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Growth Inhibition of Xenotransplanted Human Carcinomas by a Monoclonal Antibody Directed Against the Epidermal Growth Factor Receptor

H.-G. Schnürch, M. Stegmüller, A. Vering, M.W. Beckmann and H.G. Bender

In the athymic nude mice model with xenotransplanted human carcinomas, the effect of a monoclonal antibody (MAb 425), directed against the human epidermal growth factor (EGFR), on tumour growth was studied. Five different solid human breast carcinomas and one vulvar epidermoid cancer cell line (A431) were transplanted in nude mice, and treated with MAb 425 2.2 mg intraperitoneally (i.p.) on day 7 post-transplantation. Tumours with EGFR concentrations of ≥ 16 fmol/mg soluble cytosolic protein showed growth inhibition, whereas the growth pattern of EGFR-negative tumours was unaffected. Variation of MAb 425 dosage (1.1 versus 2.2 mg) revealed no difference in the growth inhibiting effect. Different application schedules (application on day 0, 12 or 26) showed different onsets and durations of tumour growth inhibition. Repeated application (1.1 mg, day 0 and 12) was followed by a prolonged inhibitory effect. Our results suggest that growth inhibition of EGFR-positive tumours by MAb 425 may lead to an additional treatment option for patients with EGFR-positive cancer.

Key words: monoclonal antibody, EGF receptor, neoplasms, xenotransplantation, nude mice
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INTRODUCTION

THE PROLIFERATIVE stimulus of epidermal growth factor (EGF) on human cells and tumours is mediated by its binding to the external domain of a specific transmembrane receptor, the epidermal growth factor receptor (EGFR). The EGFR content of tumour tissues is correlated to the growth rate [1]. Similar results were reported on cancer cells *in vitro* [2]. EGFR is overexpressed in different human solid malignant tumours: carcinoma of the uterine cervix, oesophagus, stomach, urinary bladder and female breast.

Biochemical analysis of breast cancer tissues revealed positive EGFR measurements in 10–67% of tumour samples analysed [3, 4]. Until now, neither a standardised EGFR determination method nor a specific cut-off point has been defined [4, 5]. Comparison of the results from different EGFR analysing methods is, therefore, difficult and often not feasible. EGFR expression could also be demonstrated by immunohistochemical staining in 20–60% of breast cancer specimens [6–8].

High EGFR content is correlated with low oestrogen receptor (ER) and progesterone receptor (PR) values [4, 7–10] and low EGFR content with higher differentiation and low histological grade. The EGFR status seems to be an independent prognostic factor for tumour recurrence and overall survival [10, 11]; high EGFR concentrations are correlated with a poor prognosis.

The monoclonal antibody 425 (MAb 425) binds to a part of the extracellular domain of the EGFR, which is different from the EGF binding site but close to it [12]; MAb 425 does not induce tyrosine kinase activity but inhibits the binding of EGF and transforming growth factor (TGF)α to the EGF receptor, and thus potentially prevents cellular proliferation *in vitro* [13]. In addition, according to recent evidence, the antibody-dependent cellular cytotoxicity (ADCC) reaction could mediate the growth inhibitory effect of MAb 425 [14, 15].

The aim of our study was to analyse the effect of the murine IgG_{2a} MAb 425 on the growth patterns of human carcinomas and an epidermoid cancer cell line in the nude mouse xenotransplantation model.

MATERIALS AND METHODS

Monoclonal antibody

MAb 425 was first described by Murthy and colleagues [12]. The antibody for this study was obtained from E. Merck (Darmstadt, Germany). The iodinised EGF was obtained from Amersham Buchler (Braunschweig, Germany) ([^{125}I]EGF, specific activity 3.7–4.8 MBq/μg).

