Pergamon

European Journal of Cancer Vol. 31A, No. 12, pp. 2067–2072, 1995
Elsevier Science Ltd
Printed in Great Britain
0959–8049/95 $9.50+0.00

0959-8049(95)00422-X

Original Paper

Cytotoxicity of TGFα–PE40 and Correlation to Expression of Epidermal Growth Factor Receptor

D.P. Berger,[1] L. Herbstritt,[1] U. Hellerich,[2] S. Schulzke,[1] W.A. Dengler,[1] M. Gallo,[3] I. Pastan,[3] R. Mertelsmann[1] and H.H. Fiebig[1]

[1]Department of Internal Medicine I, Haematology/Oncology, University of Freiburg, Germany; [2]Institute of Pathology, University of Freiburg, Germany; and [3]National Cancer Institute, Bethesda, Maryland, U.S.A.

TGFα–PE40 is a chimeric protein composed of transforming growth factor alpha (TGFα) linked to a modified *Pseudomonas* exotoxin (PE40). We tested the *in vitro* cytotoxicity of TGFα–PE40 on 23 different solid human tumour xenografts established in nude mice and human bone marrow cells from healthy donors, utilising a modified clonogenic assay. In order to distinguish non-specific toxicity from the targeted effects of TGFα–PE40, epidermal growth factor receptor (EGFR) expression of the tumours studied was assessed by Northern blot, slot blot and immunohistochemistry. TGFα–PE40 demonstrated differential cytotoxicity on human tumour xenografts in the clonogenic assay. No toxicity on human bone marrow cells was observed. *In vitro* activity of TGFα–PE40 showed a significant correlation with the expression of EGF receptors as determined by immunohistochemistry and slot blot. Further studies will be performed in order to determine the *in vivo* activity of this compound in tumour-bearing nude mice.

Key words: growth factors, TGFα, TGFα–PE40, recombinant toxins, nude mice, xenografts, clonogenic assay
Eur J Cancer, Vol. 31A, No. 12, pp. 2067–2072, 1995

INTRODUCTION

NATURAL TOXINS produced by bacteria or plants, such as Pseudomonas exotoxin, diphtheria toxin and ricin, have demonstrated a highly cytotoxic effect in mammalian cells and may be useful as possible therapeutic agents. In order to utilise the cytotoxic potential of natural toxins in cancer therapy, the molecules have to be selectively targeted to malignant cells. Initially, immunotoxins consisting of monoclonal antibodies chemically coupled to different cytotoxic molecules were developed, designed to bind specific antigens on the cell surface [1]. In recent years, recombinant toxins have been formed using genetic engineering techniques. Different receptor binding proteins such as transforming growth factor alpha (TGFα), basic and acidic fibroblast growth factor, insulin-like growth factor-1, interleukin-2, interleukin-4, interleukin-6, CD4 and binding portions of selected antibodies have been used to target toxins to malignant cells or cells infected with the human immunodeficiency virus type 1 [2, 3].

Correspondence to D.P. Berger at the Department of Internal Medicine I, Haematology/Oncology, University of Freiburg Medical Center, Hugstetterstr. 55, D-79106 Freiburg, Germany.
Revised 27 Apr. 1995; accepted 12 May 1995.

Pseudomonas exotoxin A (PE) is a bacterial toxin from *Pseudomonas aeruginosa* that kills mammalian cells by inactivating protein synthesis. After binding to α_2-macroglobulin receptors, the toxin is internalised and processed in several intracellular steps to produce an active 37 kDa C-terminal fragment, which ADP-ribosylates elongation factor 2. PE40 consists of a 40 kDa fragment of PE lacking the cell recognition domain [4].

TGFα is a 50 amino acid polypeptide with mitogenic properties in a variety of vertebrate cells. It shows considerable sequence homology with epidermal growth factor (EGF) and exerts its biological effects by binding to the EGF receptor (EGFR) [5]. EGFR expression, frequently accompanied by a parallel increase in TGFα expression, has been demonstrated in various human tumours and cell lines, including cancers of the head and neck, lung, stomach, breast, ovary, uterine cervix, kidney and bladder [6–9]. High levels of EGFR predict a poor clinical prognosis in squamous cell lung cancer, bladder cancer and mammary adenocarcinoma [10, 11].

