Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 863–867, February 1993
Cell Biology

Tumor necrosis factor α induces the expression of transforming growth factor α and the epidermal growth factor receptor in human pancreatic cancer cells

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Communicated by Elwood V. Jensen, October 15, 1992 (received for review August 14, 1992)

ABSTRACT **Recombinant human tumor necrosis factor (TNF)-α increased the expression of epidermal growth factor receptor (EGFR) mRNA and protein in all of six human pancreatic carcinoma cell lines tested. In addition, TNF-α increased the expression of an EGFR ligand, transforming growth factor (TGF)-α, at the mRNA and protein level in all cell lines. Increased expression of EGFR protein was associated with elevated steady-state EGFR mRNA levels. Nuclear run-on analysis showed that increase in EGFR mRNA was due to an increased rate of transcription. Induction of EGFR mRNA expression by TNF-α was abrogated by cycloheximide but occurred independently of TNF-α-induced production of TGF-α protein. Protein kinase A or G_i-type guanine nucleotide-binding proteins were not involved in this process as assessed by using appropriate stimulators and inhibitors of these signal transduction pathways. By contrast, staurosporine, an inhibitor of protein kinase C, partially inhibited, and 4-bromophenacyl bromide, a phospholipase inhibitor, completely inhibited TNF-α-dependent EGFR mRNA expression. The phospholipase C-specific inhibitor tricyclodecan-9-yl xanthogenate did not alter TNF-α-dependent EGFR mRNA expression, suggesting that phospholipase A_2 is involved in the modulation of EGFR expression by TNF-α. The simultaneous induction of a ligand/receptor system by TNF-α suggests that this cytokine modulates autocrine growth-regulatory pathways in pancreatic cancer cells.**

Transforming growth factor (TGF)-α is a polypeptide that interacts with the same receptor as the epidermal growth factor (EGF) and induces mitogenic and/or cell differentiating responses by binding to and activating the tyrosine kinase activity of the 170-kDa cell surface EGF receptor (EGFR; for review see refs. 1 and 2). Coordinate expression of high levels of TGF-α and EGFR is frequently found in human tumors and transformed cells (3), including those of the pancreas (4). However, coexpression of TGF-α and the EGFR is not restricted to neoplasms of the pancreas but has also been observed in benign inflammatory diseases such as chronic pancreatitis (5, 6). This finding suggests that TGF-α/EGFR expression in pancreatic tissue is sensitive to regulation by exogenous agents. Expression of the EGFR on different normal and malignant cell types has been shown to be induced by exogenous agents like hormones, vitamins, growth factors such as EGF/TGF-α and TGF-β, and cytokines such as interferon (IFN)-γ (7–13).

In this study, we examined the effect of tumor necrosis factor (TNF)-α on the expression of EGFR and TGF-α by pancreatic carcinoma cell lines. TNF-α is a polypeptide that has pleiotropic biological effects (14) and has been shown to upregulate the expression of the EGFR protein (15) and to downmodulate the EGFR affinity in human normal diploid fibroblasts (16). We report that, in pancreatic carcinoma cells, TNF-α induces not only the expression of EGFR but also the expression of TGF-α at the mRNA and protein levels. We have also identified phospholipases and protein kinase C activation as critical elements in the signal transduction pathway leading to TNF-α-dependent EGFR upregulation.

MATERIALS AND METHODS

Human Pancreatic Cancer Cell Lines and Culture Conditions. The cell lines Capan-2 and SW850 were supplied by J. Fogh (Sloan–Kettering Institute, New York) and HPAF was from R. Metzgar (Duke University, Durham, NC). The cell lines 818-1, 818-4 and 818-7 have been established in our laboratory. The cells were cultured as described (17).

Cytokines and Other Reagents. Recombinant human TNF-α and IFN-γ were generously supplied by G. R. Adolf (Bender, Vienna, Austria). The specific biological activities were 5×10^7 units/mg for TNF-α, and 2×10^7 units/mg for IFN-γ (stated by the supplier). Neutralizing anti-TGF-α monoclonal antibody (mAb), Ab-3, was purchased from Oncogene Science/Dianova (Hamburg, F.R.G.), and anti-EGFR mAb 425/EMD55900 (18) was made available by Merck. Cycloheximide and staurosporine were from Boehringer Mannheim, and 4-bromophenacyl bromide (4-BPB) was purchased from Sigma. Tricyclodecan-9-yl xanthogenate (D-609) was kindly provided by G. Quack (Merz, Frankfurt, F.R.G.). The applied concentrations are indicated in the figures.

