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TGF-α-Driven Tumor Growth Is Inhibited by an EGF Receptor Tyrosine Kinase Inhibitor

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The simultaneous presence of the EGFR and its ligand TGF-α in human tumor tissues suggests that autocrine TGF-α stimulation drives tumor growth. Here we show that autocrine TGF-α stimulation does cause increased tumor growth *in vivo*, an effect that was proven to be mediated via EGFR activation, and that this TGF-α/EGFR autocrine loop was accessible to an EGFR specific tyrosine kinase inhibitor. Clones of the EGFR expressing glioma cell line U-1242 MG were transfected with TGF-α cDNA using a tetracycline-inhibitory system for gene expression. TGF-α expression was inhibited by the presence of tetracycline, and subcutaneous tumors forming from cell lines injected into nude mice could be inhibited by feeding mice tetracycline. We confirmed that TGF-α mRNA and protein were present in these tumors and that, subsequently, the endogenous EGFR was activated. Tumor growth could be inhibited by an EGFR specific tyrosine kinase inhibitor of the type 4-(3-chloroanilino)-6,7-dimethoxy-quinazoline, administered daily by intraperitoneal injection, thereby interrupting the autocrine loop. © 2002 Elsevier Science

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Transforming growth factor alpha (TGF-α) is a potent mitogen structurally and functionally related to epidermal growth factor (EGF). The mature TGF-α is a 50-amino-acid peptide of 5.5 kDa that is derived from a cell surface precursor protein (1). TGF-α is a ligand for the epidermal growth factor receptor (EGFR), which is a 170-kDa transmembrane glycoprotein that exhibits a ligand-inducible protein tyrosine kinase activity (2, 3).

Many human malignant tumors are known to express both TGF-α and EGFR, and an autocrine TGF-α/EGFR stimulatory loop has been suggested to play a role in several situations of normal and neoplastic growth both *in vitro* and *in vivo* (4–8). *In vivo* studies showed for example that TGF-α increased the tumorigenicity of human colon cancer cells and led to quicker formation of tumors in nude mice (9). Attempts have also been made to interrupt the endogenous TGF-α/EGFR autocrine stimulation of tumor cells by using inhibitory EGFR antibodies (10–13).

Recently, the increased understanding of the EGFR-mediated signaling pathways has encouraged the use of different tyrosine kinase inhibitors in order to interrupt the EGFR mediated signals. Tyrosine kinase inhibitors represent a class of putative therapeutic agents for treatment of cancer that includes more or less selective inhibitors of cell surface protein tyrosine kinase receptors. Different organic and synthetic compounds have been examined for their effect on EGFR-tyrosine phosphorylation and EGFR-dependent cell proliferation (14). Tyrphostins are a class of low-molecular-weight compounds that have been widely used to inhibit protein tyrosine kinases, including the EGFR kinase (15–17). Kondapaka and Reddy (18) showed that tyrphostin RG-13022 could inhibit TGF-α induced phosphorylation of the EGFR in cultured human prostate carcinoma cells, while Miyaji *et al.* (19) showed the same inhibitory effect in EGF-stimulated glioma cell lines. Penar *et al.* (20) showed that tyrphostin A25 can inhibit glioblastoma invasion of the brain tissue.

In cultures of human glioma cell lines, the simultaneous expression of EGFR and TGF-α was demonstrated, indicating the possibility of endogenous TGF-α/EGFR autocrine growth stimulation (21). We have previously used the tetracycline off system for expressing and studying the effects of TGF-α in human glioma cells *in vitro* (22, 23) and demonstrated that the introduced TGF-α increased the motility of single glioma cells by creating a "private" autocrine loop. In the present study we have used the same system to study the effect of autocrine TGF-α on cell proliferation *in vitro* and on subcutaneous tumor growth in nude mice.

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TGF-α expression *in vivo* was controlled by adding tetracycline to the mice drinking water.

It is known that a majority of human brain tumors co-express TGF-α and EGFR *in vivo* (24) and this means that there is a chance of endogenous EGFR activation also in the absence of the EGFR gene amplification and rearrangement commonly seen in glioma. In this work we have tested whether such an autocrine stimulatory loop changes the growth properties of glioma cells *in vivo*. The aim was also to test whether such an autocrine loop was accessible for treatment with a tyrosine kinase inhibitor. It was found that tumors of TGF-α transfected clones grew much larger in the absence of tetracycline compared to when tetracycline was present. Furthermore, in order to test the specificity of the TGF-α/EGFR autocrine pathway in promoting tumor growth an intraperitoneally injected tyrosine kinase inhibitor of the type 4-(3-chloroanilino)-6,7-dimethoxy-quinazoline was tested for its effect on tumor growth and EGFR tyrosine phosphorylation was tested. Quinazolines are known to be quite specific inhibitors of the EGFR kinase, acting as efficient reversible inhibitors and competitors at the ATP binding site (25, 26).

