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Production of transforming growth factor α in human pancreatic cancer cells: Evidence for a superagonist autocrine cycle

(RNA blot hybridization/epidermal growth factor/anchorage-independent growth)

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ABSTRACT **Previous work showed that cultured human pancreatic cancer cells overexpress the epidermal growth factor (EGF) receptor. In the present study, we sought to determine whether some of these cell lines produce transforming growth factor α (TGF-α). Utilizing a radiolabeled TGF-α cDNA in hybridization experiments, we determined that ASPC-1, T_3M_4, PANC-1, COLO-357, and MIA PaCa-2 cell lines expressed TGF-α mRNA. Serum-free medium conditioned by T_3M_4 and ASPC-1 cells contained significant amounts of TGF-α protein. Although unlabeled TGF-α readily competed with ^{125}I-labeled EGF for binding, each cell line exhibited lower surface binding and internalization of ^{125}I-labeled TGF-α as compared to ^{125}I-labeled EGF. Both TGF-α and EGF significantly enhanced the anchorage-independent growth of PANC-1, T_3M_4, and ASPC-1 cells. However, TGF-α was 10- to 100-fold more potent than EGF. These findings suggest that the concomitant overexpression of EGF receptors and production of TGF-α may represent an efficient mechanism for certain cancer cells to obtain a growth advantage.**

Epidermal growth factor (EGF) is a mitogenic polypeptide that binds to the EGF receptor and regulates a variety of cellular processes (1). Following binding, the EGF receptor is autophosphorylated at tyrosine residues (2). This receptor exhibits a strong sequence homology with the product of the avian erythroblastosis virus v-*erbB* oncogene (3). An overabundance of EGF receptors has been reported in a number of human cancers, including breast (4), bladder (5), glial (6), epidermoid (7), and pulmonary (8) malignancies. EGF receptor overexpression also occurs in cultured human carcinoma cells (9–13), including a number of pancreatic cancer cells (14–16).

The mechanisms whereby EGF receptor overexpression may provide a growth advantage to cancer cells remain uncharacterized. It has been suggested, however, that unrestricted activation of the tyrosine kinase may allow cells to proliferate in the absence of EGF (17). Alternatively, malignant transformation may be associated with the production of growth-promoting polypeptides that bind and activate the EGF receptor. Thus, a number of cancer cell lines have been shown to produce transforming growth factor α (TGF-α), a 50 amino acid polypeptide that is structurally similar to EGF (18). Because there is no evidence for a distinct TGF-α receptor, it is generally accepted that the actions of TGF-α are mediated through the EGF receptor (19). The present study was undertaken to determine whether cultured human pancreatic carcinoma cells produce TGF-α, which could then activate the overexpressed EGF receptors in these cells. In addition, we compared the effects of TGF-α on cell growth with the actions of EGF. Our data suggest that these cell lines produce TGF-α, which may act in an autocrine manner as a potent growth promoter.

MATERIALS AND METHODS

Cell Culture. PANC-1, ASPC-1, and MIA PaCa-2 human pancreatic carcinoma cells were obtained from the American Type Culture Collection (Rockville, MD). T_3M_4 (20) and COLO-357 (21) cells were obtained from R. S. Metzgar at Duke University. Cells were routinely propagated in monolayer culture at 37°C in a 5% CO_2/95% humidified-air atmosphere. PANC-1, MIA PaCa-2, and COLO-357 cells were grown in Dulbecco's modified Eagle's medium (DMEM). T_3M_4 and ASPC-1 cells were grown in RPMI 1640. Media were supplemented with antibiotics and 10% fetal bovine serum.