Radioligand Binding Assay. Live-cell radioligand binding assays were performed in homologous displacement analyses with mAb 425 and recombinant human EGF (Amersham, Braunschweig, F.R.G.), as reported (19). Affinity constants as well as receptor concentrations were estimated by using the computer program LIGAND (20).

RNA Isolation and Northern Blot Analysis. Preparation of total cellular RNA and Northern blot hybridizations were performed as described (17). In addition to ethidium bromide staining, autoradiographic signals obtained with the c-*raf-1* probe served as control for equal loading of the gel slots.

DNA Probes. Probes were as follows: EGFR, 2.4-kb *Cla* I fragment derived from cDNA clone pE7 (American Type Culture Collection); TGF-α, 925-bp *Eco*RI human cDNA fragment of clone phTGF1-10-925 (kindly donated by C. Knabbe, University Hospital, Hamburg, F.R.G.); interleukin 1β (IL-1β), antisense 40-base oligonucleotide corre-

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Abbreviations: 4-BPB, 4-bromophenacyl bromide; EGF, epidermal growth factor; EGFR, EGF receptor; IFN, interferon; IL, interleukin; PDGF, platelet-derived growth factor; TGF, transforming growth factor; TNF, tumor necrosis factor; mAb, monoclonal antibody.

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sponding to a sequence from the human IL-1β first exon (Oncogene Science/Dianova); platelet-derived growth factor B chain (PDGF-B)/c-*sis*, 1.0-kb *Bam*HI–*Pst* I human genomic DNA fragment containing part of the c-*sis* seventh exon ("*sis*-Amprobe," Amersham); c-*myc*, 1.5-kb *Sst* I human genomic DNA fragment carrying the c-*myc* second exon (Amersham); c-*raf-1*, 1.6-kb *Hin*dIII–*Eco*RI human cDNA fragment (Amersham).

The double-stranded probes were labeled with [α-^{32}P]-dCTP by using the multiprime kit (Amersham). Oligonucleotides were end-labeled by using [γ-^{32}P]ATP and a 5'-end-labeling kit (Boehringer). The probes were purified by using "nick columns" or "NAP-5" columns (Pharmacia LKB).

Nuclear Run-On Transcription Assay. Nuclei were prepared from TNF-α-treated and untreated 818-4 cells as described (21). The run-on transcription and subsequent hybridization reactions were done according to Greenberg and Ziff (22). ^{32}P-labeled transcripts were hybridized to denatured DNA probes immobilized on Hybond-N membranes (Amersham) by slot blotting (Minifold II; Schleicher & Schüll, Dassel, F.R.G.). Band intensities of autoradiographs were quantitated by densitometric scanning with an Ultroscan laser densitometer (Pharmacia LKB).

[^{35}S]Methionine Labeling of Cells and Immunoprecipitation of EGFR. Tumor cells (818-4) at 80–90% confluence were metabolically labeled and solubilized as described (23). Amounts of soluble extracts corresponding to 10^6 cpm were immunoprecipitated with an anti-EGFR polyclonal antiserum (kindly donated by W. Weber, University Hospital, Hamburg, F.R.G.) or, as controls, with normal rabbit serum. The immunoprecipitates were subjected to denaturing PAGE, the dried gels were autoradiographed, and the bands were subsequently assayed for radioactivity in a scintillation counter.

TGF-α Bioassay. A clonogenic growth assay using TGF-α-dependent normal rat kidney cells was performed as described (24). Units of EGF equivalents/ml of conditioned medium were calculated from standard reference curves obtained by using various concentrations of EGF. TGF-α/EGF activity was assayed in the presence of 2 ng of TGF-β (R & D Systems, Minneapolis) per plate. The specificity of the observed effects was proven by competition of the TGF-α activity with anti-TGF-α antibodies at 20 μg/ml.