In summary, this work shows that an active TGF-α/EGFR autocrine loop leads to increased tumor growth *in vivo* in nude mice and that such an autocrine loop is well accessible for treatment with an EGFR specific tyrosine kinase inhibitor.

METHODS

Cells. The human glioma cell clones used in this work were derived from the U-1242 MG cell line after transfection with a TGF-α cDNA using the tetracycline off system as previously described (22). The TGF-α expressing clones used in the present study were clones 4, 71, and 72. TGF-α mRNA and protein were expressed in culture only in the absence of tetracycline. For comparison, we used other clonal cells derived from the same original line, i.e., clones 5, 7, and 8, that expressed the neomycin resistance marker only as well as the non-transfected original cell line U-1242 MG. U-1242 MG original cells were grown routinely in Eagle's minimum essential medium (MEM) supplemented with 10% bovine calf serum (GIBCO) and antibiotics (100 units of penicillin and 50 μg of streptomycin per milliliter), and the transfected clones were grown in the same medium with the addition of G418 (0.5 mg/ml) and tetracycline (1 μg/ml).

In vitro growth curves. Three different sets of experiments were performed. In a first set, aliquots of cells from the TGF-α expressing clones and control clones were thawed and grown for 5 days in routine medium in the presence of tetracycline (1 μg/ml) and then seeded in the presence and absence of tetracycline in 35-mm dishes at a low cell density (25×10^3 cells/dish). Media were changed every 48 h and cell numbers in duplicate dishes were determined by trypsinization and counting in a Coulter counter (Coulter Electronic, Herpendon, UK). In a second set of experiments, cells from the TGF-α expressing clone 4 and from control clone 8 were seeded as mentioned above in the presence and absence of tetracycline and in the presence and absence of the EGFR blocking monoclonal antibody 225 (4 μg/ml) (kind gift from Dr. Mendelsohn, University of Houston, TX). In a third set of experiments clone 4 and clone 8 cells were seeded as mentioned above in the presence and absence of tetracycline and also in the presence and absence of a commercially available EGFR tyrosine kinase inhibitor of the type 4-(3-chloroanilino)-6,7-dimethoxyquinazoline (RTKI) (25, 26). The inhibitor was dissolved in dimethyl sulfoxide (DMSO) and added daily to the cultures at the concentration of 4 μM. Control cultures received only DMSO.

In vitro testing of the EGFR tyrosine kinase inhibitor. The RTKI was first tested *in vitro* for its ability to inhibit EGFR tyrosine kinase activation mediated by the induced autocrine TGF-α and activation mediated by exogenously added EGF. Subconfluent clone 4 cells, grown in the absence and presence of tetracycline, were serum-starved in Eagle MEM/F10 (1:1) overnight. The RTKI was dissolved in DMSO and added to the culture media at final concentrations of 2 and 10 μM, and cultures were incubated on ice for 5 min. Cells were then scraped off from the dishes in phosphate-buffered saline (PBS) and lysed in 1% Nonidet P-40, 0.15 M NaCl, 20 mM Tris, pH 7.5, 5 mM EDTA, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% aprotinin, 1 mM sodium orthovanadate (Na_3VO_4), and 100 μM phenyl arsine oxide (PAO) for 15 min at 4°C. After centrifugation, protein concentration was measured using Bio-Rad assay and equal amounts of protein were immunoprecipitated with agarose conjugated PY20 (Transduction Laboratories, Affiniti Research Products, Ltd.) or with WGA–Sepharose (Pharmacia) for 3 h at 4°C. After three washes with lysis buffer, the precipitates were heated for 5 min at 95°C in SDS-sample buffer and fractionated by electrophoresis in 7.5% SDS–polyacrylamide gels. Proteins were then transferred onto nitrocellulose filters using Semi-dry Transfer Cell (Bio-Rad). Filters were blocked and first probed with a rabbit polyclonal EGFR antibody raised against peptides corresponding to residues 1005 to 1016 of the human EGFR protein (sc-o3; Santa Cruz Biotechnology, Inc.) at 1:100 dilution. After detection of the signal with enhanced chemiluminescence (Amersham), filters were stripped by incubation with 100 mM β-mercaptoethanol, 2% SDS, 20 mM Tris–HCl, pH 6.8, for 30 min at 50°C with occasional agitation and reprobed with the PY20 antibody at 1:2500 dilution. For exogenous stimulation, cells received EGF (Becton–Dickinson Labware) at 50 ng/ml and were incubated for 1 h on ice, scraped off, and treated as above.

