

## Cancer of the Uterine Cervix is Susceptible to Anti-EGF-R Antibody EMD 55,900 Therapy

CHRISTINE SOLBACH<sup>1\*</sup>, MARC ROLLER<sup>1\*</sup>, MARIA NICOLETTI<sup>1</sup>,  
KAI BUDISCHEWSKI<sup>2</sup>, RAINALD KNECHT<sup>3</sup> and MANFRED KAUFMANN<sup>1</sup>

<sup>1</sup>Department of Gynaecology and Obstetrics, <sup>2</sup>Department of Radiology and

<sup>3</sup>Department of Otorhinolaryngology, Johann Wolfgang Goethe University,  
School of Medicine Frankfurt am Main, Germany

**Abstract.** *Background:* Among a variety of cancer therapeutics targeting the epidermal growth factor receptor (EGF-R), monoclonal antibodies have shown some therapeutic potential in the treatment of various tumours of different entities. Nevertheless, several high EGF-R-expression carcinomas show no response to this treatment. Tumours of the uterine cervix represent a group, in which the response to anti-EGF-R treatment is hardly predictable, despite a relatively high expression of the receptor. *Materials and Methods:* To assess the antitumour activity of anti-EGF-R monoclonal antibody EMD 55,900 *in vivo*, a series of experiments were performed in the nude mouse model using xenotransplanted primary carcinomas of the uterine cervix. *Results and Conclusion:* EMD 55,900 was found to be capable of inhibiting the growth of primary carcinomas of the uterine cervix at different stages of tumour development. The therapeutic response was not dependent on EGF-R expression solely, but also on the pre-treatment microvessel density.

Overexpression of the epidermal growth factor receptor (EGF-R) is known to have an impact on prognosis in a variety of neoplasms (1). This discovery has led to the development of several strategies targeting this protein in the treatment of solid tumours (2). Among them, monoclonal antibodies have proven their antitumour activity in a series of clinical phase I/II trials (3). The monoclonal antibody EMD 55,900 binds with high affinity to the N-terminal extracellular domain of the 170 kDa human

EGF-R, close to the ligand binding domain, without inducing a tyrosine kinase activity by itself. However, the precise mechanism of the antitumour activity *in vivo* remains to be elucidated. In addition to the abrogation of an EGF-R-ligand-mediated downstream signalling cascade, several mechanisms are currently being discussed. Recent studies have demonstrated a capability of EMD 55,900 to induce antibody-dependent cell cytotoxicity (ADCC) *in vitro* (4). In our recent studies, we were able to demonstrate that the treatment efficiency *in vivo* does not depend solely on EGF-R protein expression. Therefore, a series of xenograft tumours were generated in a nude mouse model. In addition to some carcinomas of the uterine cervix, squamous cell carcinomas of the head and neck, ovarian cancer, breast cancer and thyroid carcinomas were also investigated and it was found that the pre-treatment CD31 score, reflecting tumour vascularization, and the relative proportion of connective tissue were parameters which influenced the *in vivo* therapy response (5), whereby tumours expressing a low pre-treatment CD31 score were more susceptible to the antibody treatment. Together with previous investigations, demonstrating the down-regulation of neo-angiogenic factors in response to anti-EGF-R treatment, these findings led us to the perception that EMD 55,900 is also capable of inhibiting neo-vascularization in solid tumours (6, 7). Interestingly, these parameters did not have such impact in low EGF-R-expressing tumours of the female breast, where it was demonstrated that anti-EGF-R treatment might be a therapeutic option, especially in those cases where tumours were Her2/neu-negative and, therefore, therapy with Herceptin was not applicable (8, 9).

In the previous studies, tumours of the uterine cervix showed the most controversial response to EMD 55,900 treatment *in vivo*. For this reason, the aim of the present investigation was the verification of our previously drawn conclusions, that the tumour environment, especially the pre-treatment vascularization status and the relative degree of connective tissue, are predictive factors for successful

\*Both authors contributed equally.