The recombinant toxin TGFα–PE40 has been generated by fusion of a complementary DNA encoding TGFα to the *PE40* gene. The chimeric gene was expressed in *E. coli* and the chimeric protein has been purified to near homogeneity. The combination of cell killing and cell targeting properties of

TGFα–PE40 suggests a possible role as a selective antineoplastic agent. In various *in vitro* models, the compound has demonstrated a clear cytotoxic potential in mammalian tumour cell lines expressing epidermal growth factor receptors [12]. Recently, Pai and coworkers demonstrated *in vivo* efficacy of TGFα–PE40 in nude mice bearing subcutaneous (s.c.) tumour xenografts of A431, a human epidermoid carcinoma, and DU145, a prostate carcinoma. For both tumour lines, a high level of EGFR expression was demonstrated [13].

To complement the observations in selected cell lines, experiments in highly predictive models of human disease are necessary. Here we report on the antineoplastic effect of TGFα–PE40 in human tumour xenografts established in athymic nude mice, demonstrating the cytotoxic activity of the compound in model systems of slowly growing malignancies with close resemblance to patient tumours. All tumours were extensively characterised for growth pattern, histology and chemosensitivity both *in vitro* and *in vivo* [14]. In order to distinguish non-specific toxicity from targeted effects of TGFα–PE40, EGFR expression of the tumours was assessed by Northern blot, slot blot and immunohistochemistry.

MATERIALS AND METHODS

Human tumours and nude mice

Human tumour xenografts established in serial passage in athymic nude mice were used as tumour material. Athymic nude mice (outbred NMRI nu/nu strain) grown in our own breeding facilities were used. The animals were housed under specific pathogen-free conditions with free access to food and acidified water. Room temperature was controlled at 24 ± 1°C, relative humidity was above 70%. The human origin of the tumours was confirmed by isoenzymatic and immunohistochemical methods. All tumours were derived from different patients. Characterisation of these models has been described previously, including patient data, histology, growth behaviour, chemosensitivity to standard anticancer drugs *in vitro* and *in vivo*, isoenzyme phenotype analysis, hormone receptor analysis, surface markers, and DNA histogram as generated by flow cytometry [14, 15].

Drug

TGFα–PE40 was developed and synthesised by Ira Pastan and coworkers (Bethesda, Maryland, U.S.A.) [16, 17]. TGFα–PE40 stock solution was protected from light and stored at −80°C for a maximum of 6 months. The protein was diluted with 0.2% human serum albumin, and further dilution steps were performed with saline. Final solutions were prepared freshly for each experiment.

Clonogenic assay

In vitro activity of TGFα–PE40 was studied in a modification of the soft agar culture system introduced by Hamburger and Salmon [18]. The methodology applied has been described previously [19]. Briefly, solid human tumour xenografts were mechanically and enzymatically disaggregated to a single cell suspension. Human bone marrow cells were aspirated from the iliac crest of healthy volunteers, mononuclear cells with a density of less than 1.007 g/ml were separated by density centrifugation in Ficoll paque. Culture plates consisted of 35 mm Petri dishes with two layers of soft agar. The bottom layer contained culture medium and agar only, for the top layer $0.2–1 \times 10^6$ tumour or bone marrow cells were added. Cytostatic drugs were applied by continuous exposure in 1.0 ml of medium on to the top layer (drug overlay). In each assay, six control plates received the vehicle only, drug-treated groups (TGFα–PE40 at concentrations of 0.1, 0.3, 1, 3 and 10 ng/ml) were plated in triplicate. Cultures were incubated at 37°C and 7% CO_2 in a humidified atmosphere for 6–21 days and monitored closely for colony growth. Within this period, *in vitro* tumour growth led to the formation of colonies with a diameter of >60 μm. At the time of maximum colony formation (after 5–12 days of incubation), counts were performed with an automatic image analysis system (Bausch & Lomb Omnicon FAS IV). Drug effects were expressed in terms of percentage survival. A compound was considered as cytotoxic at a given concentration, if the mean number of colonies in treated plates was reduced to <30% of the mean colony count of untreated controls. Individual IC_{70} values (inhibitory concentration reducing colony formation by 70%) were calculated from dose–response curves of each single tumour line. The overall median IC_{70} was calculated for all xenografts studied. A xenograft was considered to be responsive to TGFα–PE40 *in vitro* if the individual IC_{70} of the tumour line was equal to or lower than the median IC_{70}.