The functional stability of the RTKI was also tested *in vitro*. Clone 4 cells were grown in the absence of tetracycline for 5 days after which the inhibitor was added at 4 μM, diluted in DMSO. Parallel control cultures received only DMSO. Cells were collected and lysed 24 and 48 h after the addition of the inhibitor. Lysates were treated as described above.

Animal experiments. Athymic nu/nu fertile female Balb/c mice were purchased from Bomholt gard, Denmark. They were kept in cages in a separate ventilated/humidified rack at a temperature of 22°C and a 12 h day/night cycle and were fed *ad libitum* standard laboratory food (Lactamin R36) and water.

The cells to be injected were grown routinely as described above. Before injection, cell cultures were grown in the absence of tetracycline for 5 days. Cells were trypsinized, collected with media and pelleted by centrifugation at 2000 rpm for 5 min, resuspended in PBS and centrifuged a second time. The cells were finally resuspended in PBS and counted, and using a Hamilton syringe, 25 μl of the cell suspension containing 5×10^6 cells was injected subcutaneously on the right thigh or on the back of the mice.

Tetracycline was dissolved at the concentration of 2.2 mg/ml in water made acidic (pH 2.7) with acetic acid. The drinking water was replaced with acidic water containing tetracycline, or acidic water only, a few days prior to tumor cell injection. The RTKI was administered by intraperitoneal injections beginning when tumors were palpable, that is, when their sizes reached about 30 mm^3. The inhibitor was dissolved in DMSO and injected daily starting with a low dose of 10 mg/kg/day after which the dose was increased daily to 20, 30, and finally 40 mg/kg/day. Tumor growth was followed two to three times weekly by measuring perpendicular tumor diameters in millimeters by means of vernier calipers. Tumor volume was calcu-

lated using a previously described formula (27). Animals were killed by cervical dislocation after 4–8 weeks or when tumor volume exceeded 2 cm^3. The tumors were removed and divided into several parts. One part was fixed in formaldehyde and the other pieces were snap frozen in liquid nitrogen and stored at −70°C for later mRNA and protein analysis.

Design of in vivo experiments for assessing the effect of a TGF-α/EGFR autocrine loop on tumor growth. Four different sets of experiments were performed by injecting the different clones subcutaneously in nude mice.

In the first set of experiments (cf. Fig. 6a), a total of 20 mice were injected with the TGF-α expressing clones 4 ($n = 3$), 71 ($n = 3$), and 72 ($n = 4$), the control clones 5 ($n = 2$), 7 ($n = 2$), and 8 ($n = 2$) and the non-transfected cell line U-1242 MG ($n = 4$). In these experiments, mice were not given tetracycline in the drinking water. The duration of the experiment *in vivo* was 7 weeks. In a second set of experiments (cf. Fig. 6b) a total of 10 mice were injected with clone 4 cells. When the largest tumors were palpable, approximately 10 days after injection, half of the mice were given tetracycline in their drinking water, while the other half continued with acidic water. Mice were sacrificed after 4 ($n = 6$) and 3.5 ($n = 4$) weeks in two otherwise similar series. In a third set of experiments (cf. Fig. 6c) 8 mice were injected with clone 4 cells and another 8 mice injected with the clone 8 cells. After injection, half of the mice in each group were given tetracycline in acidic water and the other half were given only acidic water. Mice were sacrificed after 6 weeks. In a fourth set of experiments (cf. Fig. 6d), 25 mice were used but 2 died of unrelated causes after a few days and were not included in the study. Clone 4 and clone 8 cells were injected and half of the mice in each group received daily intraperitoneal injections as described above with the RTKI suspended in DMSO and the other half received only DMSO. The mice were sacrificed after 3 weeks. On the last day of the experiment, the mice were given injections of the RTKI 3 h prior to tumor removal.

