Tumor Necrosis Factor α Sensitizes Low Epidermal Growth Factor Receptor (EGFR)-expressing Carcinomas for Anti-EGFR Therapy

Markus Hambek, Christine Solbach, Hans-Georg Schnuerch, Marc Roller, Manfred Stegmueller, Anja Sterner-Kock, Jan Kiefer, and Rainald Knecht

Department of Otorhinolaryngology [M. H., M. R., J. K., R. K.], Department of Obstetrics and Gynecology [C. S., M. S.], Central Research Unit [A. S.-K.], School of Medicine, J. W. Goethe University, 60590 Frankfurt, Germany, and Lukas Krankenhaus [H-G. S.], 41460 Neuss, Germany

ABSTRACT

Analysis of 1060 xenotransplants derived from cancer cell lines as well as spontaneously occurring tumors from the larynx, pharynx, mammary gland, uterine cervix, and vulva revealed that tumor regression induced by treatment with monoclonal antibodies (EMD 55900 and EMD 72000) against the epidermal growth factor receptor (EGFR) could be enhanced by tumor necrosis factor α (TNF-α) treatment in vivo. Moreover, tumors that primarily do not respond to antibody treatment can be made susceptible by additional TNF-α treatment. To investigate the in vivo effects of monoclonal antibodies, we treated tumors derived from cell lines (A431 and Detroit 562) as well as spontaneously occurring squamous cell carcinomas and adenocarcinomas (transplanted on NMRI-nu/nu mice) generally with EMD 55900 (40 μg/g mouse) and its humanized version EMD 72000 (40 μg/g mouse). When treated with EMD 55900 and EMD 72000, carcinomas with an EGFR concentration of ≥70 fmol/mg protein showed significant reduction in tumor size compared with untreated controls. The degree of tumor regression correlated with the EGFR concentration of the tumor. In mice treated with TNF-α (0.5 μg/g mouse) and EMD 55900/72000 simultaneously, we observed enhanced antitumor effects up to complete tumor eradication. Carcinomas with an EGFR concentration <70 fmol/mg protein could be made susceptible to treatment with EMD 55900 and EMD 72000 by simultaneous treatment with TNF-α, resulting in a significant reduction in tumor size.

INTRODUCTION

There is recent evidence in the literature for the interaction of TNF-α with the EGFR (1). Because TNF-α has been identified as a potential cytokine for inducing apoptosis, its apoptotic effects may be used therapeutically. TNF-α binds to specific cell surface receptors and activates a cell death program as described previously (1, 2). The stimulated TNF-α receptor induces apoptosis through the stimulation of intracellular caspases. In contrast, the inhibition of these proteases (caspases) suppresses TNF-α-induced apoptosis (1). EGFR expression has been strongly increased by TNF-α via the p55 TNF-α receptor (3, 8). Furthermore, TNF-α improves the vascular permeability of the tumor microvasculature and increases the uptake of immunoglobulins and monoclonal antibodies into the tumor (4). Recent studies (1) revealed evidence that TNF-α reduces EGFR tyrosine phosphorylation by stimulating a protein tyrosine phosphatase. It has also been demonstrated that EGF inhibits TNF-α-induced apoptosis (5). This suggests a sensitizing effect between the blocking of the EGFR and the effects of TNF-α.

It is well known that different malignant tumors overexpress the EGFR or amplify genes encoding the EGFR. In tumors of the head and neck, EGFR gene amplification occurs in 10–20% of squamous cell carcinomas, whereas overexpression of the EGFR occurs in a much higher proportion (6). In mammary carcinomas, gene amplification has been observed only rarely (6). Overexpression of the EGFR on cellular surfaces in adenocarcinomas of the mammary gland also occurs rarely (6). There have been a few studies showing that targeting the overexpressed EGFR with monoclonal antibodies leads to a reduction in tumor growth in vivo (2, 7–11). For strong antitumor activity of the monoclonal antibodies, high expression of the EGFR seems to be required (12, 13).

Referring to former studies (1–3) characterizing the interactive effect of TNF-α and monoclonal antibodies against the EGFR, we investigated possible new implications for anti-EGFR monoclonal antibody treatment. In this report, we first described the observation, in a large series of xenotransplant experiments, of a sensitizing effect between monoclonal antibody treatment against the EGFR and treatment with TNF-α. The combination of both agents resulted in enhanced tumor regression.