RNA isolation and blotting

Total cellular RNA was prepared according to the guanidium isothiocyanate method. Briefly, human solid tumour xenografts were dissected under sterile conditions, and necrotic tissue was removed. Vital tissue specimens were frozen in liquid nitrogen and homogenised. The samples were lysed in 4 M guanidium isothiocyanate, followed by ultracentrifugation through a caesium chloride cushion for 20 h. Pelleted RNA was recovered in ethanol, resuspended and extracted with phenol and chloroform: isoamylalcohol. RNA was stored in sodium acetate/ethanol, the concentration was measured photometrically using the optical density at 260 and 280 nm [20].

For Northern blot analysis, total RNAs (10 μg each) were denatured at 65°C in morpholinopropanesulphonic acid, formaldehyde, formamide and ethidium bromide, and loaded on to 1.25% agarose gels containing formaldehyde and morpholinopropanesulphonic acid. The gels were run in morpholinopropanesulphonic acid buffer at 70 V for 3–4 h and transferred to nitrocellulose membranes by capillary blotting overnight. For RNA fixation, the membranes were dried at 80°C for 2 h. Slot blot analysis was performed as described previously [20]. Total RNAs (5 μg) were denatured at 65°C in formamide, formaldehyde and 20× standard saline citrate (SSC) and applied to nitrocellulose membranes using a slot blot apparatus.

Probes, labelling and hybridisation

EGFR mRNA expression was studied using the probe pE7 (American Type Culture Collection, Rockville, Maryland, U.S.A.). This consists of an internal cDNA fragment covering bases 600–3000 approximately of the EGFR mRNA, coding for approximately 709 amino acids (2.4 kb insert). For quantification of the RNA amount loaded in individual lanes, expression of the housekeeping gene *GAPDH* was assessed using a 1.12 kb *GAPDH*–cDNA fragment.

Nitrocellulose filters were prehybridised for 2–4 hours at 65°C with 2 × SSC, 10 × Denhardt's solution, 2.5% dextrane sulphate, 0.1% SDS, 0.1% Na_2PO_4, 2 mM EDTA and 30 μg/ml salmon sperm DNA followed by an overnight incubation at 65°C with a random prime labelled 2.2 kb ClaI EGFR–cDNA fragment of pE7. The filters were washed 2 × 15 min with 2 × SSC, 0.1% SDS, 0.1% Na_2PO_4, 2 mM EDTA, followed by 1 × SSC, 0.1% SDS, 2 mM EDTA and 0.4 × SSC, 0.1% SDS, 2 mM EDTA at 65°C. Subsequently, blots were dried and

exposed to X-ray sensitive films at −70°C for 1–3 weeks. The filters were stripped and rehybridised with the *GAPDH* probe.

Evaluation of mRNA analysis

Specificity of the DNA probes was determined by analysis of Northern blots. For EGFR-positive xenografts, the characteristic 10 kb EGFR transcript was observed, some tumours additionally showed one or two smaller transcripts. As positive controls, the prostate cancer line DU145 and the squamous cell carcinoma line A431 (data not shown) were used. For both tumours, clear expression of EGFR was observed.

Quantitative evaluation of slot blot autoradiograms was performed by laser scanning densitometry. The positive control tumour DU145 was used as 100% standard, expression levels of all other specimens were calculated relative to the positive control.

Immunohistochemistry

Representative tissue sections were embedded in paraffin. Serial sections of 4 μm thickness were stained according to the ABC method (avidin–biotin complex) [21], using the Vectastain® system (Camon, Wiesbaden, Germany). Briefly, after dewaxing and rehydration, slides were placed in absolute ethanol. Endogenous peroxidase activity was blocked with 70% methanol and 33% H_2O_2 (v/v 100/1). The primary antibody (polyclonal rabbit antibody, anti-EGFR Ab-4, Oncogene Science, Manhasset, New York, U.S.A.), directed against the intracellular domain of the EGF receptor, was prepared by dilution in 0.1% bovine serum albumin in phosphate buffered saline (PBS) at a ratio of 1:20, found to stain the antigen adequately. After application of the primary EGFR antibody, slides were incubated with the biotinylated second antibody (goat-antirabbit) for 60 min. ABC was applied for 60 min at room temperature. Finally, AEC (aminoethylcarbazole) was added for enzyme staining. All slides were counterstained with Mayers haematoxylin for 5 min and coverslipped with water-soluble mounting medium. As a positive control, the human prostatic carcinoma cell line DU145 was used (American Type Culture Collection, Rockville, Maryland, U.S.A.) As a negative control, human bone marrow specimens from healthy donors were embedded in paraffin and prepared as above.