Northern blot analysis. RNA was prepared from the frozen tumor tissue as described (28). Total RNA (15 μg/lane) in a buffer containing ethidium bromide was fractionated by formaldehyde-agarose gel electrophoresis, transferred to nitrocellulose membranes and probed with a TGF-α cDNA fragment as described previously (22). Filters were stripped and reprobed using a glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) (29) probe as a control of equal loading. Control cells were non-transplanted clone 4 cells that had been incubated *in vitro* in the absence and presence of tetracycline and U-563 MG glioma cells that are known to express the endogenous TGF-α mRNA (21). As an additional control we used U-343 MGa Cl2:6 cells (21) that had been transfected with a TGF-α cDNA fragment in a regular expression vector without using the tetracycline repressible system, and where the transfected cells were known to express TGF-α mRNA both in the presence and absence of tetracycline (El-Obeid, unpublished data). Total RNA was prepared from the cultured cells using the LiCl/urea method (30) and was otherwise processed the same way as above. Total RNA was also prepared in the same way from all the TGF-α expressing clones and control clones cultured in the presence and absence of tetracycline. Northern blot analysis was performed to confirm their TGF-α expression levels before use in animal experiments.

Detection of TGF-α protein in explanted tumor cells. To detect TGF-α protein in the tumor cells of the xenografts, tumor tissue was explanted and grown *in vitro*. Tumors were excised 3 weeks after injection, disassociated mechanically and cultured in routine medium with 1 μg/ml tetracycline and 0.5 mg/ml G418 for 2 weeks. Cells were pooled and seeded at a density of 5×10^5 cells/100 mm dish and parallel cultures were incubated for 5 days with and without tetracycline. Media were thereafter changed to Eagle's MEM/F10 1:1, collected after 24 h, and analyzed for TGF-α protein by using an ELISA kit (Oncogene Science, Manhasset, NY) as described (22). As a positive control, keratinocyte-conditioned medium was analyzed in parallel.

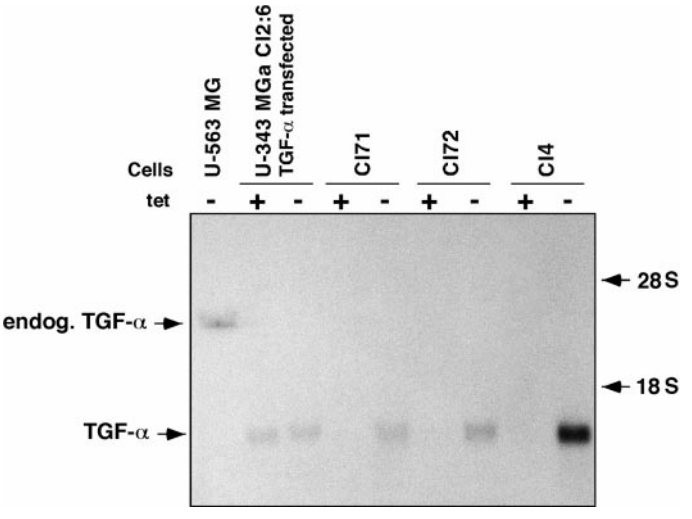

FIG. 1. Tetracycline regulated TGF-α mRNA expression in U-1242 MG glioma cell clones *in vitro*. Messenger RNA was prepared from the TGF-α expressing clones 4, 71, and 72, that had been grown *in vitro* for 5 days in the presence and absence of tetracycline. As controls, mRNA was prepared from the TGF-α producing U-563 MG human glioma cells and from U-343 MGa Cl2:6 human glioma cells transfected with the human TGF-α cDNA contained in a regular expression vector. The blot was probed with the ^{32}P-labeled TGF-α cDNA.

Western blot analysis of EGFR tyrosine phosphorylation in tumors in vivo. Tumor tissue stored at −70°C was thawed on ice and immediately suspended in lysis buffer containing 10 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 5 mM EDTA, 1 mM PMSF, 1% aprotinin, 1 mM benzamidine, and 1 mM Na_3VO_4. Samples were sonicated with a microtip sonicator (Soniprep 150) and protein content was determined using BCA protein assay kit (Pierce). Equal amounts of protein were incubated with a polyclonal EGFR antibody (sc-03) or with agarose conjugated antiphosphotyrosine antibody PY20 for 3 h at 4°C. In certain experiments, as in the one shown in Fig. 9a, lysates were pre-incubated with normal rabbit serum prior to incubation with the EGFR antibody. After three washes with lysis buffer, the precipitates were heated for 5 min at 95°C in SDS sample buffer and fractionated by electrophoresis in 4–12% Tris-Glycine gels or 3–8% Tris–acetate gels (Novex). As a positive control for EGFR activation, cell lysate of the human vulval carcinoma cell line A431 stimulated with EGF (Transduction Laboratories) was used. Proteins were transferred onto Hybond ECL nitrocellulose membranes (Amersham Life Science) or PVDF membranes (Novex). Membranes were blocked and probed with the polyclonal EGFR antibody, PY20 (Transduction Laboratories), or another antiphosphotyrosine antibody, PY99 (Santa Cruz Biotechnology, Inc.). After incubation with horseradish peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulin (Amersham), immunocomplexes were detected by enhanced chemiluminescence (Amersham).

