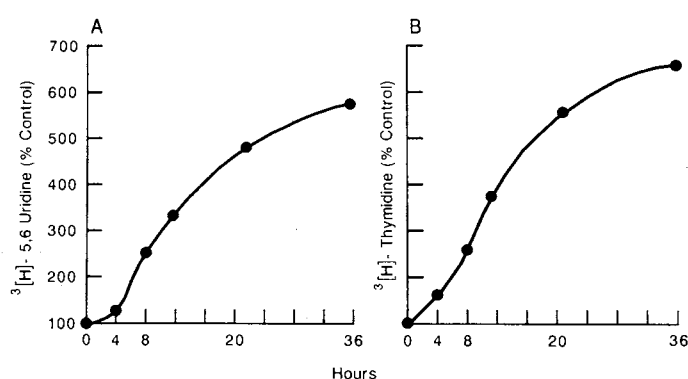


Fig. 6 Uptake of 3 [H]-5,6 uridine and 3 [H]-thymidine by WI-38 cells incubated in the presence or absence of ECGS and heparin. The cells were grown to confluency in 24-well Linbro culture dishes, washed, and maintained on serum-free RPMI medium for 7 days. The cells were then refed with fresh serum-free medium (1 ml/well) or with the same medium supplemented with ECGS (50 μ g/ml) and heparin (90 μ g/ml). At 0 h, 4 h, 8 h, 18 h, and 32 h, replicate wells were pulsed with either (A) 3 [H]-5, 6 uridine (1 μ C/well) or (B) 3 [H]-thymidine (1 μ C/well). Four h after each pulse, the cells were washed three times with ice cold PBS and precipitated with cold 10% TCA. The precipitated cells were washed with distilled water and harvested with 0.3% NaOH and 1% SDS as described in Materials and Methods. The acid precipitable radioactivity was counted, analyzed and expressed as % radioactivity incorporated in ECGS and heparin-treated cultures versus control cultures

bands appeared when anti-UK IgG was employed to precipitate proteins from Bowes melanoma conditioned medium (Fig. 5, lane 5); nor did any PA bands appear when non-immune IgG was employed to precipitate proteins from either the Bowes melanoma or WI-38 samples (Fig. 5, lanes 8, 9 and 10). The fact that neither the 63–65 K nor the 55 K PA species dissociated to light chain, (Mr 34 K) under reducing conditions argued that both PAs were secreted *primarily* in the single chain form.

Effect of ECGS and Heparin on RNA and DNA Synthesis

Quiescent well cultures of WI-38 cells were pulse labeled with 5,6 3 H-uridine or 3 H-thymidine for 4 h at various times during a 36 h incubation period in the presence and absence of ECGS and heparin. At appropriate intervals, cells were harvested and counted as described in Materials and Methods. Beginning at about 4 h after exposure of WI-38 cells to ECGS and heparin, there was a dramatic rise in both RNA and DNA synthesis as

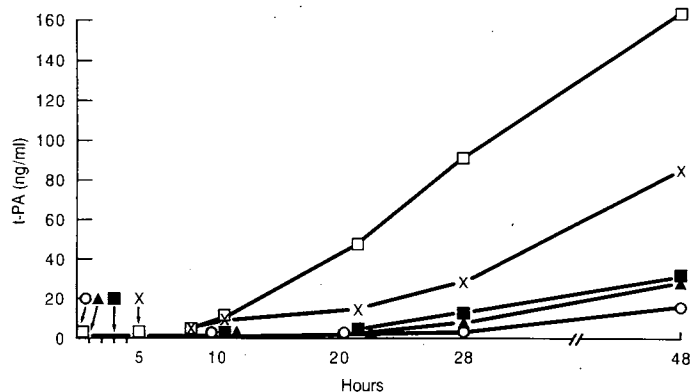


Fig. 7 Effect of actinomycin D on the induction of t-PA synthesis by ECGS and heparin. WI-38 cells were grown to confluency in 24-well Linbro dishes as described in Materials and Methods. At 0 time, cells were rinsed twice with PBS and fresh serum-free RPMI medium supplemented with ECGS (50 μ g/ml) and heparin (90 μ g/ml) was added. At times shown by the arrows, actinomycin D (1 μ g/ml) was added and the secreted t-PA was measured by ELISA at periodic intervals. Actinomycin D was added at 0 h (open circle), 1 h (closed triangle), 3 h (closed square), and at 5 h (X). The controls consisted of no addition of actinomycin D (open squares) and no addition of actinomycin D, ECGS, and heparin. T-PA levels produced in RPMI medium alone did not exceed those observed when actinomycin D was added before 5 h (data not shown)

reflected by a five to six fold increase in the uptake of uridine and thymidine versus control cultures incubated in the absence of factors (Fig. 6 A and B). The increase in DNA synthesis was not accompanied by cell proliferation, since cell numbers increased only marginally from the beginning to the end of the incubation period (data not shown). Thus, the data suggest that quiescent, confluent, diploid cultures were driven by ECGS (and heparin) through S phase, but not through G₂ of the cell cycle. The possibility that only a percentage of the cells took up thymidine and subsequently divided, however, cannot be ruled out.