Each slide was evaluated by two of us (S.S., U.H.) based on the following criteria: −, no staining; +, moderate staining (10–50% of tumour cells positive) and minimum background staining; ++, maximum staining (>50% of tumour cells positive) with no background staining.

Correlation and statistics

Clonogenic assay results were entered into a database and evaluated using specifically designed software. For comparison of clonogenic assay and immunohistochemistry, results were dichotomised. A tumour was considered to be responsive to TGFα–PE40 *in vitro* if the individual IC_{70} was equal to or lower than the median IC_{70}. In immunohistochemistry, at least moderate staining with minimum background staining was required for expression of EGFRs. Statistical significance was assessed by use of the χ^2-test (modified according to Yates for $20 \leq n < 60$). Clonogenic assay results and slot blot data were compared by use of the Wilcoxon test. Statistical significance was assumed to be $P < 0.05$.

RESULTS

Clonogenic assay

The *in vitro* cytotoxicity of TGFα–PE40 was studied using a panel of solid human tumour xenografts of different histological entities established in nude mice and human bone marrow cells (CFU-GM) from two different healthy donors (Table 1). Drug concentrations ranged between 0.1 and 10 ng/ml with continuous exposure. In human tumour xenografts, a clear dose–response relationship was observed. At concentrations of 0.1, 0.3, 1, 3 and 10 ng/ml, the IC_{70} was reached in 0% (n = 0/23), 9% (2/23), 30% (7/23), 39% (9/23) and 65% (15/23) of the tumours studied, respectively. In contrast, colony growth of human bone marrow progenitor cells (CFU-GM) was not affected by TGFα–PE40.

Further analysis of the IC_{70} values demonstrated a median IC_{70} of 3.0 ng/ml. IC_{70} values <3.0 ng/ml indicating responsiveness were observed for a variety of tumour lines of different histology (Table 1). Differential cytotoxicity was demonstrated, with activity in gastric cancer (one of the three xenografts studied), non-small cell (2/4) and small cell lung carcinomas (1/3) as well as in tumours of the breast (1/3), ovary and kidney, in a soft tissue sarcoma and in the positive control prostate tumour DU145. Colorectal tumours (n = 3) as well as melanomas (n = 2) and a testicular cancer xenograft were not responsive.

Expression of EGFR

Analysis of EGFR expression was performed at the mRNA and the protein level, using Northern as well as slot blot technique and immunohistochemistry, respectively.

Expression of EGFR mRNA. The specificity of the EGFR–cDNA used was determined by Northern hybridisation. Examples of EGFR expression demonstrating the characteristic 10 kb transcript in gastrointestinal tumours are given in Figure 1. Only few tumour lines demonstrated an additional 4 kb transcript (GXF 251 and DU 145).

Quantitative evaluation of EGFR mRNA expression was performed using the slot blot technique. EGFR mRNA signals were observed in 13/19 tumours studied (Table 1). Positive or highly positive tumours were found for all histiotypes studied. The positive control tumour line, DU145, was highly positive for EGFR mRNA, whereas human bone marrow cells did not express the message for EGFRs.

Immunohistochemistry. Immunohistochemical analysis revealed a similar expression pattern (Table 1). Twelve of the 21 xenografts studied showed expression of the EGFR protein, as defined by at least moderate staining with minimum background staining. Staining was membrane-associated and intracellular (cytoplasmic) in all cases. Adenocarcinomas showed typical fine granular staining of membranes and submembranous cytoplasm. The distribution of positive cells appeared heterogeneous, with focal or diffuse staining patterns. Two xenografts showed maximum staining without background staining, i.e. the gastric carcinoma line GXF 209 and the positive control prostatic carcinoma DU145. A variety of xenografts showed moderate staining with minimum background, i.e. tumours of the colon, stomach, lung, breast, ovary, kidney and melanoma. Human bone marrow specimens were negative for EGFR. Three tumours (colon carcinoma CXF 886, gastric cancer GXF 97 and breast cancer MAXF 401) showed EGFR mRNA expression in Northern and slot blots, but immunohistochemical staining for EGFR protein was negative.