Binding Studies. Biologically active EGF was prepared from mouse submaxillary glands (22). Purified and refolded human TGF-α was synthesized in *Escherichia coli* (23). The peptides were iodinated by a modification of the chloramine-T method (24) to a specific activity of 120 μCi/μg (EGF) or 150 μCi/μg (TGF-α) (1 μCi = 37 kBq). To measure binding, cells that were 75–80% confluent were washed once in binding medium (DMEM supplemented with 20 mM Hepes, pH 7.4) and incubated at 4°C in binding medium in the presence of labeled ligand. Nonspecific binding, determined in the presence of a 2000-fold excess of unlabeled EGF (2 μg/ml), did not exceed 5% of total binding. Incubations were stopped by washing cells six times in Hanks' balanced salts solution containing 0.1% bovine serum albumin. To monitor ligand internalization, washed cells were incubated for 4 min at 4°C with 0.5 M NaCl (pH 2.5). Radioactivity removed by this incubation was taken to represent the surface-bound ligand, whereas the radioactivity remaining with the cells was considered as internalized ligand (25).

RNA·cDNA Hybridization Studies. RNA was extracted from confluent cells by the guanidinium isothiocyanate method (26). Poly(A)$^+$ mRNA was prepared according to the method of Werner *et al.* (27), using poly(U) paper (Hybond-mAP, Amersham). Total RNA and poly(A)$^+$ mRNA were fractionated in 0.8% agarose gels and electroblotted onto Nytran membranes (Schleicher & Schuell). The filters were probed with the human TGF-α cDNA contained in plasmid pTGF-C1 (23), according to the method of Thomas (28). Prehybridization and hybridization were carried out in 50% (vol/vol) formamide at 42°C.

TGF-α Assay. All five cell lines were grown in T175 tissue-culture flasks in the presence of fetal bovine serum. Cells were allowed to reach confluence before the medium was replaced with fresh medium devoid of serum. Following an additional 48-hr incubation the supernatants were collect-

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Abbreviations: TGF-α, transforming growth factor α; EGF, epidermal growth factor; ^{125}I-, ^{125}I-labeled.

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ed, dialyzed extensively against 1 M acetic acid, and lyophilized. TGF-α was assayed by a highly specific double-sandwich enzyme-linked immunosorbent assay (ELISA) that does not detect TGF-α containing incorrectly formed disulfide bridges and does not react with human EGF at 10 μg/ml (29).

Anchorage-Independent Growth. Freshly trypsinized cells were suspended in 0.33% agar (Difco Laboratories) in medium supplemented with antibiotics, 10% fetal bovine serum, and 0, 0.1, 1.0, or 10.0 ng of murine TGF-α (Peninsula Laboratories) or EGF per ml. The batches of EGF and TGF-α used in the present study were equipotent in displacing ^{125}I-labeled (^{125}I-EGF) in a radioreceptor assay. Cells were layered in 35-mm plates (2×10^5 cells per plate) onto a base of 0.5% agar containing the medium and the corresponding growth factor. Plates were incubated for 2 weeks at 37°C in a 5% CO_2/95% humidified-air atmosphere. Cumulative counts of colonies greater than 86 μm and 104 μm in diameter were quantitated with an optical image analyzer (Omicon Fas-II, Bausch and Lomb). Colony volumes (in μm^3) were calculated from the diameter of each colony. Statistical analysis was assessed by Student's *t* test.

RESULTS

TGF-α Levels. TGF-α protein was present in the supernatant of T_3M_4 and ASPC-1 cells at concentrations of 4.5 and 2.2 pg/ml, respectively (Table 1). This corresponded to a production rate of 7.8 (T_3M_4) and 4.9 (ASPC-1) pg per 10^6 cells per 48 hr, assuming complete recovery of the protein and absence of peptide degradation. TGF-α protein was not detected in the supernatant of the other three cell lines (Table 1).

Blot hybridization analysis of electrophoretically fractionated poly(A)$^+$ mRNA revealed a single band corresponding to TGF-α mRNA of ≈4.6 kilobases (kb) in ASPC-1, COLO-357, MIA PaCa-2, and T_3M_4 cell lines (Fig. 1). ASPC-1 cells exhibited the greatest amount of TGF-α mRNA. Because poly(A)$^+$ mRNA could not be readily isolated from PANC-1 cells, total RNA from this cell line was fractionated and compared with total RNA from MIA PaCa-2 cells. Blot analysis of total RNA indicated that TGF-α mRNA levels were comparable in the two cell lines (Fig. 1).