RESULTS

TNF-α-Induced Upregulation of EGFR Protein. Binding studies using EGFR-specific mAb 425 showed that TNF-α increased the constitutive expression of EGFR protein in six out of six pancreatic cancer cell lines by 1.6- to 4.6-fold without apparent change in affinity in five of six cell lines (Table 1). These results were confirmed when specific binding of ^{125}I-labeled EGF to 818-4 cells was analyzed by Scatchard plot (results not shown). Because TNF-α-mediated upregulation of EGFR was most significant in 818-4 cells—i.e., 4.6-fold when assessed by binding of ^{125}I-labeled mAb 425 (Table 1) and 7.5-fold when determined by binding of ^{125}I-labeled EGF (data not shown)—we focused on these cells to characterize TNF-α-dependent effects in greater detail. Consistent with the results of binding assays, more EGFR protein was immunoprecipitated upon TNF-α exposure of 818-4 cells, and autoradiographs (Fig. 1) showed higher levels of the nascent (160-kDa) and glycosylated mature (170-kDa) EGFR molecule in TNF-α-treated vs. untreated 818-4 cells or 818-4 cells treated with human recombinant IFN-γ.

Table 1. Analysis of TNF-α inducible EGFR expression by pancreatic carcinoma cells

Cell line	K_a, nM^{-1}		EGFRs per cell: No. × 10^{-5}		Fold increase
	−	+	−	+	
818-1	13	8	1.3	3.0	2.2
818-4	13	13	1.2	5.7	4.6
818-7	9	7	1.4	3.8	2.7
Capan-2	8	7	0.4	0.8	2.0
HPAF	8	7	0.3	0.5	1.7
SW850	8	6	0.9	1.5	1.6

Cells were cultured in the absence (−) or presence (+) of TNF-α (1000 units/ml, 48 hr). EGFR expression was assessed by radioligand binding assay using EGFR-specific mAb 425.

Effects of TNF-α on EGFR mRNA Expression. Upregulation of steady-state levels of EGFR mRNA by TNF-α (1000 units/ml, 48 hr) was evident in all six pancreatic carcinoma cell lines (Fig. 2). The cytokine-induced increase in EGFR mRNA occurred in a dose-dependent manner and was selective for the EGFR because TNF-α treatment did not affect steady-state levels of mRNA encoding other receptor tyrosine kinases such as HER-2/neu, which is structurally related to the EGFR (25); the insulin-like growth factor I receptor; or the PDGF receptor (data not shown). Run-on transcription analysis of nuclei from TNF-α-treated and untreated 818-4 cells revealed a transcriptional activation of the EGFR gene after TNF-α treatment, resulting in a 3.8-fold increase over the constitutive transcription level after 16 hr of TNF-α exposure (Fig. 3). Moreover, steady-state levels of EGFR transcripts (analyzed by a 4-hr actinomycin D treatment after TNF-α exposure) remained nearly unchanged, in contrast to untreated cells indicating an mRNA-stabilizing effect achieved by TNF-α (data not shown).

Signal Transduction Pathways in TNF-α-Stimulated EGFR Synthesis. TNF-α treatment of pancreatic carcinoma cells induced not only EGFR mRNA levels but also expression of a number of growth-regulatory proteins, including IL-1β, PDGF-B, and TGF-α. We addressed a possible role of TGF-α in upregulation of EGFR expression because TGF-α has been found to induce expression of EGFR in various cell types (8), and TGF-α mRNA expression was induced by TNF-α in all cell lines that were examined for TNF-α-dependent EGFR upregulation. Furthermore, cycloheximide treatment abrogated the effect of TNF-α on EGFR transcription, suggesting a role of protein mediators in the signal transduction pathway (Fig. 4). In contrast, cycloheximide treatment alone drastically upregulated TGF-α mRNA levels, whereas a combination of cycloheximide with TNF-α, as described previously for PDGF-A-chain regulation (17), reduced this effect.

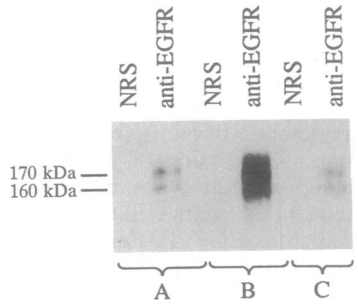

FIG. 1. SDS/PAGE of immunoprecipitated EGFRs. Cells (818-4) were untreated (lanes A), or treated for 24 hr with TNF-α (1000 units/ml) (lanes B) or IFN-γ (10 units/ml) (lanes C) and then metabolically labeled with [^{35}S]methionine. Soluble extracts were immunoprecipitated with normal rabbit serum (NRS) or specific anti-EGFR antibodies and subjected to denaturing SDS/PAGE with subsequent autoradiography. Molecular size determination was performed by using ^{14}C-labeled molecular weight markers (Amersham).