Table 1. *Comparison of* in vitro *colony inhibition by TGFα–PE40* (IC_{70} *values) and expression of EGFR as detected by immunohistochemistry and slot blot in human tumour xenografts*

Tissue	Histology	TGFα–PE40 IC_{70} (ng/ml)	EGFR expression IHC*	EGFR expression Slot blot (%)†
HBM No. 1	Human bone marrow, donor 1	>10.0‡	−	0
HBM No. 2	Human bone marrow, donor 2	>10.0‡	−	0
CXF 280	Colorectal cancer	13.8	−	0
CXF 886	Colorectal cancer	>10.0‡	−	76
CXF 1103	Colorectal cancer	>10.0‡	+	24
GXF 97	Gastric cancer	13.8	−	27
GXF 209	Gastric cancer	3.0	++	116
GXF 251	Gastric cancer	1.2	+	>100
LXFA 629	Lung cancer, adenocarcinoma	0.2	+	nd
LXFE 409	Lung cancer, squamous cell carcinoma	6.5	−	0
LXFL 529	Lung cancer, large cell carcinoma	0.1	+	43
LXFL 1029	Lung cancer, large cell carcinoma	6.8	−	0
LXFS 538	Lung cancer, small cell carcinoma	0.4	+	78
LXFS 605	Lung cancer, small cell carcinoma	4.7	−	0
LXFS 650	Lung cancer, small cell carcinoma	17.8	−	0
MAXF 401	Breast cancer	11.8	−	116
MAXF 1162	Breast cancer	>10.0‡	+	27
MAXF 1322	Breast cancer	0.9	+	nd
MEXF 276	Melanoma	>10.0‡	−	0
MEXF 514	Melanoma	5.6	+	14
OVXF 899	Ovarian cancer	0.9	+	22
RXF 1220	Renal cancer	1.3	+	41
SXF 587	Soft tissue sarcoma	0.8	nd	nd
TXF 593	Testicular cancer	7.5	nd	nd
DU 145	Prostate cancer	0.7	++	100

* As determined by immunohistochemistry: −, no staining; +, moderate staining and minimum background staining; ++, maximum staining with no background staining; † as compared to positive control prostate cancer cell line DU 145 (expression level 100%); ‡ IC_{70} was not reached at the concentrations used. The highest concentration studied *in vitro* is given (10 ng/ml). Tumour line was not included in the calculation of median IC_{70}. nd, not done.

Correlation of EGFR expression to TGFα–PE40 efficacy in vitro. A clear relationship between EGFR protein expression and sensitivity to TGFα–PE40 was observed. In 12/21 tumours, expression of EGFR was demonstrated by immunohistochemical staining with a polyclonal antibody and nine of these tumours were considered sensitive (IC_{70} < 3.0 ng/ml) to TGFα–PE40 *in vitro*. Nine other tumours did not show EGFR expression, and all were resistant in the clonogenic assay (IC_{70} > 3.0 ng/ml). This difference was statistically significant ($P = 0.003$, χ^2-test modified according to Yates, $n = 21$). A comparison of TGFα–PE40 cytotoxicity and quantitative EGFR mRNA expression as determined by slot blot analysis also revealed a significant correlation ($P = 0.007$, Wilcoxon test).

DISCUSSION

In this study, we demonstrated cytotoxic activity of the recombinant toxin TGFα–PE40 in highly predictive models of slow growing human solid tumours. The efficacy of TGFα–PE40 was evaluated utilising a modified clonogenic assay with human tumour xenografts established in serial passage in athymic nude mice. The compound displayed differential cytotoxicity and a clear dose–response relationship at concentrations between 0.1 and 10 ng/ml. Colony formation of non-small cell lung cancer, breast and ovarian cancer specimens as well as one soft tissue sarcoma and the positive control prostate carcinoma DU145 was reduced significantly with IC_{70} values below 3.0 ng/ml (continuous exposure). In contrast, growth of human bone marrow cells from two different healthy donors was not affected by TGFα–PE40, even at the high concentration of 10 ng/ml. Similar cytotoxicity profiles have been observed by Von Hoff and associates [22], who studied 107 patient tumour specimens in a human tumour colony-forming assay for sensitivity to TP40, a similar recombinant toxin consisting of the entire TGFα protein fused to a 40 kDa segment of the *Pseudomonas* exotoxin A. Sensitive tumour types included breast, colorectal, endometrial, head and neck, non-small cell lung, gastric and pancreatic as well as sarcoma. Other authors demonstrated activity of the drug in protein synthesis inhibition assays using clonal tumour cell lines [17].