*Correspondence to:* Dr. med. Christine Solbach, Johann Wolfgang Goethe University, School of Medicine, Theodor Stern Kai 7, 60590 Frankfurt am Main, Germany. Tel: +49-69-6301-5238, Fax: +49-69-6301-83469, e-mail: Solbach@em.uni-frankfurt.de

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therapy *in vivo*. A large series of xenotransplanted carcinomas of the uterine cervix were examined.

## Materials and Methods

**Monoclonal antibody EMD 55,900.** The monoclonal antibody EMD 55,900 is a murine IgG2a directed against the N-terminal extracellular domain of the human epidermal growth factor receptor and has been described elsewhere (10). The antibodies used in this study were kindly provided by Merck KGaA (Darmstadt, Germany).

**EGF-R quantitative ELISA.** For the detailed method of approach please refer to reference (5). In brief, excised tumour samples were pulverized in liquid nitrogen using a microdismembrator (B. Braun, Melsungen, Germany). The obtained powder was further suspended in lysis buffer, centrifuged and the obtained protein solution was used for further ELISA analyses. The protein solution was subsequently normalized to a protein content of 50 µg/ml, and 200 µl were used for the ELISA, according to the manufacturer's instructions (Immunodiagnostik, Bensheim, Germany).

**Immunohistochemistry.** To assess pre-treatment vessel density, immunohistochemistry was performed on excised control tumours. The excised tumours were fixed in 4% buffered formalin solution for a minimum of 24 hours followed by standard paraffin embedding. Sections of 3-4 µm were deparaffinized and boiled 5 times for 3 minutes in citric buffer (10 mM citric acid, 10 mM sodium citrate, pH 6.0). Rat anti-mouse-CD31 (Dianova, Hamburg, Germany) was used for primary incubation for 1 hour. Detection was performed using the anti-rat super sensitive AP-kit (BioGenex, Hamburg, Germany), and the sections were counterstained with hematoxylin. The results were recorded in a semi-quantitative fashion and scored from 0 to 5, where 0 showed no immunoreactivity and 5 showed a strong reaction.

**Determination of connective tissue.** The relative proportion of connective tissue was determined by standard staining according to Goldner (11) for estimation of the proportion of connective tissue to solid tumour (5).

**Animal experiments.** Tumour specimens from patients treated at the University Hospital Frankfurt am Main, Germany, were transplanted on athymic nude mice (NMRI nu/nu) and kept as xenotransplants. For therapy experiments, tumours were transplanted subcutaneously as tissue fragments of 2 mm<sup>2</sup> onto 4- to 5-week-old nude mice. The tumour growth was monitored weekly with vernier calipers. The largest diameter was multiplied by the perpendicular diameter to assess the tumour area. In the treatment groups, the animals received a single injection of 100 mg/kg body weight of EMD 55,900 by an intraperitoneal injection.

**Statistics.** The Mann-Whitney *U*-test was performed for the evaluation of significant growth patterns in the animal experiment. Probabilities were considered significant at  $p \leq 0.05$ .

## Results

**EGF-R expression in primary cervical cancer.** To generate a representative collective of xenotransplanted tumours, the

EGF-R expression was first examined on a series of primary surgical specimens. Overall, 65 consecutive primary carcinomas of the uterine cervix were analysed for EGF-R expression by fully quantitative enzyme-linked immunosorbant assay (ELISA). A median EGF-R expression of 81.8 fmol/mg total protein (t.p.) was found and the mean expression was 99.7 fmol/mg t.p., with an expression range of 2.3 to 482.6 fmol/mg t.p. (Figure 1).