MATERIALS AND METHODS

TNF-α and Antibodies. The murine antibody EMD 55900 and its humanized version EMD 72000 were generously provided by E. Merck KGaA (Darmstadt, Germany). Monoclonal antibody EMD 55900 was induced by immunization of BALB/c mice with cells of the human carcinoma cell line A431 (12). Phase I trials revealed that the immune systems of patients receiving EMD 55900 were inducing antibodies (human antimouse antibodies) against the drug because of its murine origin. For creating EMD 72000, mouse-complementary-determining regions have been reconstituted from EMD 55900 into a human IgG1 framework as described previously (12). The monoclonal antibodies are highly specific for the human EGFR and recognize the deglycosylated region of the EGFR M,, ~110,000 EGF-binding domain, but not the other peptides of the EGFR. Binding of both antibodies correlates with the number of EGFR-binding sites and is strongest with A431 carcinoma cell membranes. Scatchard’s analyses of 125I-labeled EMD 55900 binding to A431 membranes revealed the presence of two binding components: (α) a high-affinity, low-capacity component (K, ~ 10 pM, 5 × 10⁸ sites/μg membrane protein); and (β) a relatively low-affinity, high-capacity component (K, ~ 1 nM, 2 × 10¹⁰ sites/μg membrane protein). Plasma-elmination half-lives of the murine (EMD 55900) and reshaped (EMD 72000) version were similar: short in the Cynomolgous monkey (26 h for EMD 55900 and 31 h for EMD 72000) and long in rats (240 h for EMD 55900 and 225 h for EMD 72000; Ref. 12). Biodistribution studies of 125I-EMD 72000 in xenografted nude mice revealed a tumor/blood ratio of 1:2 on day 1 and 5:1 on day 18, respectively (12). TNF-αgenerously was provided by G. R. Adolf, Bender (Boehringer Mannheim), Vienna, Austria.

Cell Lines. Cell lines A431 (squamous cell carcinoma of the vulva) and Detroit 562 (squamous cell carcinoma of the pharynx) were obtained from the American Type Culture Collection (Nr.CRL-1555 and CCL-138). For establishing cell lines as tumors on NMRI nu/nu mice (6–8 weeks of age), 2 × 10⁶ cells of A431 and Detroit 562 squamous cell carcinoma cell lines were injected s.c.

Tumors. Spontaneously occurring tumors derived from gynecological and head and neck patients were established on nude mice by direct transplantation of small tumor pieces (~2 mm³). We used squamous cell carcinomas of the larynx, pharynx, and uterine cervix. Adenocarcinomas were derived from tumors of the mammary gland. Established tumors on nude mice were transplanted again onto NMRI nu/nu mice as described previously (14).
**Treatment Course.** Xenotransplants (n = 1060) derived from squamous cell carcinomas of the uterine cervix and vulva (n = 180), mammary adenocarcinomas (n = 420), and squamous cell carcinoma of the larynx (n = 200) and pharynx (n = 260) were investigated. Tumor size was measured with vernier calipers every 5 days. Tumor size was calculated by the product of length × width. One week after transplantation, nude mice were randomly divided into groups of 10 animals [(a) control group; (b) antibody group; (c) TNF-α group; and (d) combined treatment group]. Treatment was started when tumors of all groups reached a mean size of 25 mm³. Typically, treatment of nude mice with EMD 55900/EMD 72000 and TNF-α consisted of a fixed regimen. Nude mice were injected i.p. a total of two times with the monoclonal antibody at a dose of 40 μg antibody/g body weight on days 1 and 7 of the treatment course. This concentration of EMD 55900/EMD 72000 has been established previously as the dose of monoclonal antibodies required for prevention of tumor growth in the nude mouse model (14). TNF-α was given five times once a day (on days 2–6 of the treatment course between antibody treatments) at a dose of 0.5 mg/g body weight. In the combined treatment group, animals received the described compounds according to the regimen described above. Controls were treated accordingly with the vehicle. The mice were followed up for tumor growth for at least 42 days.

**Analysis of EGFR Concentration.** Tissues obtained from transplanted tumors were dissected and then pulverized in liquid N₂ by a microdispermator (Braun, Melsungen, Germany). The tissue powder was suspended in a phosphate buffer [0.14 M NaCl, 2.6 mM KCl, 8 mM Na₂HPO₄ (pH 7.4), and 1% Tween 20], then homogenized in a Teflon/glass homogenizer. Ultracentrifugation for 1 h at 100,000 × g yielded a supernatant containing the cytosolic fraction and soluted cell membranes. Protein content was measured in the supernatant by Bio-Rad Protein Assay (Bio-Rad, Munich, Germany). The fraction and soluted cell membranes. Protein content was measured in the samples as well as the Mann-Whitney test. All results were expressed as mean ± SD and analyzed by an analysis of variance (ANOVA). Differences between groups were considered significant at P < 0.05.