Study groups

Tumour specimens, from primary breast carcinomas of patients who were treated between 1975 and 1978 in the Department of Gynaecology and Oncology (University Hospital, Frankfurt/Main, Germany) had been primarily transplanted into athymic nude mice and kept in animal passages since then [16]. Our experiments started in February 1990 with five of those tumours and the number of passages in nude mice from first xenotransplantation to our experiments are given in Table 1. Five histopathologically different breast carcinomas were chosen for our

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Table 1. Biochemical analyses of the EGFR content of six different carcinomas

Tumour	Abbreviation	EGFR concentration*	Histology	Passage no.
Breast carcinoma 1	BC1	0	IDC, solidum simplex	62
Breast carcinoma 2	BC2	0	Medullary carcinoma	73
Breast carcinoma 3	BC3	3	IDC, dedifferentiated	83
Breast carcinoma 4	BC4	16	IDC, solidum, dedifferentiated	71
Breast carcinoma 5	BC5	30	IDC, solidum simplex	94
Epidermoid carcinoma	A431	65	Squamous cell carcinoma	7

*fmol/mg soluble cytosolic protein, at the beginning of the experiments. IDC, invasive ductal carcinoma.

experiments (Table 1). Small pieces (2 mm^3) of these five different tumours were transplanted subcutaneously to the milk line on both sides of healthy nude mice (day 0). Solid specimens of a tumour in nude mice passage, derived from a vulvar epidermoid carcinoma cell line (A 431), were transplanted to a sixth group of nude mice. EGFR content of these six tumour entities were analysed biochemically as described below. The five breast cancer tissues were chosen because of their difference in EGFR content. As mentioned above, 10–67% of breast cancer specimens show positive EGFR values. The results of our analyses range from 0 to 180 fmol/mg soluble cytosolic protein [17].

Tumour measurements

The tumour growth in the nude mice was measured by a caliper. A virtual tumour area was calculated by multiplication of the greatest diameter with the perpendicular diameter. Measurements were taken twice a week for the first 3 weeks and then once a week. Measurements of all tumours within one group were represented by the mean value. Mean values (mm^3) were plotted against time (days) post-transplantation, resulting in growth curves. Wilcoxon–Mann–Whitney tests were performed for statistical evaluation of significant differences in growth patterns between two study groups; Kruskal–Wallis tests were used if more than two groups were compared.

EGFR quantitative assay

EGFR concentrations of the six tumour tissues were analysed with modifications according to Nicholson [15]. In brief, the tissue samples were dissociated using a micro-dismembrator (B. Braun, Melsungen, Germany), homogenised, dissolved in phosphate buffered saline (PBS) buffer and centrifuged (1 h, >105 000 *g*, 1°C). Pellets were stored frozen (−70°C). For the biochemical analyses, these frozen pellets were thawed out in a Teflon-glass homogeniser with the addition of Tris bovine serum albumin (BSA) buffer. After the first centrifugation (5 min, 800 *g*, room temperature), the supernatant was collected and recentrifuged (1 h, >105 000 *g*, 1°C). The remaining pellets were resuspended in Tris/BSA and the binding capacity of the membrane fraction for radiolabelled EGF ([^{125}I]EGF) was measured. Aliquots were incubated with five different concentrations of [^{125}I]EGF with or without unlabelled EGF in excess (100×, 1 h, 20°C). Using a Brandel Cell Harvester (Dunn Ltd, Germany), the suspension was filtered through FC filters (Dunn Ltd.) to collect the dissolved membranes including the ligand–receptor complex ([^{125}I]EGF–EGFR). Filters were washed twice with 10 ml ddH_2. The remaining radioactivity of the [^{125}I]EGF–EGFR complex in the filter paper was counted in a gamma counter (ANSR, Abbott, Germany). Values from different measurements were analysed by Scatchard analysis [18]. Protein content was measured in the second supernatant by the method of Biostad Comp. (Munich, Germany) using human albumin as a standard.