Binding Characteristics. Both ^{125}I-TGF-α and ^{125}I-EGF readily bound to each cell line used in the present study. In each instance, the amounts of surface-bound and internalized radioactivity were greater for ^{125}I-EGF than for ^{125}I-TGF-α (Table 2). However, unlabeled TGF-α readily competed with ^{125}I-EGF for binding to these cells, and unlabeled EGF readily competed with ^{125}I-TGF-α (data not shown).

Table 1. Comparison of EGF receptor number and TGF-α level

Cell line	EGF receptors, no. per cell	TGF-α level, pg/ml
T_3M_4	1.2×10^6 (14)	4.5
ASPC-1	2.2×10^5 (16)	2.2
PANC-1	4.0×10^5 (14)	<1.0
COLO-357	2.5×10^5 (14)	<1.0
MIA PaCa-2	1.7×10^5 (16)	<1.0

The number of surface EGF receptors per cell was obtained by carrying out saturation binding studies at 4°C with increasing concentrations of ^{125}I-EGF, as previously reported (14, 16). Numbers in parentheses represent the respective references. TGF-α levels were determined after collecting 100 ml of medium per cell line and reconstituting the lyophilized supernatants in 1 ml of 150 mM NaCl/50 mM Tris, pH 7.4/2 mM EDTA/0.05% Tween 20/0.5% bovine serum albumin.

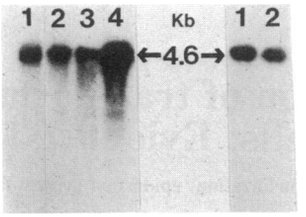

FIG. 1. RNA blot hybridization analysis. (*Left*) Poly(A)$^+$ RNA (4 μg) from each cell line was electrophoresed in 0.8% agarose, transferred to nylon membrane, and probed with a TGF-α cDNA (2×10^6 cpm). Lanes: 1, T_3M_4 cells; 2, MIA PaCa-2 cells; 3, COLO-357 cells; 4, ASPC-1 cells. (*Right*) Total RNA (5 μg) was electrophoresed in 0.8% agarose, transferred to nylon membrane, and probed with a TGF-α cDNA (2×10^6 cpm). Lanes: 1, PANC-1 cells; 2, MIA PaCa-2 cells. RNA size (in kilobases, kb) was determined by comparison with ribosomal RNA markers.

Anchorage-Independent Growth. The effects of TGF-α and EGF on colony formation in soft agar were compared in three cell lines. The reproducibility of the counts, determined for several colony sizes, was excellent; triplicate determinations of colony number for five plates fell within 1.1 standard deviations of the mean. TGF-α exerted a dose-dependent effect on colony formation in PANC-1 cells, peak growth enhancement occurring 5–8 days after plating (Fig. 2). For colonies greater than 86 μm in diameter, a threshold stimulatory effect was observed at 0.1 ng/ml (16%), and a highly significant effect was seen at 1.0 ng/ml (44%, $P < 0.002$). Maximal stimulation (118%, $P < 0.001$) occurred with TGF-α at 10 ng/ml (Fig. 2*A*). In contrast, EGF at 0.1 and 1.0 ng/ml did not significantly alter the growth of PANC-1 cells. However, EGF at 10 ng/ml enhanced colony formation by 90% ($P < 0.002$). For colony sizes greater than 104 μm, TGF-α again exerted a highly significant effect at 1.0 ng/ml (61%, $P < 0.003$), and maximal effects at 10 ng/ml (179%, $P < 0.001$) (Fig. 2*B*). Further, EGF at 10 ng/ml enhanced colony formation by 133% ($P < 0.001$).