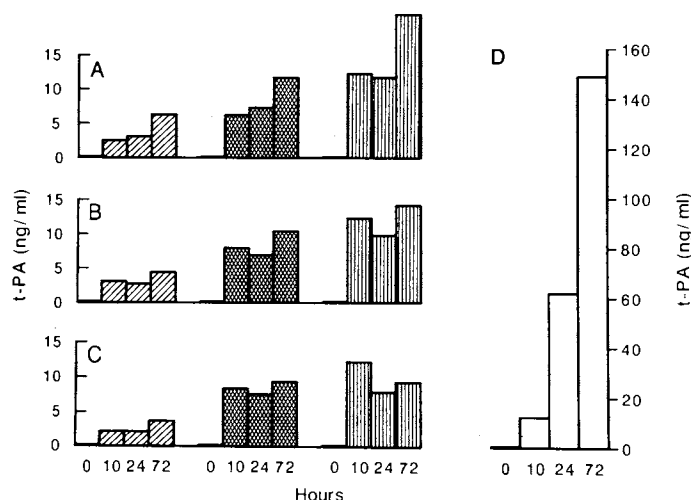


Fig. 8 Effect of cyclohexamide on the induction of t-PA synthesis by ECGS and heparin. At confluency, WI-38 well cultures were rinsed twice with PBS and fresh serum-free RPMI medium supplemented with ECGS (50 μ g/ml) and heparin (90 μ g/ml) was added. At 0 h (bars with diagonal lines), 6 h (cross-hatched bars), and 10 h (bars with vertical lines), replicate wells were treated with cyclohexamide at 2 μ g/ml (A), 10 μ g/ml (B), or 50 μ g/ml (C) and t-PA secreted into the medium at intervals during a 72-h incubation was measured by ELISA. Controls consisted of media with ECGS and heparin (D) and RPMI alone. The cultures in RPMI alone did not produce more than 40 ng/ml t-PA in 72 h (data not shown)

Effect of Metabolic Inhibitors on ECGS/Heparin Stimulated PA Synthesis

Addition of the RNA synthesis inhibitor, actinomycin D, to confluent WI-38 cultures at time zero and at 1, 3, and 5 h after addition of ECGS and heparin precluded full expression of t-PA determined at various time intervals (Fig. 7). An increase from little or no t-PA synthesis for actinomycin added at zero, 1 and 3 h to nearly one-half the control level for actinomycin added at 5 h, indicated not only a dependency of t-PA production on RNA synthesis but confined the time of initiation of RNA synthesis to between 3 and 5 h.

Addition of the protein synthesis inhibitor, cyclohexamide, to confluent WI-38 cultures also precluded full expression of t-PA synthesis. A dose-dependent inhibition of t-PA production by cyclohexamide was manifest in conditioned medium from cultures to which the inhibitor was added at zero, 6, and 10 h after the addition of ECGS and heparin (Fig. 8).

Dot Blot Analysis of mRNA

Aliquots of total RNA (2, 10 and 50 μ g) from WI-38 cultures that had been incubated for 48 h in the presence and absence of ECGS (and heparin) were applied to duplicate nitrocellulose membranes. One set of blots was hybridized with a 1.3 kb (bp 517–1805) ³²P-DNA probe from a t-PA cDNA clone and the other set, with a 0.34 kb (bp 283–623) ³²P-DNA probe from a u-PA cDNA clone as described in Materials and Methods. One each of the plasmid DNAs was also applied to one of the nitrocellulose membranes in 2, 10, and 50 ng amounts. Upon subsequent exposure of the nitrocellulose membranes to X-ray film, the radiograms revealed a five to ten fold greater level of t-PA and u-PA mRNA present in total RNA isolated from ECGS (and heparin)-treated cells than in the same quantity of total RNA isolated from control cells (Fig. 9). The sensitivity of the probes for plasmid DNA was approximately 4 to 5 ng for t-PA cDNA and about 2 ng for u-PA cDNA.