**RESULTS**

**Tumor Growth Reduction Induced by Anti-EGFR Monoclonal Antibody EMD 55900 Correlates with EGFR Concentration.** Analysis from xenotransplantation experiments (Table 1) demonstrated that antibody efficacy depends on EGFR concentration rather than on the histogenetic origin of the tumor. As seen in Fig. 1, 35 days posttreatment with EMD 55900, tumors were reduced in size when the EGFR concentration of the tumor tissue was high (>70 fmol/mg protein). In contrast, tumors with low EGFR concentration (<70 fmol/mg protein) were nonsignificantly reduced in size. Animals in experiment A with high EGFR-expressing squamous cell carcinomas (mean EGFR value, 360 fmol/mg protein) showed maximum reduction of tumor size after treatment with EMD 55900 (Fig. 1). In experiment G (high EGFR-expressing adenocarcinoma; mean value, 320 fmol/mg) tumor growth was strongly (P < 0.001) inhibited by the monoclonal antibody EMD 55900 (Fig. 1). In contrast, tumors in experiment M had low EGFR concentrations (mean value, 75 fmol/mg protein) and were only moderately growth-inhibited (Fig. 1). On the basis of those results, we subdivided tumors into two groups as follows: (a) high EGFR-expressing tumors with significant growth reduction in response to EMD 55900 antibody treatment; and (b) low EGFR-expressing tumors with nonsignificant growth reduction in response to EMD 55900 (Fig. 2).

**TNF-α Monotherapy Leads to Moderate Growth Reduction.** As seen in Fig. 2, TNF-α monotherapy moderately reduces tumor size in either high or low EGFR-expressing tumors. However, those effects were only significant in high EGFR-expressing tumors (P < 0.01).

**TNF-α Enhances Anti-EGFR Effects and Sensitizes Cancer Cells to Antibody Treatment.** To examine whether the antitumor effects of monoclonal antibodies could be enhanced by TNF-α, we decided to apply TNF-α to nude mice bearing xenografts with variable concentrations of the EGFR. Antibody application resulted in significant growth inhibition only in the high EGFR-expressing group. In experiment G, 35 days posttreatment, tumor size in the EMD 55900 group showed a reduction to 5% of the control size (Fig. 1); in

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* Mamma, mammary gland.

Fig. 1. Tumor growth inhibition by EMD 55900 on different tumor xenografts compared with the tumor EGFR content. Letters refer to experiments described in Table 1. Tumors ≥2 mm in size were transplanted s.c. into the nude mice. Treatment was started when tumors reached a mean size of 25 mm³. Nude mice were injected i.p. with EMD 55900 (n = 10 for each tumor) twice, on days 1 and 7 of the treatment course. Control mice received no treatment. On day 42 after the start of treatment, tumor size was measured and calculated as the percentage of control. The EGFR content of different tumors was determined by ELISA before the treatment course. Ps for reduced tumor size were <0.001 in experiments above the dotted line, and >0.15 in experiments below the dotted line. The data represent the mean tumor size in percent of control.
susceptible to growth inhibition by TNF-α alone (Fig. 3). However, treatment of low-EGFR-level tumors with the combination of TNF-α and EMD 55900 showed highly significant ($P < 0.001$) tumor growth reduction compared with the control group. In tumors with low EGFR-expression, combination treatment led to a tumor size reduction of 50% of the control size (Fig. 2). For example, in experiment I, which is shown in Fig. 6, tumors were neither susceptible to treatment with EMD 55900 nor with TNF-α, but showed a highly significant tumor growth reduction to 30% of the control size ($P < 0.0001$) in response to combination treatment, suggesting a synergistic effect between TNF-α and anti-EGFR monoclonal antibodies.

**EMD 72000 Effects.** We were able to confirm the described results even with the humanized antibody EMD 72000 in the treatment of tumors from experiments A-G (data not shown). To compare directly both antibodies and to evaluate the dose-dependence of xenotransplants on antibody treatment, animals with a mean weight of 25 g were treated in a dose-escalating manner with increasing doses of EMD 55900 and EMD 72000: 0.02 mg, 0.2 mg, and 2 mg/25 g body weight. We observed that with each higher dose, tumor size decreased experiment A, antibody treatment reduced tumor size to 0.025% of the control size (Fig. 3). In experiments B and F, antibody treatment reduced tumor size to 16% and 22% of the control size ($P < 0.001$; Figs. 4 and 5), respectively. Combination treatment showed the maximum reduction of tumor size, which was significantly lower than treatment with either substances alone ($P < 0.001$; Fig. 2). In experiment F, as shown in Fig. 5, combination treatment led to tumor size reduction to 5% of the control size. In experiment B, we observed tumor growth reduction by combination treatment of up to complete eradication of tumors (Fig. 4). In experiments where EMD 55900 treatment alone resulted in the maximum tumor size reduction (EGFR level, >350 fmol/mg), combining treatment with TNF-α could not enhance those effects any further (Fig. 3).