Treatment protocols

Experiment 1 was planned to detect the overall growth inhibitory effect of MAb 425 in the six tumour entities; the individual growth pattern of each tumour was examined in 14 animals, seven being treated with MAb 425, and seven with PBS only to serve as the control group. The treatment protocol started on day 7 after tumour implantation; 2.2 mg of MAb 425 dissolved in 0.45 ml PBS buffer were injected intraperitoneally (i.p.) into each mouse of the six treatment groups. Control groups received 0.45 ml PBS i.p. only.

Experiments 2, 3 and 4 (Table 2) were performed to evaluate the impact of variation of the application schedule of MAb 425 on tumour growth pattern. These dose modification experiments were performed with one xenotransplanted tumour tissue (BC5, EGFR content: 30 fmol/mg soluble cytosolic protein) which proved to be the most sensitive to MAb 425 treatment in Experiment 1. Experiment 2 assessed the MAb 425 effect after two different dosages (2.2 versus 1.1 mg) applied on day 0. Experiment 3 assessed the growth patterns related to different time intervals between transplantation of the tumour (day 0) and the application day (i.e. day 12, 26, ...). Application days were chosen arbitrarily. Experiment 4 assessed the effect of a fractionated application of MAb 425 (1.1 mg on day 0 and day 12). In Experiment 5, the growth stimulatory potential of the physiological ligand EGF (day 1 to 5.5 μg daily) was tested. This dosage was given according to Gregory [19]. The untreated group served as the control.

Table 2. Variation of dosage and application schedule performed on tumour BC5

Experiment	Day(s) of application*	Dosage	Drug applied
2	0	1.1†	MAb 425
	0	2.2†	MAb 425
3	0	2.2†	MAb 425
	12	2.2†	MAb 425
	26	2.2†	MAb 425
4	0	1.1†	MAb 425
	12	1.1†	MAb 425
5	1–5	5‡	EGF

*Day(s) of application post-transplantation. †mg i.p. ‡μg/day. EGF, epidermal growth factor.