Preparation of tumors for histological analysis. One-third of each tumor was fixed in 4% paraformaldehyde in PBS at pH 7.4 for 24 h. Specimens were dehydrated and embedded in paraffin wax. Deparaffinized sections were stained with hematoxylin and eosin.

RESULTS

Autocrine TGF-α Increases the Proliferation of Glioma Cells in Vitro

The effect of autocrine TGF-α on glioma cell growth was first tested *in vitro*. It was confirmed that the

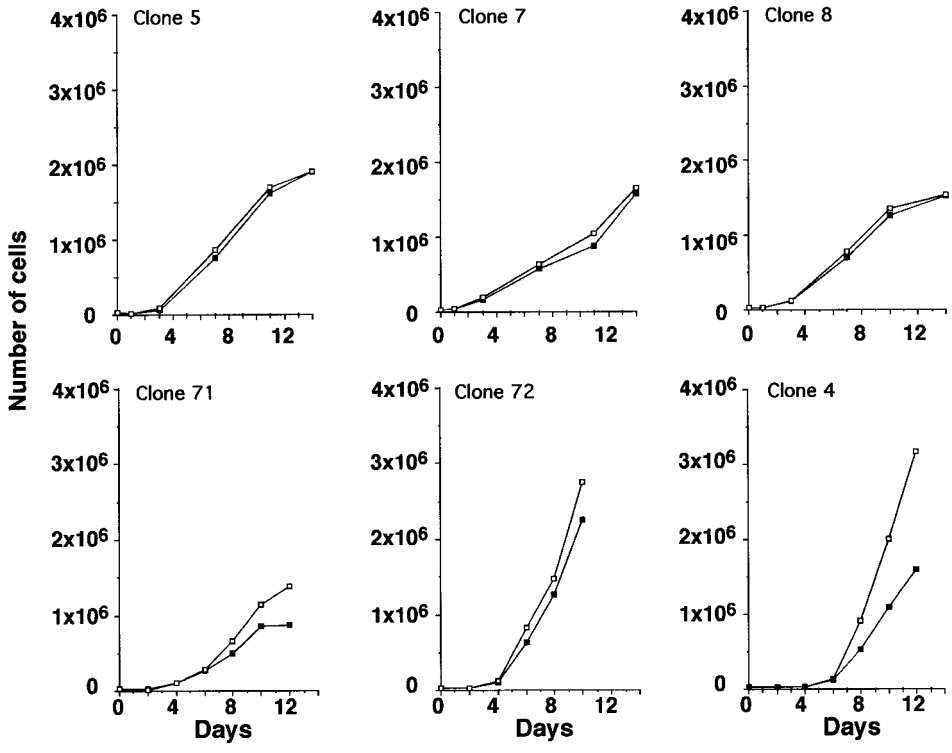

FIG. 2. *In vitro* growth curves for the TGF-α expressing clones 4, 71, and 72 and for the control clones 5, 7, and 8. Growth curves were performed *in vitro* as described under Methods. Cells were pooled, seeded sparsely, and incubated in routine media with 10% serum in the presence and absence of tetracycline (1 μg/ml). Cells were trypsinized and counted in an electronic cell counter. A clear increase in proliferation was observed for clones 4, 71, and 72 in the absence of tetracycline (open symbols) compared to in its presence (filled symbols), while very small differences were observed for the control clones 5, 7, and 8 in the absence and presence of tetracycline. Results are representative of four separate experiments performed with duplicate dishes.

selected clones 4, 71, and 72 expressed TGF-α mRNA in the absence of tetracycline (Fig. 1), while the control clones 5, 7, and 8 did not (data not shown). TGF-α mRNA of 1 kb was also detected in the TGF-α transfected U-343 MGa Cl2:6 cells. In contrast, the endogenous TGF-α mRNA is usually present as a 4.5-kb transcript, as exemplified in U-563 MG cells (Fig. 1).