**Growth inhibition of xenotransplanted primary cervix carcinomas by EMD 55,900 antibody therapy.** As previously demonstrated, the prediction of therapy response of anti-EGF-R antibody treatment is difficult, especially in carcinomas of the uterine cervix. To further investigate these observations, a collective of primary tumours was generated as xenotransplants in nude mice, expressing EGF-R levels in the physiological range, as assessed by EGF-R expression in primary surgically excised tumours. Initially one tumour (Cer1), expressing EGF-R at a relatively low level for this type of carcinoma (mean EGF-R expression 39.3 fmol/mg total t.p., range 30-50 fmol/mg t.p.) and one carcinoma with an intermediate EGF-R expression in comparison to the entire clinical collective (Cer2, mean EGF-R expression 76.8 fmol/mg t.p., range 50-100 fmol/mg t.p.) were treated. The tumours were transplanted, and the animals received a single injection of EMD 55,900 intraperitoneally at a dose of 100 mg/kg body weight. The antibody was applied one week following tumour transplantation, when the mean tumour size had reached about 25 mm<sup>2</sup>. To assess antitumour activity, tumour growth was recorded for 8 weeks by weekly measurement of the tumour size in the largest diameters (Figure 2). As observed previously, a significant growth inhibition was found in Cer1 ( $p=0.004$ ,  $n=28$ ), a tumour expressing relatively low levels of EGF-R. In contrast, the Cer2 tumour, with EGF-R expression two-fold higher than that of Cer1, showed no significant response to EMD 55,900 antibody treatment ( $p>0.05$ ,  $n=36$ ).

**Growth inhibitory effects of EMD 55,900 at different stages of tumour development.** The growth inhibitory effect of EMD 55900 at different stages of tumour development was subsequently investigated. Two additional primary carcinomas were established as stable xenotransplants in nude mice and the Cer1 tumour served as a control for the anti-tumoral activity of EMD 55,900 in the early stages of tumour development. All tumours were treated in two different treatment schedules. One group received the antibody injection 1 week following tumour transplantation (d0), and the second group when the mean tumour size was approximately 50 mm<sup>2</sup> (dx). The treatment was kept constant with a single intraperitoneal antibody injection of 100 mg/kg body weight. Tumour growth was monitored for a minimum of 7 weeks, whereby the largest diameters were recorded.