Xenotransplants with EGFR concentrations of <70 fmol/mg were not significantly growth-inhibited by EMD 55900 and only a little
of TNF-α (1–3, 21–23), we introduced this cytokine into our investigations to enhance antibody efficacy if antibody treatment as monotherapy did not lead to maximum tumor size reduction. It has been shown that EGFR expression has been increased by TNF-α via the p55 TNF receptor (1). Furthermore it was demonstrable that vascular permeability of the tumor microvasculature has been increased by TNF-α, leading to improved uptake of monoclonal antibodies into the tumor (4). It is also known that proinflammatory cytokines such as TNF-α are able to activate a cell death program (1, 2). Therefore, apoptosis is being induced by the TNF-α receptor through receptor clustering and stimulation of caspases. Inhibition of these caspases suppresses TNF-α-induced apoptosis (1). Recently it has been shown that TNF-α reduces EGFR tyrosine phosphorylation via stimulating a protein tyrosine phosphatase, leading to blockade of the EGFR-mediated signal transduction (1). It also has been demonstrated that EGF inhibits TNF-α-induced apoptosis (5).

In conclusion, there seems to exist a sensitizing or synergistic effect resulting from the interaction between the EGFR blocked by monoclonal antibodies and TNF-α, resulting in tumor size reduction. The combination treatment of monoclonal antibodies and TNF-α led to a highly significant and synergistic tumor growth reduction in xenografts with high EGFR expression values. However, if EMD 55900 or EMD 72000 treatment alone led to maximum tumor size reduction, additional TNF-α application could not enhance those effects any more. Xenografted tumors with low EGFR protein concentrations (<70 fmol/mg) showed only little tumor size reduction when treated with monoclonal antibodies or TNF-α alone. A putative mechanism for these observations could be an enhancement of EGFR expression induced by TNF-α resulting in increased occupancy of EGFRs by anti-EGFR antibodies. This thesis is supported by the fact that combination treatment led to significant tumor size reduction, even if monotherapy of either antibodies or TNF-α showed very little effect by itself. Therefore we hypothesize that TNF-α leads to an increase in the vascular permeability of the connective tissue stalk supporting the neoplastic epithelial cells of the tumor. This could lead to an enhanced extravascular concentration of the applied monoclonal antibodies in the tumor environment resulting in increased occupancy of EGFRs. Furthermore the combination treatment could lead to an increase in apoptosis of the neoplastic cells. These mechanisms are probably predominant in tumors with low EGFR concentration. To summarize, the observed effects of antibodies against the EGFR can reduce tumor growth even in cancers with low EGFR expression in the presence of TNF-α.

It is well known that overexpression of receptors of the erbB family such as the EGFR on malignant transformed cells is strongly associated with the ability to induce proliferation in neoplastic and normal tissues (4, 15–19). As a result, those receptors became the target of a variety of monoclonal antibodies with therapeutic implications for the treatment of cancer cells (2, 7–11, 20). For strong antitumor activity of the monoclonal antibodies, a high expression in tumor cells seems to be required (12, 13). In adenocarcinomas of the mammary gland, EGFR expression of ≥50 fmol/mg protein occurs in 5% of those neoplasias, whereas squamous cell carcinomas of the vulva and the uterine cervix express the EGFR in concentrations of ≥50 fmol/mg in 33% and 62% of cases, respectively (14). In contrast, nearly 100% of squamous cell carcinomas of the larynx and pharynx express the EGFR in concentrations of ≥50 fmol/mg protein (14).

In this context we and others (14) could show that the growth inhibitory effects of the monoclonal antibodies EMD 55900 and EMD 72000 depend mainly on the EGFR expression value rather than on the tumor entity. Our data demonstrate a strong EGFR-dependent antitumor efficacy of both monoclonal antibodies on squamous cell carcinoma xenografts of the head and neck and the uterine cervix as well as adenocarcinomas of the mammary gland.

Therefore, we determined a cut-off level of EGFR expression (70 fmol/mg) in tumor xenografts to optimize the use of monoclonal antibodies as therapeutic agents. Carcinomas with low EGFR protein levels (<70 fmol/mg) were not susceptible to treatment with the antibodies. In contrast, carcinomas with high EGFR protein levels (>70 fmol/mg) were highly significantly growth-inhibited.

It has not been demonstrated thus far that carcinomas expressing low levels of the EGFR also can be made susceptible to EGFR antibody therapy. Considering earlier studies on the antitumor effects
cytokines like TNF-α. This may extend EGFR antibody treatment to a broader spectrum of carcinomas and influence future treatment protocols after our phase I/II studies with EGFR antibodies in cancers of the head and neck and other epithelial tumors.

REFERENCES


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