Various groups demonstrated activity of TGFα–PE40 in selected cell lines expressing high levels of EGFRs, for example, DU145 prostate carcinoma, A431 squamous cell carcinoma or HT 29 colorectal cancer, both in proliferation inhibition assays *in vitro* as well as in nude mice after subcutaneous injection of the cell lines *in vivo* [12, 13]. In a complementary analysis, we evaluated the compound in models of slow growing human solid tumours. In contrast to cell lines cultivated *in vitro*, human tumour xenografts established in athymic nude mice *in vivo* retain the characteristics of the original patient's neoplasms to a greater extent. These include tumour heterogeneity, growth factor expression and vascularisation pattern [14, 15]. In order to distinguish between non-specific, merely cytotoxic effects and

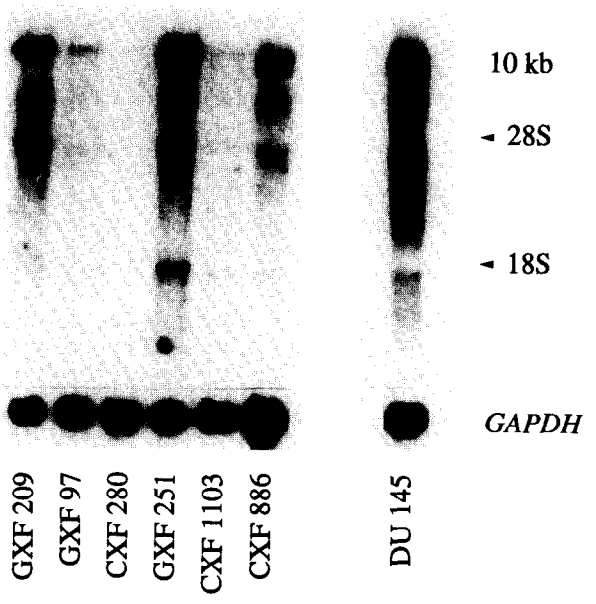

Figure 1. Northern blotting. Total RNA (10 μg) from different gastrointestinal tumour xenografts and the positive control prostatic carcinoma DU145. Hybridisation with a random prime labelled 2.2 kb ClaI EGFR–cDNA fragment of pE7. Autoradiography demonstrates the characteristic 10 kb EGFR transcript. To ensure uniform RNA loading and transfer from the gel to nitrocellulose filters, each blot was rehybridised to a human *GAPDH* probe.

selective cell kill mediated by TGFα directed drug targeting, the expression of EGFRs was assessed in the tumour lines studied.

Twenty-one of the 23 tumours as well as human bone marrow specimens were analysed for EGFR mRNA expression by Northern and slot blot hybridisation. The expression pattern observed was in accordance with the literature [5–9]. The characteristic 10 kb EGFR transcript was detected in 13 of the 21 xenografts studied. An additional 5.6 kb transcript has been reported in other systems, however, no evidence of this was found with the probe used. Aberrant signals of 4 kb were detected in the gastric carcinoma GXF 251 and in DU145 cells, but these may be due to cross-hybridisation. Similar transcript patterns have been described recently [23, 24].

EGFR protein expression was assessed by immunohistochemical staining of paraffin-embedded specimens of the same tumours. The primary antibody used detects the intracellular domain of the EGFR. Membrane-associated and intracellular (cytoplasmic) immunoreactivity was observed. Identical staining patterns have been observed by various authors with the same or other antibodies. Intracellular staining has been explained by ligand-induced receptor internalisation or by immunohistochemical detection of cytoplasmic EGFR precursors [25–27]. Comparison of slot blot data and immunohistochemistry results have revealed a clear correlation between EGFR mRNA detection and expression of the EGFR protein. However, three tumours demonstrating EGFR mRNA did not show expression of the protein. This indicates a lower sensitivity of the immunohistochemistry technique, which may be due to background staining of some of the xenografted malignancies. Alternatively, post-transcriptional regulation might influence EGFR protein synthesis.