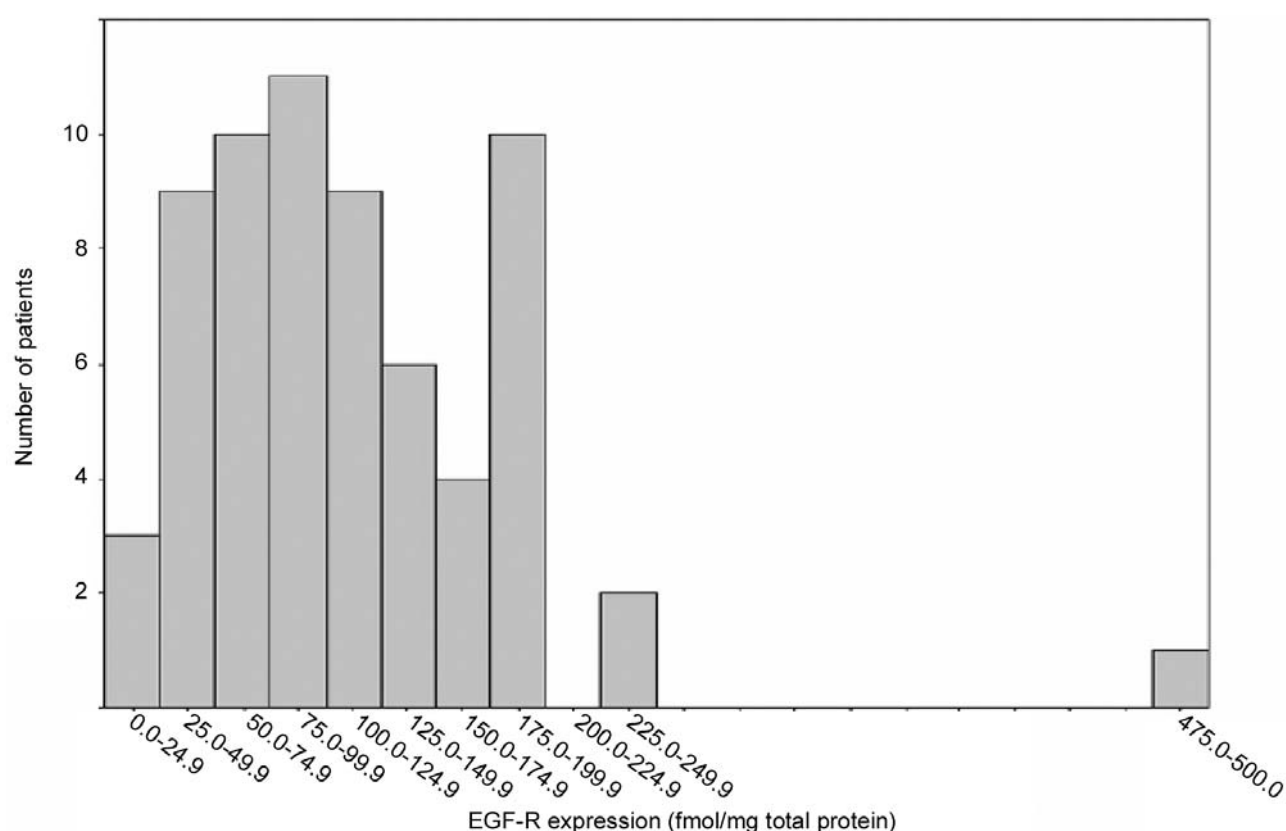


Figure 1. Distribution of EGF-R protein expression of all (n=65) analysed tumour samples of primary carcinomas of the uterine cervix.

As expected, Cer1 showed a significant growth inhibition when treatment was started one week following tumour transplantation ( $p < 0.001$ ,  $n = 26$ ). Despite the relatively low EGF-R expression of this carcinoma, treatment of well-established tumours still revealed significant growth inhibitory effects ( $p = 0.038$ ,  $n = 26$ ). In contrast, the Cer3 tumour, which expressed the highest EGF-R level (mean expression: 285.6 fmol/mg t.p., range: 250-350 fmol/mg t.p.) examined in this study, showed only significant growth inhibitory effects when the treatment was started 1 week following the transplantation ( $p = 0.001$ ,  $n = 26$ ), whereas the treatment of well-established tumours showed no significant therapeutic effect for this carcinoma ( $p > 0.05$ ,  $n = 26$ ). The third tumour tested in this setting (Cer4, mean EGF-R expression: 97.0 fmol/mg t.p., range: 60-130 fmol/mg t.p.) showed significant growth inhibition in both cases, when tumour treatment was started 1 week following transplantation ( $p < 0.0001$ ,  $n = 28$ ), as well as on relatively large tumours ( $p = 0.003$ ,  $n = 22$ ) (Figure 3).

Both tumours have comparable levels of EGF-R expression, but, in contrast to Cer 4, Cer2 showed no response to EMD 55,900 antibody treatment. *Pre-treatment*

*CD31 score and relative proportion of connective tissue predicts therapy success in vivo.* To further investigate whether these effects were also dependent on the pre-treatment vessel density and the relative proportion of connective tissue, respectively, histological examinations were performed on excised tumour samples. The previously established ratio of CD31 score divided by the relative proportion of connective tissue was employed to give the maximum vessel density in the Cer2 tumour (4+), as well as the lowest proportion of connective tissue (10%). The other tumour samples exhibited a relatively even proportion (20-30%) of connective tissue. Differences, however, were found in the pre-treatment CD 31 scores of 1+ in Cer4 and 3+ in Cer1 and Cer3 (Table I).