Growth enhancement by TGF-α and EGF was still evident 8–10 days after cell plating, but the effects of both growth factors were less prominent (data not shown). Further, TGF-α exerted a greater stimulatory effect on cumulative colony volume than EGF (Table 3) and was a more potent stimulator of colony formation than EGF in T_3M_4 and ASPC-1 cells (data not shown).

DISCUSSION

Autocrine mechanisms appear to have important regulatory roles in the control of tumor cell growth. Evidence for this hypothesis is based on the observation that transformed cells in tissue culture produce endogenous growth factors that enhance cell growth (30–32). As a result, these cells require fewer exogenous factors for optimal proliferation than nontransformed cells (30–32). Several structurally distinct peptides are now known to act in an autocrine manner. In addition to TGF-α, these include transforming growth factor β (TGF-β) (33), insulin-like growth factor I (34), platelet-derived growth factor (35), bombesin (36), and interleukin 2 (37). In rare instances, certain malignant cells produce EGF-like peptides that can ostensibly act in an autocrine manner (38). Further, transformation of rat fibroblasts with an expression vector coding for TGF-α imparts to these cells the ability to produce TGF-α and to exhibit anchorage-independent growth (39).

In the present study, we determined that five of five human pancreatic cancer cell lines that overexpress the EGF receptor synthesize TGF-α mRNA. However, the amount of TGF-α protein assayed in the supernatant of the cells did not

Table 2. Comparison of ^{125}I-EGF and ^{125}I-TGF-α binding

Cell line	^{125}I-EGF, cpm		^{125}I-TGF-α, cpm	
	Cell surface	Internalized	Cell surface	Internalized
T_3M_4	2933 ± 195	13,796 ± 665	2329 ± 78	13,801 ± 554
ASPC-1	2289 ± 245	8,632 ± 735	1427 ± 311	8,286 ± 1483
PANC-1	2818 ± 48	9,838 ± 273	1109 ± 146	6,251 ± 159
COLO-357	1382 ± 9	10,040 ± 581	847 ± 78	6,745 ± 321
MIA PaCa-2	2025 ± 26	7,869 ± 215	877 ± 51	4,391 ± 67

Cells were incubated for 60 min at 37°C in the presence of ^{125}I-EGF or ^{125}I-TGF-α (1 ng/ml; 100,000 cpm/ml). Surface-bound radioactivity was determined by elution into pH 2.5 buffer. Internalized radioactivity was determined by solubilization of cells in 0.5 N NaOH after removal of surface-bound radioactivity. Data are means ± SD of triplicate determinations from two representative experiments.

directly correspond with the amount of TGF-α mRNA detected in these cells. Further, there was no direct correlation between the number of EGF receptors on the surface of these cells and the amount of TGF-α protein or mRNA. These discrepancies may relate to different rates of degradation of TGF-α mRNA or differences in TGF-α protein half-life among these cell lines. Alternatively, it is possible that the TGF-α is associated with the cells, or that variations in the posttranslational modifications of the precursor result in different TGF-α protein moieties that are not detected by the ELISA (23).

In agreement with the hypothesis that TGF-α binds to the EGF receptor (19), TGF-α readily competed with EGF in competition-inhibition studies in these cells. Although EGF appeared to bind more efficiently than TGF-α in each cell line, the latter was 10- to 100-fold more potent than EGF in enhancing anchorage-independent growth. The reasons for these differences are not known. However, TGF-α is known to exert a greater stimulatory effect than EGF on several biological functions. These include calcium mobilization from fetal rat long bones (40), induction of cell ruffling (41), angiogenesis in the hamster cheek pouch model (42), skin wound healing (43), and stimulation of blood flow in the femoral artery of the dog (44). The proliferative effects of TGF-α in the hamster cheek pouch and on epithelial wound healing were significant at 300 ng/ml and 100 ng/ml, respectively. In contrast, in the present study TGF-α at 1 ng/ml exerted a significant stimulatory effect on anchorage-independent growth. Recent work showed that the inhibitory effect of TGF-α on the proliferation of RL95-2 cells is greater than that of EGF (45). These observations suggest that TGF-α may activate postreceptor pathways that are not activated by similar concentrations of EGF, and that certain malignant cells may be exquisitely sensitive to the growth-regulatory actions of TGF-α.