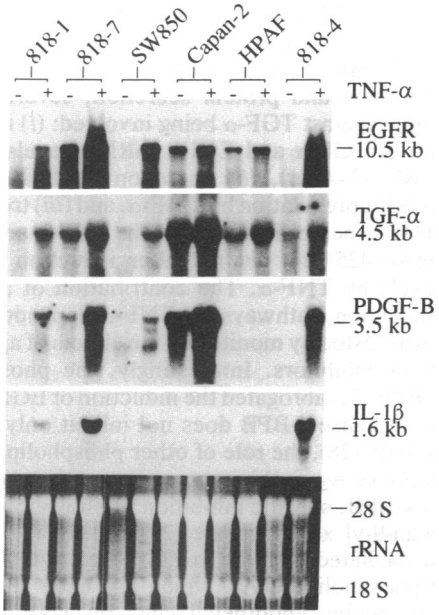

FIG. 2. Effect of TNF-α treatment (1000 units/ml, 48 hr) on the mRNA levels for several growth factors and receptors, as indicated, in six pancreatic cancer cell lines. Northern blot analysis was performed as described in *Materials and Methods*. Ethidium bromide staining of the agarose gel (*Bottom*) served as control for equal loading of the gel slots and quality of the RNA samples.

Role of TGF-α in TNF-α-Mediated Induction of EGFR mRNA. To test whether TNF-α also induced TGF-α protein synthesis, TGF-α activity was assessed in anchorage-independent growth assays (24). Colony formation by normal rat kidney cells was increased significantly in the presence of supernatants of 818-4 cells treated with TNF-α for 24 hr and 48 hr (0.3 and 1.2 ng EGF equivalents/ml, respectively; $P < 0.001$). The specificity of the mitogenic effects for TGF-α rather than other TNF-inducible mitogens, such as PDGF (17), was confirmed by use of a TGF-α-neutralizing mAb (Ab3), which reduced the TNF-α-dependent maximal mitogenic activities detected in 818-4 supernatants by 73%. To assess whether secreted TGF-α mediated the effects of TNF-α on EGFR expression, we determined the effect of TGF-α-neutralizing mAb on EGFR mRNA levels in TNF-α-treated 818-4 cells. The mAb effectively inhibited induction of EGFR mRNA by exogenous TGF-α (20 nM) but failed when given together with TNF-α to attenuate the TNF-α-induced EGFR mRNA expression in 818-4 cells (Fig. 5). Similar effects were observed for TNF-α-induced TGF-α mRNA. In addition, the EGFR-specific mAb 425 did not block the TNF-α-mediated generation of EGFR mRNA.

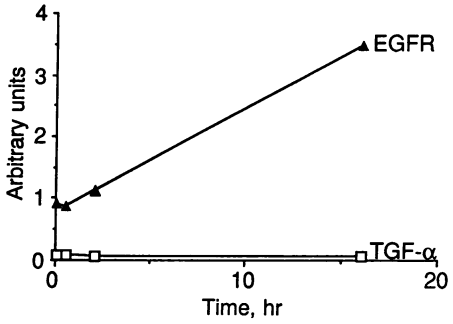

FIG. 3. Run-on transcription analysis of 818-4 cells treated with TNF-α for various times. Autoradiographic signals after hybridization of *in vitro* transcripts to EGFR- and TGF-α-specific DNA probes were evaluated by laser densitometric scanning.

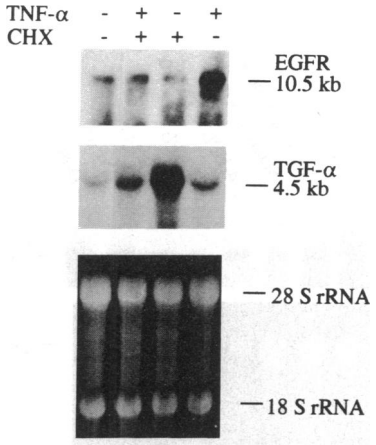

FIG. 4. Northern blot analysis of total RNA from 818-4 cells untreated or treated for 2 hr with TNF-α (1000 units/ml) plus cycloheximide (CHX, 5 μg/ml) or either drug alone. The hybridized DNA probes are indicated with their corresponding transcript sizes. Ethidium bromide staining of the agarose gel (*Bottom*) served as loading control.