## Discussion

Therapy of patients with monoclonal antibodies directed against the EGF-R has provided promising results in clinical phase I/II trials (3). However, access to this therapeutic option is still restricted to tumours that show a high expression of the receptor. This study demonstrates

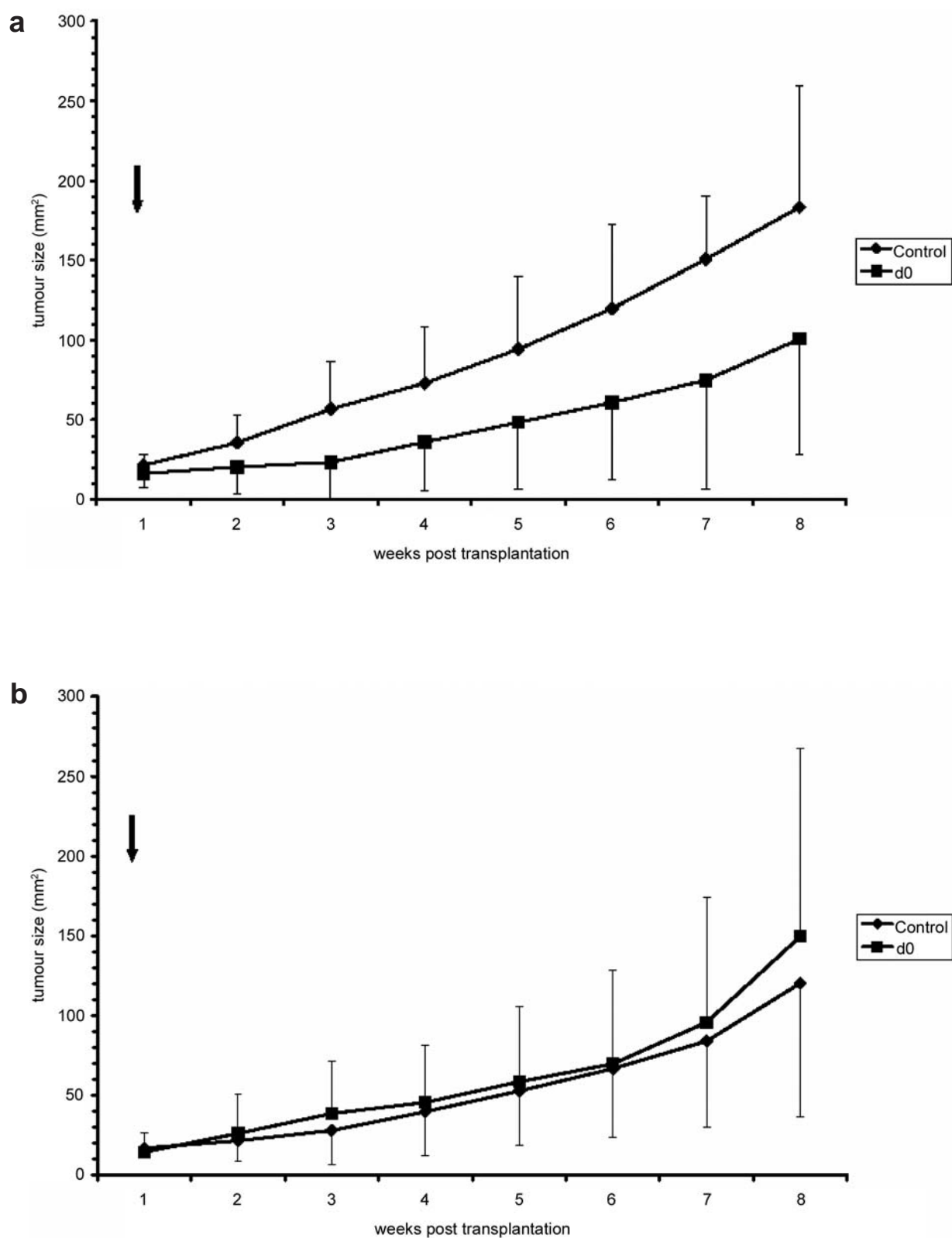


Figure 2. Growth curves of Cer1 (a) and Cer2 (b). Despite the relatively low level of EGF-R expression in Cer1, antibody therapy led to a significant growth inhibitory effect, whereas Cer2, with an approximately two-fold higher EGF-R expression, showed no significant response to the treatment. The arrows indicate the day of antibody injection 1 week following tumour transplantation.