Comparison of clonogenic assay data and EGFR protein expression demonstrated a significant correlation ($P = 0.003$). Twelve of the 21 tumours studied displayed EGFR either on the cell surface or intracellularly. *In vitro* clonogenic growth of nine of these tumours was reduced significantly by incubation with TGFα–PE40, resulting in IC_{70} values <3.0 ng/ml. Nine tumours did not express EGFR, and IC_{70} values of these tumours were well above 3.0 ng/ml. Correlation of IC_{70} values to quantitative EGFR mRNA expression data, as determined by slot blot analysis, also revealed a significant correlation ($P = 0.007$, Wilcoxon test). Efficacy of TGFα–PE40 was observed in EGFR mRNA expressing lines only. However, three tumours expressing EGFR protein and mRNA were resistant to TGFα–PE40. This may be due to high endogeneous levels of EGF or TGFα with competitive binding to the receptor or to primary resistance of tumour cells against the *Pseudomonas* exotoxin A. Furthermore, tumours showing EGFR protein or mRNA expression might be heterogeneous, consisting of EGFR-negative and EGFR-positive fractions of tumour cells. Similar to the clinical situation, the discrepancy between the numbers of receptors and sensitivity to TGFα–PE40 may thus be due to tumour heterogeneity, with a proportion of cells with high receptor numbers being killed and another proportion of cells with low receptor numbers being resistant.

In conclusion, these data confirm the specific effect of TGFα–PE40 *in vitro* in a panel of slow growing human solid tumour xenografts with close resemblance to patient neoplasms. Application of the compound should focus on histological types expressing EGFRs. Recent studies in EGFR-expressing tumour types (i.e. glioma, glioblastoma, medulloblastoma and non-small cell lung carcinoma) demonstrated activity of TGFα–*Pseudomonas* exotoxin fusion proteins in selected tumour models *in vivo* [28, 29]. Additional *in vivo* testing of TGFα–PE40 is warranted.

1. Pastan I, Willingham MC, Fitzgerald DJ. Immunotoxins. *Cell* 1986, **47**, 641–648.
2. Pai LH, Pastan I. Immunotoxin therapy for cancer. *JAMA* 1993, **269**, 78–81.
3. Theuer CP, Pastan I. Immunotoxins and recombinant toxins in the treatment of solid carcinomas. *Am J Surg* 1993, **166**, 284–288.
4. Fitzgerald D, Pastan I. *Pseudomonas* exotoxin and recombinant immunotoxins derived from it. *Ann NY Acad Sci* 1993, **685**, 740–745.
5. Derynck R, Goeddel DV, Ullrich A, *et al.* Synthesis of messenger RNAs for transforming growth factors α and β and the epidermal growth factor receptor by human tumors. *Cancer Res* 1987, **47**, 707–712.
6. Bennett C, Paterson IM, Corbishley CM, Luqmani YA. Expression of growth factor and epidermal growth factor receptor encoded transcripts in human gastric tissues. *Cancer Res* 1989, **49**, 2104–2111.
7. Atlas I, Mendelsohn J, Baselga J, Fair WR, Masui H, Kumar R. Growth regulation of human renal carcinoma cells: role of transforming growth factor α. *Cancer Res* 1992, **52**, 3335–3339.
8. Gullick WJ, Marsden JJ, Whittle N, Ward B, Bobrow L, Waterfield MD. Expression of epidermal growth factor receptors on human cervical, ovarian and vulval carcinomas. *Cancer Res* 1986, **46**, 285–292.
9. Veale D, Kerr N, Gibson GJ, Harris AL. Characterization of epidermal growth factor receptor in primary human non-small cell lung cancer. *Cancer Res* 1989, **49**, 1313–1317.
10. Harris AL, Nicholson S, Sainsbury JRC, *et al.* Epidermal growth factor receptor: a marker of early relapse in breast cancer and tumor stage progression in bladder cancer: interactions with *neu*. In Furth M, Greaves M, eds. *The Molecular Diagnostics of Human Cancer*. Cold Spring Harbor, New York, Cold Spring Harbor Laboratory Press, 1989, vol. 7, 353–357.
11. Neal DE, Bennett MK, Hall RR, *et al.* Epidermal growth factor receptors in human bladder cancer. Comparison of invasive and superficial tumors. *Lancet*, 1985, **1**, 366–368.