Furthermore, time-course experiments revealed a coincidental rather than a subsequent increase of EGFR and TGF-α transcripts starting 2 hr after TNF-α exposure of 818-4 cells (data not shown). Taken together, these results do not support a significant role for endogenous, secreted TGF-α in TNF-α-dependent upregulation of EGFR expression in 818-4 cells.

Second Messengers. TNF-α activates protein kinase A- and/or protein kinase C-dependent signal transduction pathways, as well as phospholipases A_2 and C, in several cell systems (for review see ref. 26). Neither the protein kinase A activators forskolin (50 μM) and dibutyryl-cAMP (1 mM), nor pertussis toxin (100 ng/ml) as G_i inhibitor, nor the protein kinase A inhibitor HA-1004 (250 μM) had any effect on the constitutive or TNF-α-stimulated EGFR mRNA expression of 818-4 cells (data not shown). TNF-α-induced EGFR mRNA levels were only moderately reduced by staurosporine treatment (≈30% as assessed by densitometric scanning of autoradiographs) (Fig. 6), which inhibits protein kinase C

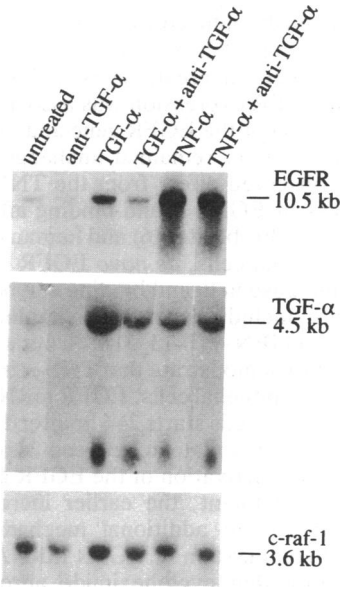

FIG. 5. Northern blot analysis of EGFR and TGF-α transcript levels in 818-4 cells, treated with TNF-α, TGF-α, and neutralizing anti-TGF-α mAb as indicated. Equal loading of the gel was determined by reprobing with c-*raf-1* (*Bottom*) as a housekeeping gene.

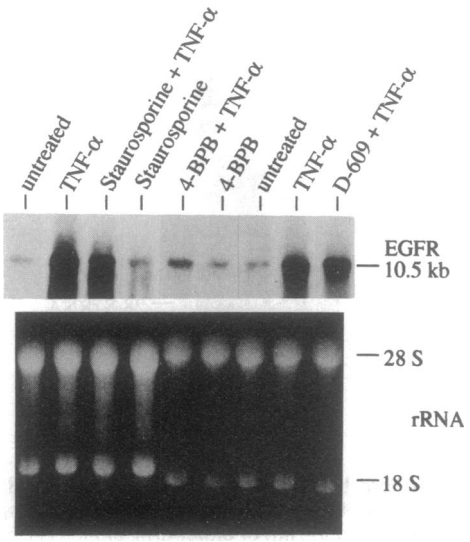

FIG. 6. Effects of TNF-α treatment on EGFR mRNA expression in 818-4 cells under protein kinase C or phospholipase inhibition. Cells were treated with TNF-α (1000 units/ml, 14 hr) in combination with the protein kinase C inhibitor staurosporine (150 nM, 14 hr) or either drug alone as indicated. The effects of the phospholipase inhibitor 4-BPB (50 μM), and the phospholipase C-specific inhibitor tricyclodecan-9-yl xanthogenate (D-609, 30 μg/ml) were also tested. Ethidium bromide staining (*Bottom*) served as loading control.

among other protein kinases (27), indicating a limited role for kinase C in TNF-α-dependent effects on EGFR mRNA levels.

Marked inhibition of EGFR mRNA upregulation by TNF-α was achieved with the phospholipase inhibitor 4-BPB (50 μM), which has been shown to inhibit phospholipases A_2 and C (28) (Fig. 6). However, the phospholipase C-specific inhibitor tricyclodecan-9-yl xanthogenate (30 μg/ml) (29) did not affect TNF-α-dependent EGFR mRNA upregulation, suggesting that inhibition of phospholipase A_2 accounts for the inhibitory effect of 4-BPB.