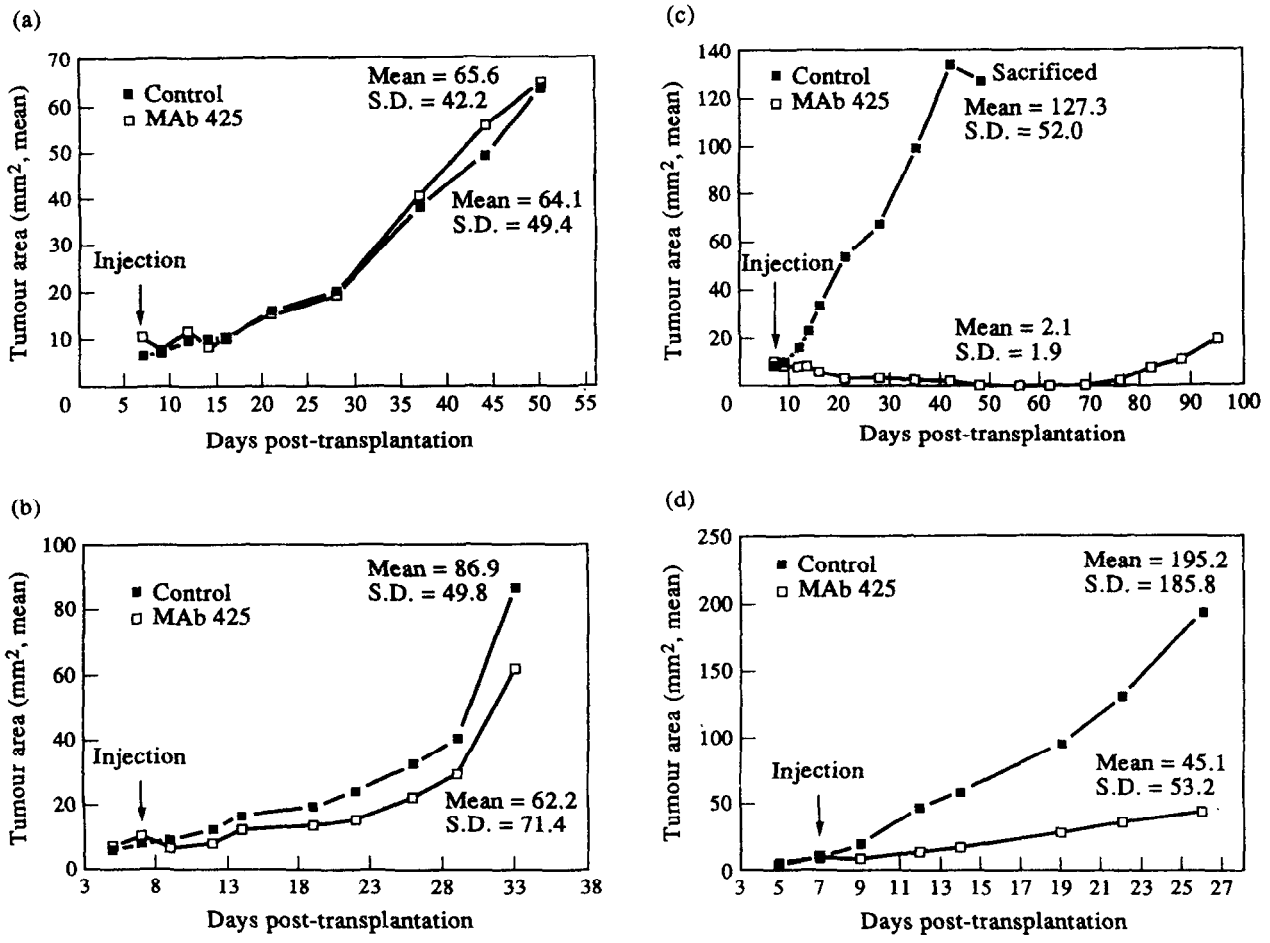

Figure 1. Results from Experiment 1. Absolute growth of different tumour entities with various EGFR concentrations with and without MAb 425 treatment on day 7. Statistical evaluation by Wilcoxon–Mann–Whitney tests. (a) Breast carcinoma (BC) 3 (3 fmol/mg protein), $P = 0.69$. (b) BC 4 (16 fmol/mg protein), $P = 0.17$. (c) BC 5 (30 fmol/mg protein), $P < 0.0001$. (d) Squamous epidermoid carcinoma cell line A 431 (65 fmol/mg protein), $P = 0.005$. S.D., standard deviation.

RESULTS

EGFR analysis

The six different tumour entities showed quantitative differences in their EGFR content, varying from 0 to 65 fmol EGFR/mg soluble cytosolic protein (Table 1)

In Experiment 1, the overall influence of MAb 425 on growth patterns of the six tumour entities was assessed. MAb 425 had no effect on absolute tumour growth in tumours with undetectable EGFR (BC1, BC2; data not shown) or with EGFR concentrations $\leq$ 3 fmol/mg soluble cytosolic protein (BC3, Figure 1a). Breast cancers with EGFR concentrations of $\geq$ 16 fmol/mg soluble cytosolic protein (BC4, Figure 1b; BC5, Figure 1c) responded to MAb 425 treatment with inhibition of tumour growth. BC5, the breast cancer with the highest EGFR value, showed the most intense response (Figure 1c). The solid tumour tissue derived from vulvar carcinoma cell line A431 had the highest EGFR concentration (65 fmol/mg soluble cytosolic protein) and was significantly inhibited by MAb 425 treatment (Figure 1d).

Comparison of the tumour growth of MAb 425-treated groups and the untreated control groups revealed that the relative tumour growth inhibition was related to EGFR concentrations of the six different tumours (Figure 2): the higher the EGFR value the stronger the inhibitory effect.