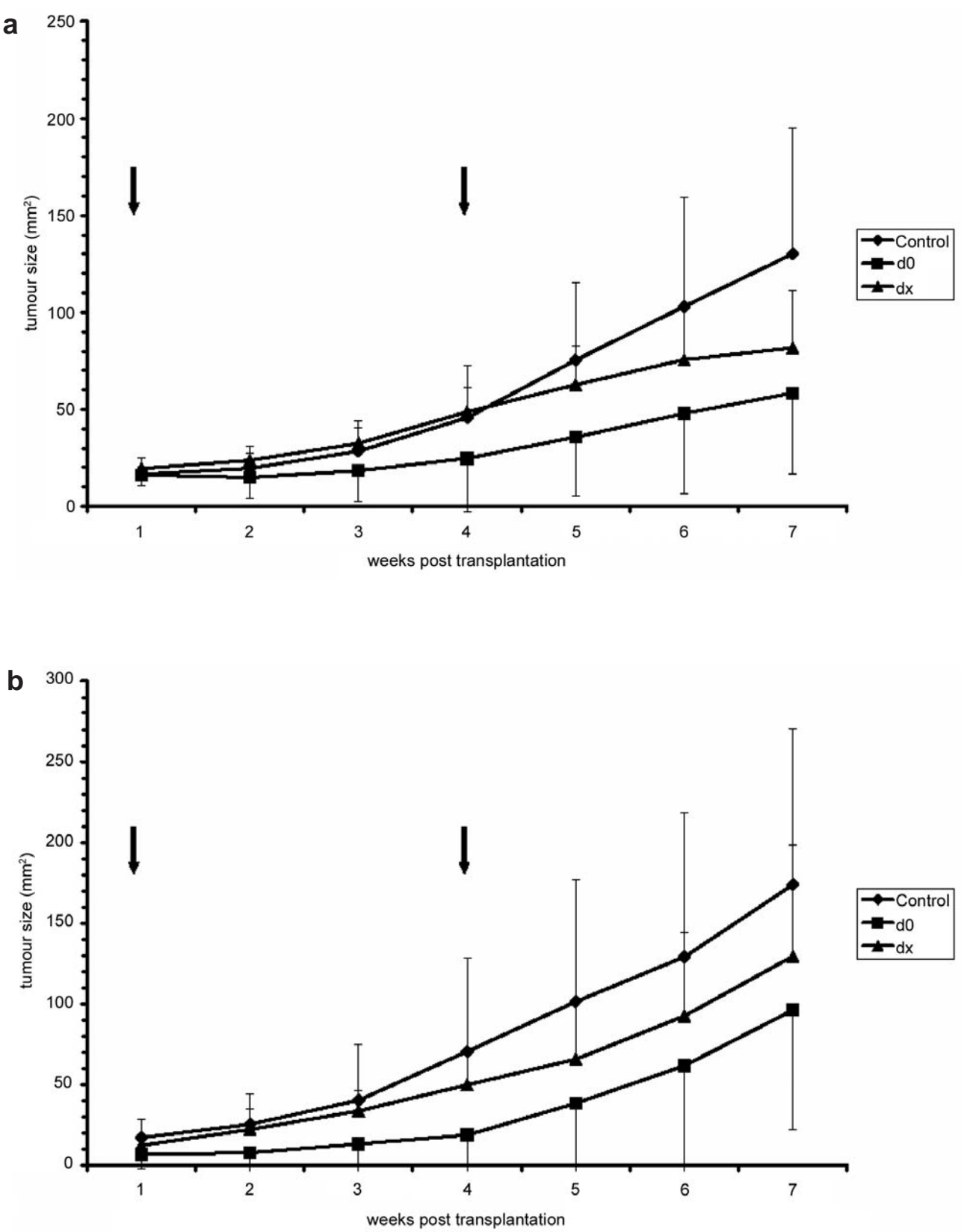


Figure 3.