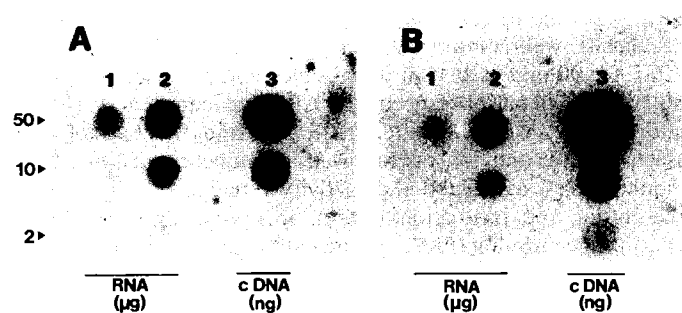


Fig. 9 Dot blot hybridization of total RNA from WI-38 cells incubated in the presence and absence of ECGS and heparin. At confluency, WI-38 cells cultivated in 150 cm² flasks were washed twice with PBS and incubated in serum-free RPMI medium alone (control) or in serum-free medium supplemented with ECGS (50 μ g/ml) and heparin (90 μ g/ml) (treated). After incubation for 48 h, the cells were scraped from the flasks and total RNA was extracted from control and treated cultures (40 \times 10⁶ cells each) by sarkosyl quantidium isothiocyanate treatment and cesium chloride centrifugation. Two μ g, 10 μ g, and 50 μ g of RNA from control (lane 1) and treated cultures (lane 2) were applied to duplicate nitrocellulose membranes and plasmid DNA containing the gene for t-PA or for u-PA was applied to one each of the nitrocellulose membranes (panels A and B, respectively). The membrane dotted with t-PA plasmid DNA was hybridized under conditions of high stringency with a ³²P-labeled nick-translated 1.3 kb fragment of the t-PA cDNA (bp 517–1805) (panel A) and the membrane dotted with u-PA plasmid DNA was hybridized under the same conditions with a similarly prepared 0.34 kb probe (283–623) from u-PA cDNA (panel B)

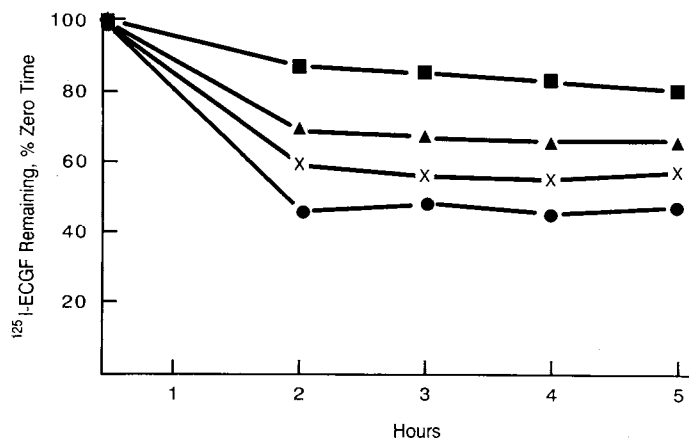


Fig. 10 Effect of heparin on dissociation of ^{125}I -ECGF from WI-38 fibroblasts. Confluent well cultures were washed three times with DMEM/BSA and labeled with ^{125}I - α -ECGF (5×10^4 cpm/well) for 90 min at 4°C in serum-free DMEM/BSA containing 0, 0.5 $\mu\text{g/ml}$, 5.0 $\mu\text{g/ml}$, and 50 $\mu\text{g/ml}$ heparin, respectively. Duplicate cultures were incubated under identical conditions except for the presence of at least a 100-fold excess of cold α -ECGF (50–100 ng/well). At the end of the incubation period, the cultures were washed three times with DMEM/BSA, lysed with 0.3 N NaOH, 1% SDS, and the amount of specifically bound ^{125}I - α -ECGF determined. Parallel cultures incubated under identical conditions were washed three times, replenished with DMEM/BSA, and shifted to 37°C . At the appropriate time, these cultures were cooled to 4°C , washed with DMEM/BSA, and solubilized with 0.3 N NaOH and 1% SDS, as before. The amount of specifically bound ^{125}I - α -ECGF remaining cell-associated as a function of time was determined for each heparin concentration, 0 (closed circles), 0.5 $\mu\text{g/ml}$ (X), 5.0 $\mu\text{g/ml}$ (closed triangles), and 50 $\mu\text{g/ml}$ (closed squares). The amount of ^{125}I - α -ECGF specifically bound at 0 time (100%) increased slightly with increasing heparin concentration, whereas non-specific binding decreased with increasing heparin concentration, ranging from approximately 33% (0 heparin) to 15% (50 $\mu\text{g/ml}$ heparin).