In a first set of growth curves the efficiency of the tetracycline regulatory system was shown as well as the effect of TGF-α on cell growth *in vitro*. The TGF-α transfected clones showed a clear increase in cell number in the absence of tetracycline, i.e., when TGF-α was produced, while only a minor difference was observed for the control clones in the absence and presence of tetracycline (Fig. 2). This experiment was repeated three times with consistent results. Next, it was tested if the induced cell proliferation was mediated via an activated EGFR. It was found that addition of the EGFR-blocking monoclonal antibody 225 caused a clear inhibition of proliferation of clone 4 in the absence, but not in the presence of tetracycline. The antibody showed no effect on the control clone, neither in the presence nor in the absence of tetracycline (Fig. 3). This experiment was repeated once with the same result.

Finally, the effect of an EGFR specific tyrosine kinase inhibitor (RTKI) was tested. Clear inhibition of the TGF-α expressing clone 4 upon addition of the RTKI was observed, while a much lower inhibitory effect was observed for the control clone. The number of cells in the RTKI-treated cultures was calculated at days 7, 10, and 14 after seeding and was found to be 46, 23, and 16%, respectively, of that in non treated cultures. In comparison, RTKI-treated cultures of the mock-transfected clone 8 contained 63, 72, and 67%, respectively, of the cell number in nontreated control. The vehicle, DMSO, did not affect the proliferation of either of the clones at the concentration used (Fig. 4).

Effect of the Tyrosine Kinase Inhibitor on EGFR Tyrosine Phosphorylation in Vitro

The results described above indicated that the RTKI indeed can interrupt the TGF-α/EGFR autocrine loop, but that it may in addition have some unrelated inhibitory activity since it decreased the proliferation rate of clone 4 cells below the level obtained in the presence of tetracycline and some effect was observed on the proliferation rate of the control clone (Fig. 4). The effect of the RTKI on EGFR tyrosine kinase activity was there-

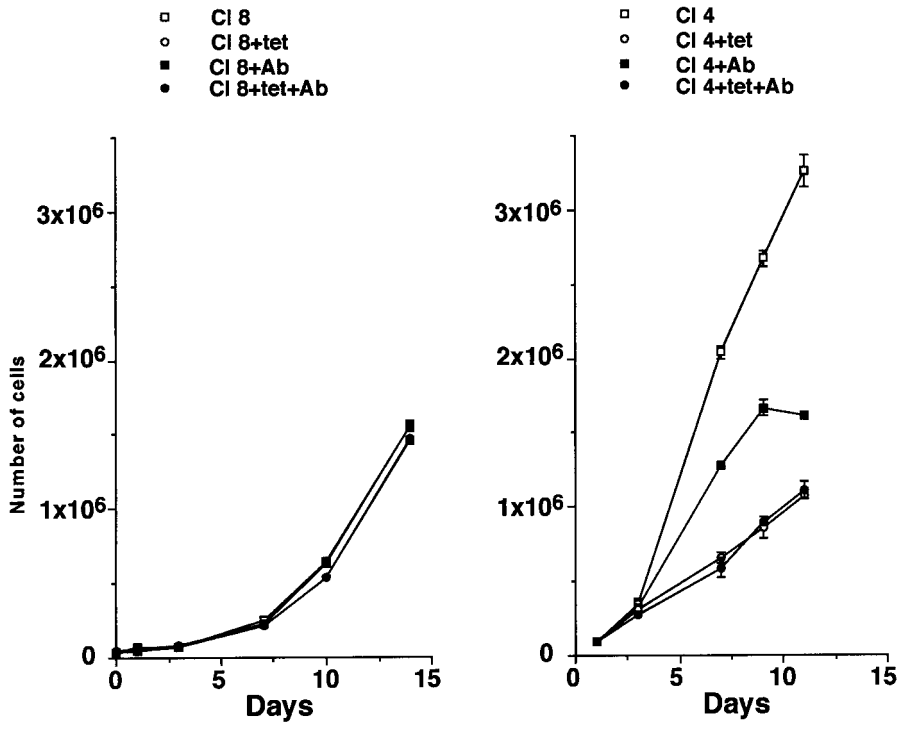

FIG. 3. Effect of an anti-EGFR monoclonal antibody. The effect of the monoclonal antibody 225 (Ab) was tested on the TGF-α expressing clone 4 (right) and on the control clone 8 (left) in an experiment otherwise performed as in Fig. 2. Addition of the antibody (4 μM) caused clear inhibition of the TGF-α induced cell proliferation in clone 4 but had no inhibitory effect on clone 8.