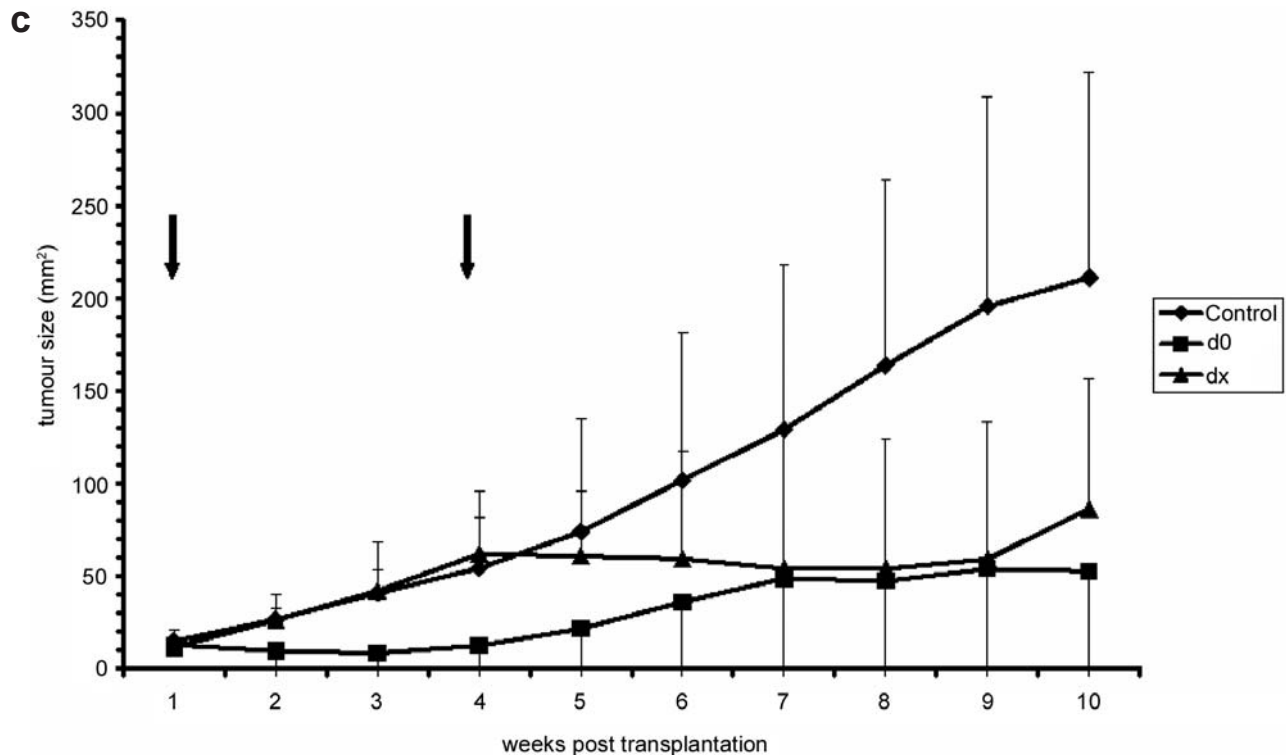


Figure 3. Growth curves of Cer1 (a), Cer3 (b) and Cer4 (c). As expected, Cer1 showed a significant therapeutic response to EMD 55,900 treatment when the antibody was applied 1 week following transplantation (d0). Moreover, a significant response was also detectable when the treatment was started 4 weeks post-transplantation (dx). In contrast Cer3 only showed significant growth inhibitory effects to antibody application when the animals were treated 1 week post-transplantation (d0). Therapy 4 weeks following transplantation had no significant growth inhibitory effects, despite the fact that the Cer3 tumour had the highest EGF-R expression tested in this study. Cer4, with EGF-R expression levels comparable to Cer2, also showed significant growth inhibitory effects on EMD 55,900 treatment.