The breast cancer tissue BC5 showed a significant response to the MAb 425 treatment in Experiment 1 and, therefore, was

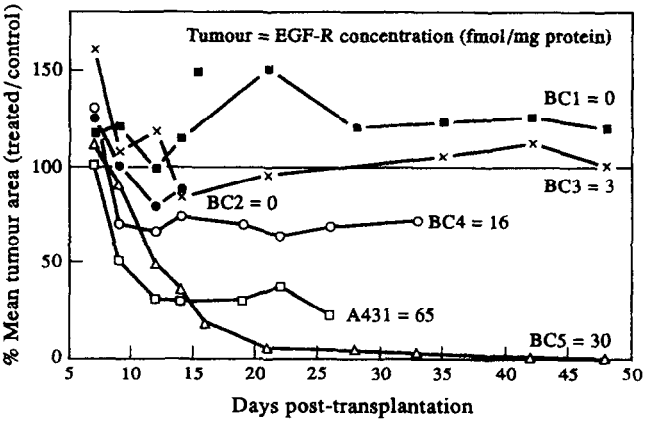

Figure 2. Relative tumour growth inhibition of six tumour entities by MAb 425 application. Each curve represents the quotient of the mean tumour area of the treatment versus the control group. Tumour identification and EGFR concentrations (fmol/mg soluble protein) are given at the end of each curve (different antineoplastic effects also correlate to different growth rates of tumors).

chosen for the first experiments addressing dose modifications (Experiments 2, 3 and 4). In Experiment 2, the effect of two different dosages (1.1 and 2.2 mg) applied on day 0 was assessed (Table 2). Figure 3a shows the results. Statistical analysis (Kruskal–Wallis) showed significant differences: D0 2.2 versus control: $P<0.0001$; D0 1.1 versus control: $P<0.00001$; during the time interval examined, no significant difference in tumour growth could be demonstrated between the two treatment groups.

Experiment 3 was designed to gain insight into the effect of different time points of MAb application. MAb 425 injected at a later time point (day 26) was still associated with significant growth inhibition (Figure 3b). This growth inhibition was not as significant as seen with MAb 425 injections at earlier time points (day 7, day 12), with: D12 versus control: $P<0.005$, D26 versus control: $P<0.05$. Nevertheless, the examined intervals between transplantation and application did not affect the time periods until the tumours re-established their primary growth pattern.

Experiment 4 provided data on repeated treatment, 1.1 mg given at day 0 and at day 12 after transplantation, compared to the single application of 2.2 mg at day 0 (Figure 3c). It showed that with D0 12 versus control: $P<0.0000001$, D0 2.2 versus D0 12 1.1: $P=0.94$. No significant difference was detected between the two treatment groups, but tumour growth inhibition seemed to last longer after the fractionated application.

Experiment 5 provided data on the enhanced growth potential of BC5 after injection of the physiological EGFR ligand, EGF. A minor, insignificant growth acceleration could be demonstrated starting at day 50, if EGF was given i.p. days 1 to 5 (Table 2; Figure 3d). Statistics were control versus D1-5 EGF: $P=0.76$.

In order to examine the phenomenon of secondary tumour progression after growth inhibition by MAb 425, we analysed the results of Experiments 2–5 semilogarithmically (Figure 4). This demonstrates that the second tumour progression started after remission intervals of 30 to 74 days. The beginning of the secondary tumour proliferation was related to the last day of MAb 425 application. As expected for malignant tumour tissues, the control group and the EGF-treated group both showed exponential growth behaviour.