fore confirmed by immunoprecipitation and Western blot analysis using lysates of glioma cell cultures. A clear inhibitory effect on EGFR tyrosine phosphorylation was found both at a concentration of 2 and at 10 μM (Fig. 5a). To explore the functional stability of the inhibitor *in vitro* and to test the effect of the vehicle, we added the RTKI to cells (4 μM) which in turn were lysed 24 and 48 h thereafter. Results showed that, after 48 h the RTKI still mediated a complete inhibitory effect on EGFR phosphorylation (Fig. 5b). The vehicle, DMSO, had no inhibitory effect on phosphorylation. From these experiments, we concluded that the RTKI had the desired effect and therefore in this respect was suitable for further use *in vivo.*

Expression of TGF-α Increases EGFR Tyrosine Phosphorylation and Tumor Growth in Vivo

It was next tested whether the tetracycline regulable system was suitable for creating a controlled TGF-α/EGFR autocrine loop *in vivo,* and if activation led to the same type of effect *in vivo* as *in vitro.* When the cells were precultured in the absence of tetracycline and no tetracycline was given to the mice, we found that the different injected clones developed into tumors that varied in size. Mice injected with the TGF-α expressing clones (4, 71, and 72) developed larger tumors than those injected with the control clones (5, 7, and 8), while no tumors developed in mice injected with the wild type cells (Fig. 6a).

We also tested whether administration of tetracycline to the mice could suppress TGF-α expression and subsequently tumor growth. Mice were therefore injected with clone 4 cells and when the tumors were palpable, half of the mice were given tetracycline in acidic water, while the other half received only acidic water. Addition of tetracycline to the drinking water resulted in a clear reduction of receptor phosphorylation (Fig. 7) and of tumor size (Figs. 6b and 6c). For clone 8 tumors, there was no size difference between tumors grown in mice receiving tetracycline or acidic water only. It should be noted though, that all clone 8 tumors were small and differences would be difficult to assess.

It was concluded from these *in vivo* experiments that larger tumors were formed when TGF-α was supposed to be expressed than when it was suppressed by tetracycline, and we further wanted to verify that TGF-α indeed was expressed *in vivo.*

Expression of TGF-α mRNA and Protein in Tumors

It was confirmed, in the following way, that TGF-α mRNA and protein were expressed in the expected way in the tumors *in vivo.* In the first set of *in vivo* experiments, where mice were injected with the TGF-α ex-

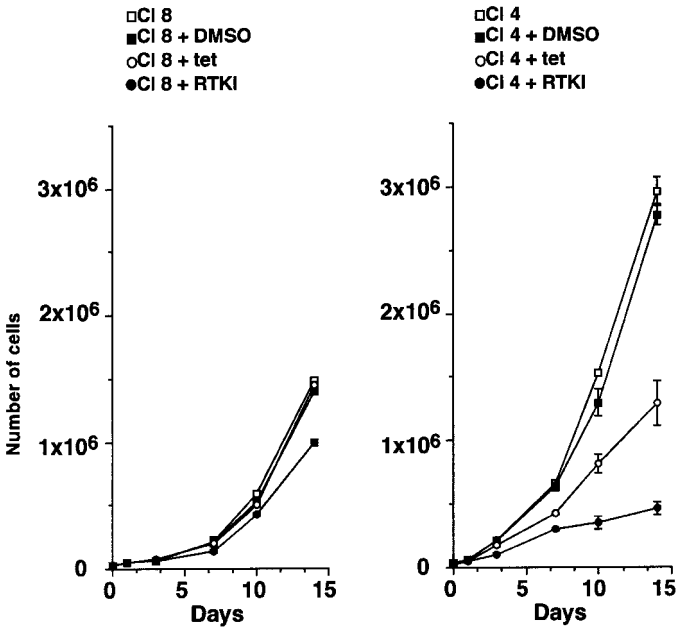

FIG. 4. Effect of an EGFR tyrosine kinase inhibitor on TGF-α induced cell proliferation *in vitro*. The effect of a 4-(3-chloroanilino)-6,7-dimethoxy-quinazoline EGFR tyrosine kinase inhibitor (RTKI) on TGF-α induced cell proliferation was tested on the TGF-α transfected clone 4 (right) and the control clone 8 (left). Clear inhibition was observed of clone 4 proliferation upon addition of the RTKI, while a much lower effect was observed for clone 8. DMSO had only a minute inhibitory effect.