ECGF Receptor Binding Studies

To assess the effect of heparin on the binding of α -ECGF to WI-38 cells, well cultures were incubated with 0.5 nM of ^{125}I - α -ECGF for 90 min at 4°C in the absence and presence of 0.5, 5, and 50 $\mu\text{g/ml}$ heparin, respectively. After incubation at 4°C , the cultures were washed, shifted to 37°C , and the fate of cell associated radioactivity was followed as a function of time. The amount of cell associated radioactivity remaining at any given time during the incubation period, increased with increasing heparin concentration (Fig. 10). The data suggest that once bound in the presence of heparin, ^{125}I - α -ECGF remains more firmly bound, possibly due to stabilization of the ECGF-receptor complex (11).

Discussion

There is abundant evidence that a wide variety of normal and malignant cells and tissues secrete t-PA, u-PA, or both and that a number of substances are capable of influencing their synthesis (31). Prominent among the stimulators of PA or PA mRNA synthesis are the polypeptide mitogens, epidermal growth factor (EGF) (32–34); platelet-derived growth factor (34); transforming growth factor-beta (TGF) (35); ECGF (36); and basic fibroblast growth factor (bFGF) (18). Several of these factors have received a great deal of attention recently in the context of their ability to promote angiogenesis via interaction with high affinity receptors on endothelial cells (18, 37). In the case of ECGF, it has been determined that there are approximately 20,000 to 40,000 receptor sites on human endothelial cells and on human fibroblasts

originating from foreskin (11). Here, we provide evidence that ECGF stimulates the synthesis of both t-PA and u-PA by confluent diploid fibroblasts originating from fetal human lung, and we show that the stimulation is potentiated by heparin. Just as in the potentiation of ECGF-induced proliferation of endothelial cells (10, 11), this is probably due to a conformational change in ECGF resulting from its interaction with heparin. It has been suggested that such a conformational change is responsible for an increase in the receptor binding affinity of ECGF (11). Our data concerning prolonged occupancy of ECGF receptor sites as a function of heparin concentration (Fig. 10) is likely a reflection of these events.

The production of PAs by human embryonic lung fibroblasts has been examined previously (32, 38–42). As early as 1979, it was recognized that the diploid cell line, IMR-90, produced multiple molecular weight forms of PA and that the two forms might be immunologically distinct (38). In the present investigation, we provide biochemical and immunological evidence that WI-38 cells produce both forms of PA in their single chain form (Figs. 4 and 5). Furthermore, we demonstrate that their synthesis can be elevated eightfold or more above basal levels when the cells are stimulated optimally by ECGF or ECGF and heparin (Figs. 1, 2 and 3, Tables 1 and 2) and that these events are dependent on *de novo* protein and RNA synthesis (Figs. 6–9). For reasons that are not clear, potentiation of ECGF stimulation by heparin was occasionally more pronounced in the case of t-PA than it was for u-PA (e.g. Fig. 3). Nevertheless, the combined factors reproducibly triggered maximal synthesis of both PAs in relatively equal amounts.

Growth factor regulation of proteolysis appears to be a fundamental control mechanism in cell proliferation, angiogenesis, reproduction, neoplasia, and a host of other normal and aberrant processes (31). The ubiquity of cells or tissues throughout the body that are capable of responding to growth factor signals by synthesis and secretion of one or both types of PA offers opportunities for exocrine or autocrine control. Recent evidence that human endothelial cells express bFGF (43), for example, suggests that when properly stimulated, these cells are capable of secreting their own growth factor, which, in turn, may act in an autocrine manner to promote t-PA synthesis and subsequent events. Here, we present evidence of endothelial cell growth factor control of PA synthesis by human diploid fibroblasts originating from fetal lung. An auxiliary role for heparin is indicated as well. Possibly, one explanation for the profibrinolytic actions attributed to heparin (44) may reside in its ability to potentiate endogenous growth factor(s).

The question arises as to what relevance, if any, these findings may have for fibroblast physiology *in vivo*. It is possible that endogenous glycosaminoglycans that exist in the extracellular matrix of responsive target cells (45–47), may serve to amplify the stimulation of PA synthesis by minute amounts of endogenous growth factor. Fibroblasts residing in lung or other organs may represent such a target population and thus could play a larger role in certain physiological processes than previously thought. The potential therapeutic utility of growth factors or growth factor analogues and heparin as regulators of endogenous PA levels in the vasculature and elsewhere remains to be explored.

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