It is not readily apparent why TGF-α can enhance the growth of cells that are already producing this growth factor. However, in the present study, the cells were plated in soft agar at very low seeding densities. It is possible that at this initial low seeding density the amount of TGF-α produced by the cells is not sufficient to rapidly stimulate their growth. Conversely, once the initial growth of these cells is supported by the exogenous addition of either TGF-α or EGF, the cells may be able to maintain this growth as a result of endogenous TGF-α production. Irrespective of the mechanisms, our findings indicate that TGF-α acts as a superagonist of EGF in stimulating the anchorage-independent growth of pancreatic

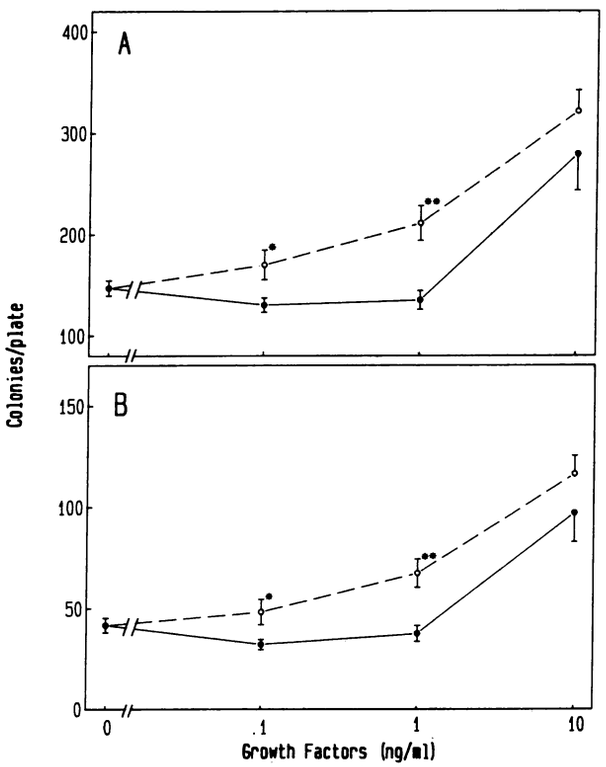

FIG. 2. Effects of TGF-α and EGF on colony formation in soft agar. PANC-1 cells were incubated with either TGF-α (○) or EGF (●), as described in the legend to Table 3. (*A*) Cumulative counts of colonies greater than 86 μm in diameter. (*B*) Cumulative counts of colonies greater than 104 μm in diameter. Each point is the mean ± SEM (n = 20) from four separate experiments. Asterisks indicate TGF-α colony-formation values that differ significantly (*, $P < 0.02$; **, $P < 0.001$) from the values obtained with the respective concentration of EGF.

Table 3. Effects of TGF-α and EGF on the growth of PANC-1 cells in soft agar

Growth factor, nM	Percent change from control	
	Colonies >86 μm	Colonies >104 μm
TGF-α		
0.017	+17	+17
0.17	+54*	+75†
1.70	+148‡	+210‡
EGF		
0.017	−15	−26§
0.17	−9	−11
1.70	+107‡	+144‡

PANC-1 cells were incubated with the indicated molar concentrations of TGF-α and EGF, corresponding to 0.1, 1.0, and 10.0 ng/ml of either growth factor. Cumulative volumes of colonies were calculated from the diameter of each colony, as determined with an image analyzer 5–8 days after plating. Values are expressed as the percent change from control plates (n = 20).

*P = 0.002.
†P = 0.004.
‡$P < 0.001$.
§P = 0.03.

cancer cells. These observations suggest, therefore, that production of TGF-α may be an efficient mechanism whereby certain malignant cells that overexpress the EGF receptor may obtain a growth advantage.

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