DISCUSSION

Treatment of cultured pancreatic carcinoma cells with TNF-α simultaneously induced expression of EGFR and its ligand TGF-α in all six cell lines tested, suggesting a common response of malignant pancreatic cells to TNF-α exposure. TNF-α-induced EGFR expression was associated with transcriptional activation of the EGFR gene and *de novo* protein synthesis. EGFR affinity remained unchanged, clearly distinguishing the observed effect from the TNF-α-dependent transient changes of EGFR ligand-binding affinity reported for human gingival fibroblasts (16) and human carcinoma cell lines (30). In epithelial cells, *de novo* EGFR synthesis associated with an increase in ligand binding can be induced by a variety of agents, including estrogen, vitamins, EGF itself, TGF-β, IFN-α, and IFN-γ (7–13, 31). To our knowledge, this is the first report to demonstrate that TNF-α induces such an effect in malignant epithelial cells. EGFR mRNA induction in pancreatic carcinoma cells starts 2–4 hr after TNF-α addition and increases to reach a plateau between 24 and 48 hr. Yet, since transcriptional activation of the EGFR gene peaked 16 hr after TNF-α treatment, the earlier increase of EGFR mRNA may be due to additional mechanisms, such as TNF-α-induced stabilization of EGFR mRNA.

The observation that cycloheximide abrogated TNF-α-dependent EGFR mRNA accumulation in 818-4 cells indicates that protein mediators participate in this process, as described for EGFR induction by estrogen in rat uterine membranes (7).

Induction of EGFR protein synthesis by its ligands EGF and TGF-α has been described previously in different cell systems (8–11). However, although TNF-α induces TGF-α mRNA expression and protein secretion, several lines of evidence argue against TGF-α being involved: (*i*) induction/upregulation of TGF-α and EGFR mRNA levels occurred simultaneously (2–4 hr), (*ii*) secretion of TGF-α protein followed EGFR upregulation by TNF-α, and (*iii*) treatment of cells with TGF-α-neutralizing Ab3 or the TGF-α-antagonistic antibody mAb 425 (24) did not alter induction of EGFR message levels by TNF-α. The contribution of alternative signal transduction pathways to TNF-α-dependent EGFR regulation was tested by monitoring the effects of appropriate stimulators or inhibitors. Interestingly, the phospholipase inhibitor 4-BPB (32) abrogated the induction of EGFR mRNA expression. Because 4-BPB does not inhibit only phospholipase C activity (28), the role of other phospholipases, such as phospholipase A_2, in this process cannot be excluded. In contrast to 4-BPB, specific inhibition of phospholipase C by tricyclodecan-9-yl xanthogenate (29) did not interfere with the TNF-α-mediated increase in EGFR mRNA. This favors a role for phospholipase A_2, which has been shown to be activated in bovine endothelial cells by TNF-α-mediated process (33). Preliminary data suggest that 55-kDa TNF receptors are involved in this phospholipase A_2-dependent signaling of TNF-α-induced EGFR mRNA upregulation (34).

The induction by TNF-α of a potential EGFR-dependent autocrine cycle in pancreatic carcinoma cells presents one of the most salient and challenging findings of this study. Palombella *et al.* (15) have speculated that TNF-α-induced expression of the EGFR on human diploid fibroblasts may be related to stimulation of fibroblast growth by TNF-α. Yet TNF-α (at the concentrations required to induce EGFR and TGF-α expression of pancreatic carcinoma cells) inhibits growth of these cells in culture (35, 36). This is not entirely unexpected, since the induction of growth factors does not necessarily imply growth promotion but may result, as shown recently, also for TNF itself in cell differentiation and negative growth control (37). Indeed, it has been pointed out that growth inhibition in tumor cells occurs at a high number of expressed EGFRs and at high concentrations of the ligand (38, 39), an observation also made with 818-4 pancreatic carcinoma cells (unpublished results).

While further studies are required to address the biological role of EGFR overexpression in pancreatic cancer cells as a function of cytokine treatment, the TNF-α-mediated upregulation of EGFR on tumor cells also attracts clinical interest. Growth factor receptors are widely used targets for antitumor therapy with mAbs (for review see ref. 40). High target-antigen density on the tumor cell membrane has been shown to be a critical determinant for a successful antibody approach (19). Further studies will show what impact the data presented in this study have on immunotherapeutical concepts for EGFR-expressing tumors.

We thank Dr. G. A. Luckenbach for providing the anti-EGFR antibody (mAb 425/EMD55900) and for helpful discussion. The excellent technical assistance of Anna Bossler, Jutta Gieseking, and Inge Humburg is highly appreciated. This work was supported by Duetsche Krebshilfe (Grant M52/KL1), by the Bundesministerium für Forschung und Technologie (Grant DLR 01GA08805/6), and by a grant from the W. W. Smith Foundation.

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