12. Heimbrook DC, Stirdivant SM, Ahern JD, *et al.* Transforming growth factor α–*Pseudomonas* exotoxin fusion protein prolongs survival of nude mice bearing human tumor xenografts. *Proc Natl Acad Sci USA* 1990, **87**, 4697–4701.
13. Pai LH, Gallo MG, FitzGerald DJ, Pastan I. Antitumor activity of a transforming growth factor alpha – *Pseudomonas* exotoxin fusion protein (TGFα–PE40). *Cancer Res* 1991, **51**, 2808–2812.
14. Fiebig HH, Berger DP, Dengler WA, Wallbrecher E, Winterhalter BR. Combined *in vitro/in vivo* test procedure with human tumor xenografts for new drug development. In Fiebig HH, Berger DP, eds. *Immunodeficient Mice in Oncology. Contrib Oncol.* Basel, Karger, 1992, vol. 42, 321–351.
15. Berger DP, Fiebig HH, Winterhalter BR, Wallbrecher E, Henss H. Preclinical phase II study of ifosfamide in human tumour xenografts *in vivo*. *Cancer Chemother Pharmacol* 1990, **26**(suppl), 7–11.
16. Chaudhary VK, FitzGerald DJ, Adhya S, Pastan I. Activity of a recombinant fusion protein between transforming growth factor type α and *Pseudomonas* toxin. *Proc Natl Acad Sci USA* 1987, **84**, 4538–4542.
17. Siegall CB, Xu YH, Chaudhary VK, Adhya S, FitzGerald DJ, Pastan I. Cytotoxic activities of a fusion protein comprised of TGFα and *Pseudomonas* exotoxin. *FASEB J* 1989, **3**, 2647–2652.
18. Hamburger AW, Salmon SE. Primary bioassay of human tumor stem cells. *Science* 1977, **197**, 461–463.
19. Berger DP, Henss H, Winterhalter BR, Fiebig HH. The clonogenic assay with human tumor xenografts: evaluation, predictive value and application for drug screening. *Ann Oncol* 1990, **1**, 333–341.
20. Sambrook J, Fritsch EF, Maniatis T. *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor, New York, Cold Spring Harbor Laboratory Press, 1989.
21. Heydermann E. Immunoperoxidase techniques in histopathology: application, methods and controls. *J Clin Pathol* 1979, **32**, 971–978.
22. Von Hoff DD, Marshall MH, Heimbrook DC, *et al.* Activity of a recombinant transforming growth factor alpha–*Pseudomonas* exotoxin hybrid protein against primary human tumor colony-forming units. *Invest New Drugs* 1992, **10**, 17–22.
23. Smith K, Fennelly JA, Neal DA, Hall RA, Harris AL. Characterization and quantitation of the epidermal growth factor receptor in invasive and superficial bladder tumors. *Cancer Res* 1989, **49**, 5810–5815.
24. Ekstrand AJ, James CD, Cavenee WK, Seliger B, Petterson RF, Collins VP. Genes for epidermal growth factor receptor, transforming growth factor α, and epidermal growth factor and their expression in human gliomas *in vivo*. *Cancer Res* 1991, **51**, 2164–2172.
25. Volm M, Efferth T, Mattern J. Oncoprotein (c-*myc*, c-*erbB1*, c-*erbB2*, c-*fos*) and suppressor gene product (p53) expression in squamous cell carcinomas of the lung. *Anticancer Res* 1992, **12**, 11–20.
26. Henzen-Logmans SC, van der Burg MEL, Foekens JA, *et al.* Occurrence of epidermal growth factors in benign and malignant ovarian tumors and normal ovarian tissues. *J Cancer Res Clin Oncol* 1992, **118**, 303–307.
27. Yasui W, Hata J, Yokozaki N, *et al.* Interaction between epidermal growth factor and its receptor in progression of human gastric carcinoma. *Int J Cancer* 1988, **41**, 211–217.
28. Draoui M, Siegall CB, FitzGerald D, Pastan I, Moody TW. TGFα–PE40 inhibits non-small cell lung cancer growth. *Life Sci* 1994, **54**, 445–453.
29. Phillips PC, Levow C, Catterall M, Colvin OM, Pastan I, Brem H. Transforming growth factor α–*Pseudomonas* exotoxin fusion protein (TGFα–PE38) treatment of subcutaneous and intracranial human glioma and medulloblastoma xenografts in athymic mice. *Cancer Res* 1994, **54**, 1008–1015.