DISCUSSION

Growth inhibition of tumours *in vitro* and *in vivo* after application of antibodies directed against growth factor receptors has been reported by several authors [2, 15, 20]. A direct relationship between EGFR concentration in the primary tumour and the effect of MAb 425 treatment has not yet been reported. In our study on xenotransplanted human breast

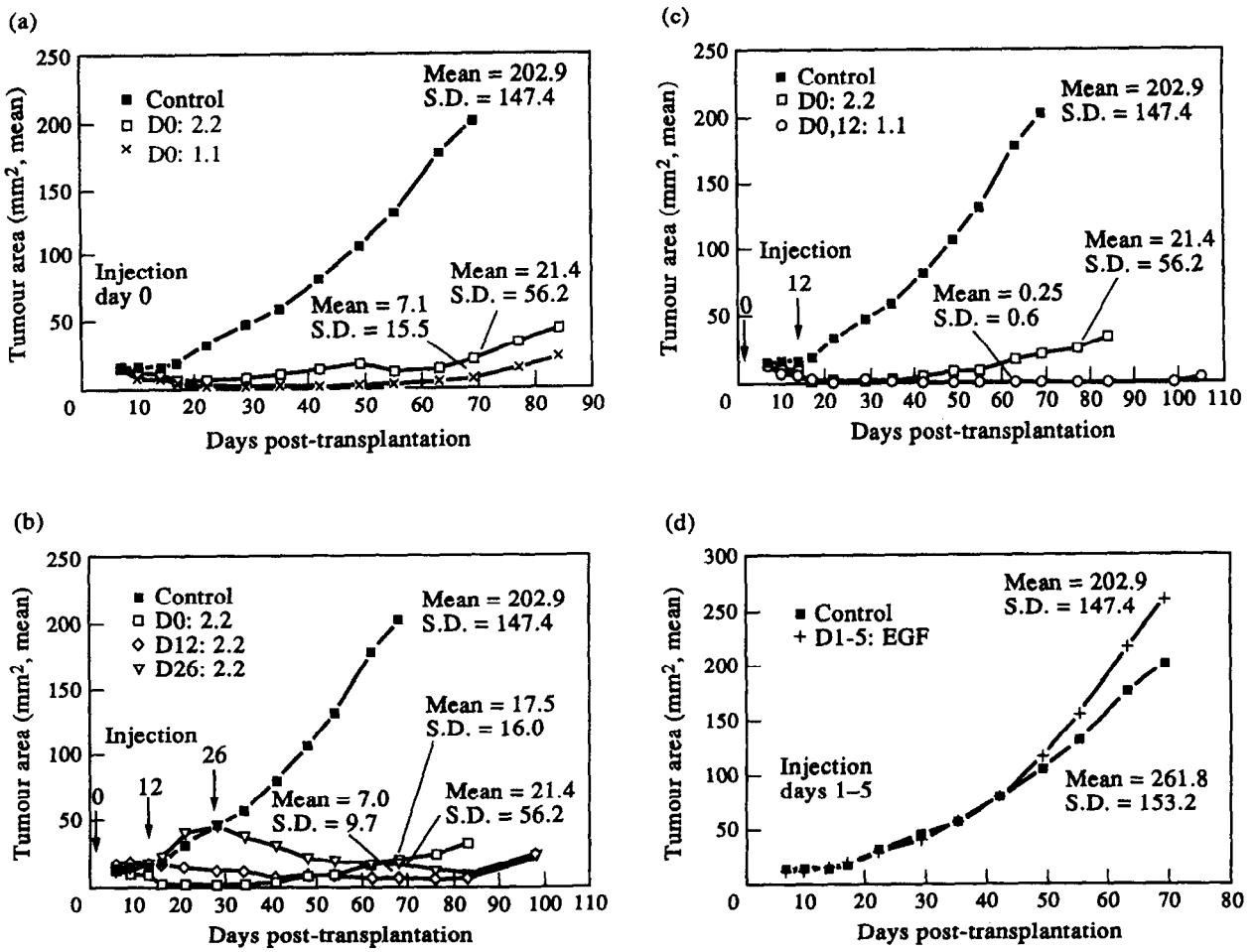

Figure 3. (a–d) Tumour growth patterns under modifications of dosage and application schedule of MAb 425 (see also Table 2). Statistical evaluation by Kruskal–Wallis test. (a) Two different MAb 425 dosages (1.1 and 2.2 mg) applied on day 0; D0 2.2 versus control: $P < 0.0001$; D0 1.1 versus control: $P < 0.00001$. (b) Application at different time points (day 0, 12 and 26 post-transplantation); D12 versus control: $P < 0.005$, D26 versus control: $P < 0.05$. (c) Fractionated dosage (1.1 mg on day 0 and 12); D012 versus control: $P < 0.0000001$, D0 2.2 versus D0 12 1.1: $P = 0.94$. (d) EGF (5 μg/day on days 1–5). S.D., standard deviation.

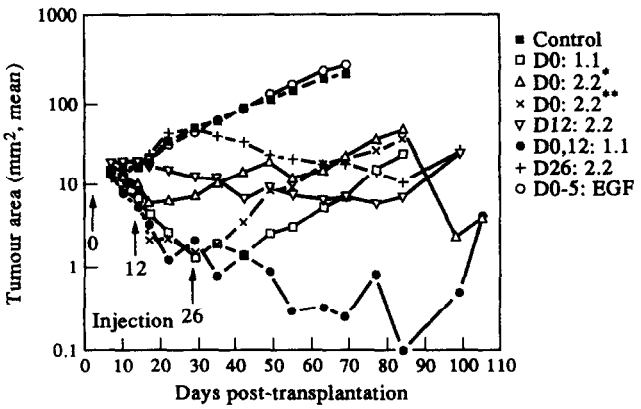

Figure 4. Modifications of dosage and application schedule in nude mice with BC5 tumour tissue transplanted: identical groups as seen in Figures 3a–d, mean tumour areas of the treated groups and the control group are presented with a logarithmic *y*-scale. *Results of Experiment 2; **, results of Experiment 4.

carcinomas, we demonstrated that the effect of MAb 425 treatment was related to the EGFR concentration. Xenotransplanted breast tumours with high EGFR concentrations showed significant tumour growth inhibition after antibody administration, whereas the growth of tumours without measurable of with low EGFR concentrations remained unaffected.