Table I. Characteristics of tumours investigated in the study.

Tumour	EGF-R	MGI	P values d0	P values dx	CD31
Cer1	30-50	59	0.001	0.038	+++
Cer2	50-100	n.a.	>0.05	n.a.	++++
Cer3	250-350	74	0.001	>0.05	+++
Cer4	60-130	80	0.0001	0.003	+

Table I reflects the EGF-R expression (fmol/mg total protein), the maximal growth inhibition of treated tumours against the corresponding control in % (MGI), the level of significance of growth reduction when treatment was started 1 week following transplantation (d0) or when the mean tumour size had reached 50 mm<sup>2</sup> (dx), respectively. CD31 reflects the semi-quantitative determination of pre-treatment microvessel density. (n.a.= not applicable).

that tumours with high EGF-R expression patterns do not always respond to a higher degree to this treatment. Moreover, tumours with two-fold higher EGF-R expression than others showed no therapeutic response at all, as

demonstrated in the Cer2 tumour. Interestingly, we were not able to demonstrate this effect in tumours of the female breast, where the therapeutic response *in vivo* seemed to depend mainly on EGF-R expression (8). One might speculate that the current observations might be due to the different type of tumours in comparison to breast cancer. However, it is known that tumours of the uterine cervix exhibit a broad range of EGF-R expression, in contrast to tumours of the breast where most were low EGF-R expressing. Furthermore, epithelia of the uterine cervix might be more responsive to EGF than epithelia of the breast, where predominantly the Her2/neu receptor plays a key role in the development of this tissue. In contrast to our previous study (8), we found the degree of connective tissue in this type of tumour to be relatively evenly distributed, indicating a secondary role. This seems to be a major difference in the tumours investigated. Nevertheless, our data clearly demonstrate that the pre-treatment vessel density of the tumour has a strong impact on EMD 55,900-induced growth inhibition *in vivo*. The detailed mechanism



of action of this antibody still remains speculative. However, the disruption of autocrine stimulation processes induced by EGF is generally accepted. Inhibition of the EGF pathway leads to hypophosphorylation of the Rb protein, resulting in G1 arrest of tumour cells (12). Moreover, a recent study has demonstrated that anti-EGF-R treatment also induces apoptosis in keratinocytes immortalized by human papilloma virus 16, associated with cervical cancer (13). Furthermore, EMD 55,900 is also capable of inducing antibody-dependent cell-mediated cytotoxicity (ADCC) *via* the Fc-fragment (4).

Recapitulating the findings of this and our previous investigations, we conclude that EGF-R protein expression is not a predictor for therapy response to EMD 55,900 treatment in cervix carcinomas. The detailed mechanism for this effect needs further investigation. As far as we can speculate, the data presented in this investigation strengthen the findings that a high pre-treatment CD31-score significantly decreases the antitumour activity of anti-EGF-R antibodies. This might indicate an anti-angiogenic effect of EMD 55,900. This anti-angiogenic effect might be due to disruption of paracrine stimulation processes triggered by tumour cell-derived EGF stimulation. Nevertheless, further investigations into the complex interaction between tumours, stroma and vascularization might shed light on this question. However, this work, along with other studies, demonstrates that antitumour therapy which targets EGF-R, results in down-regulation of tumour-derived angiogenic factors, thus indicating that combination therapy might lead to a significant response in those tumours that do not respond to anti-EGF-R therapy (1, 14-15).

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