Comparison of these results with the effect of MAb 425 on grafts derived from a vulvar carcinoma cell line (A431), which is known to have high EGFR concentration per mg soluble cytosolic protein, revealed that, in this study, the inhibitory effect did not strictly correlate to the absolute tissue concentration of EGFR. Different immunological effects, i.e. the ADCC reaction [14, 15], may also be relevant in addition to direct growth inhibition. Tumour-specific growth patterns or the heterogeneity of the EGFR distribution in the tissue could also contribute to the different MAb 425 effects observed in *in vivo* tumours.

The first successful experiments with the MAb 425 in nude mice were performed in the Wistar Institute (Philadelphia, U.S.A.) with 11 injections i.p. each with 0.2 mg (D. Herlyn, personal communication). In our experiments, the total dosage of 2.2 mg was applied with a single injection on day 0 post-transplantation. Experiments with dose reduction were performed down to 0.055 mg as a single application. The growth inhibitory effect did not change with doses down to 0.1 mg with a single i.p. application in nude mice (M. Sproll, personal communication). Toxicity was never seen in nude mice, even with higher doses, probably because the MAb 425 does not interact with the murine EGF receptor. First clinical phase I/II studies in patients with EGFR-rich tumours are under way, using 20–100 mg MAb 425 per day, 5 days a week, for 4 weeks; no sign of acute toxicity has been seen [21].

For *in vivo* application of antibodies, pharmacokinetic and pharmacodynamic studies must be performed. We, therefore, designed *in vivo* animal experiments with varying administration schedules of MAb 425. We studied two different dosages of MAb 425; the results with respect to growth inhibition differed only slightly and were not significant. Further investigations are in progress.

Tumour growth was inhibited by administering MAb 425 at the day of transplantation (day 0), and also by application of MAb 425 after a longer time interval. There was no significant difference in the absolute growth inhibition by MAb 425 after different application dates, suggesting a constant sensitivity to the MAb 425 treatment, independent of the tumour size. In all cases, the effect of MAb 425 was time limited. Tumours regained their proliferative activity after a variable remission interval.

The tumour growth inhibiting effect of MAb 425 could be reinforced by fractionation of the MAb 425 dose in a 12-day interval. In this particular experiment (Experiment 3), the transplanted tumours showed proliferative activity no earlier than 12 weeks after the last treatment, suggesting an intensified growth inhibition. For the clinical use of a growth inhibiting MAb, this finding should be particularly interesting, because the optimal application strategy is considered to be an important aspect of further research in this field.

Further *in vivo* investigations are needed to gain insight into the mechanism of action of MAb 425, and to determine the possible use of this antibody for cancer treatment in patients. Tumour characteristics, predicting the response to EGFR antibodies, are of particular relevance. High histological grade, high proliferative activity (Ki67, S-phase fraction), aneuploidy, negative ER and PR status [22], as well as high EGFR content are well known as indicators of poor prognosis; further data from ongoing studies will help to predict the sensitivity of individual tumours to MAb 425 treatment. In particular, patients with rapid progression of their disease could potentially benefit from this additional treatment option. MAb 425 is currently being tested in phase I/II studies in patients with glioblastoma with, as yet, promising results (Stasiecky, E. MERCK Comp., Germany).

The antibody uptake per weight in mice may be relatively higher than in humans, however, in the human studies mentioned above the total dosage applied was up to 2.0 g. This is clearly more (909-fold) than the usual 200-fold amount calculated for the relation mouse to human. Therefore, the interpretation of the results appears to be realistic.

The results of this study support the suggestion that monoclonal antibodies against growth factor receptors may develop into a specific treatment option for certain patients with EGFR positive tumours.

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Acidic and Basic Fibroblast Growth Factors in Human Breast Tissue

J. Smith, A. Yelland, R. Baillie and R.C. Coombes

Previously we have reported changes in fibroblast growth factors (FGF) in conditioned medium (CM) derived from rat mammary tumours undergoing remission. We have used a similar approach to assay for the presence of FGFs in human breast tissue and cell lines. The majority of cancer tissues (35/50), benign tissues (8/9) and all cancer adjacent normal tissues (20/20) released heat labile, NR6 transforming activity which coeluted from heparin with acidic FGF (aFGF) at 0.9–1.1 M NaCl and was neutralised by antibodies to aFGF. The conclusion that the majority of breast cancers contain active aFGF was supported by immunoblotting. The CM of a minority (15/50) of cancers and one benign tissue had highly transforming activity for NR6 cells, and was mitogenic for a breast cancer cell line, was heat labile, and strongly heparin binding, eluting at 1.5–2.0 M salt. It was not immunoreactive with antibodies to aFGF, basic FGF (bFGF) or Kaposi's FGF (kFGF) and its activity was reduced by the presence of aFGF, suggesting competition for the same receptor. Very little aFGF was observed in the CM of these tumours, and neither aFGF nor other FGF activity was detected in CM of breast cell lines.

Key words: breast cancer, fibroblast growth factors, breast cancer cell lines, acidic FGF, basic FGF
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INTRODUCTION

HUMAN BREAST carcinoma is the most common solid tumour of females in the western world. An effective treatment regime of oestrogen receptor (ER)-positive patients involves the use of endocrine therapy. Unfortunately, in the majority of patients, endocrine therapy ultimately becomes ineffective and the cancer regrows [1]. This process may be mediated by changes in growth factor secretion or by loss or gain of their receptors. The fibroblast growth factor (FGF) family of growth factors, of which to date there are seven members, are important in