pressing clones 4, 71, and 72, control clones 5, 7, and 8, and U-1242 MG, RNA was prepared from the different tumors. TGF-α mRNA of 1 kb was detected in tumors that developed from clones 4, 71, and 72 but not in tumors that developed from control clones (data not shown). In addition, when clone 4 tumors were further tested, TGF-α mRNA was detected only in tumors where mice had not received tetracycline in drinking water. A representative result with clone 4 in the second set of *in vivo* experiments is shown in Fig. 8. For detection of TGF-α protein, tumor cells were explanted and grown in the presence and absence of tetracycline, and conditioned media were assayed by using an ELISA kit for TGF-α protein. TGF-α was detected in the media conditioned by cells from a clone 4 tumor, and the concentration was found to be 490.3 pg/ml, equivalent to 0.4 ng/10^6 cells/24 h. A low level of protein (71.5 pg/ml) was detected when the same explanted cells were grown in the presence of tetracycline. TGF-α protein was detected in the keratinocyte-conditioned control media at a concentration of 125 pg/ml.

Inhibition of Tumor Growth and of EGFR Tyrosine Phosphorylation by the RTKI in Vivo

After having confirmed the presence of an active TGF-α/EGFR autocrine loop in the tumors, the *in vivo* effect of the RTKI was assessed. Mice were injected with clone 4 and clone 8 cells. Half the mice received the RTKI dissolved in DMSO and the other half received only DMSO. There was a distinct decrease of clone 4 tumor growth in the presence of RTKI resulting in tumors measuring 26% of tumors in nontreated mice. Clone 8 tumors in mice given RTKI measured 82% of tumors in non-treated mice (Fig. 6d). The effect of the RTKI on EGFR tyrosine phosphorylation in tumors *in vivo* was also analyzed. After immunoprecipitation and Western blot of tumor lysates, an EGFR antibody and the phosphotyrosine antibody PY99 were used to identify EGFR expression and activation respectively (Fig. 9a). Results showed a complete inhibition of the EGFR tyrosine phosphorylation by RTKI. A very slight activation of the EGFR in the clone 8 tumor seen in the absence of RTKI was extinguished as well in the presence of the inhibitor. This may explain the slight inhibitory effect of the RTKI on clone 8 tumor growth (Fig. 6b).

Histological Analysis of the RTKI-Treated Tumors

Histological analysis showed that the tissue pattern was similar in all the sectioned tumors. All tumors showed mitotic activity and there were areas of necrosis mainly in the center of the tumors. Larger tumors tended to have larger areas of dead cells. The tumors

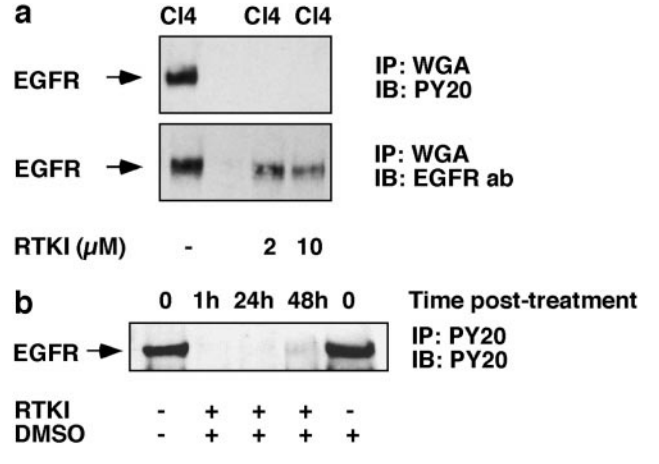

FIG. 5. Effect of the RTKI on EGFR tyrosine phosphorylation in the TGF-α expressing clone 4 *in vitro*. Clone 4 cells were cultured *in vitro* with different additions and prepared for Western blot analysis as described under Methods. (a) Immunoprecipitation was with wheat germ agglutinin and blotting with the PY20 antibody followed by the anti-EGFR-1005 antibody. The RTKI showed a clear inhibitory effect on EGFR tyrosine phosphorylation at concentrations of 2 and 10 μM. (b) Test of the functional stability of the EGFR tyrosine kinase inhibitor (RTKI) *in vitro*. The RTKI dissolved in DMSO (4 μM) and DMSO itself were initially added to the cells daily, after which cells were harvested 24 and 48 h after the last addition of the RTKI and prepared as described under Methods for analysis of EGFR tyrosine phosphorylation by Western blot. Immunoprecipitation and blotting was with the PY20 antibody. Results showed a complete inhibition of the EGFR tyrosine phosphorylation both after 24 and 48 h. DMSO had no effect.

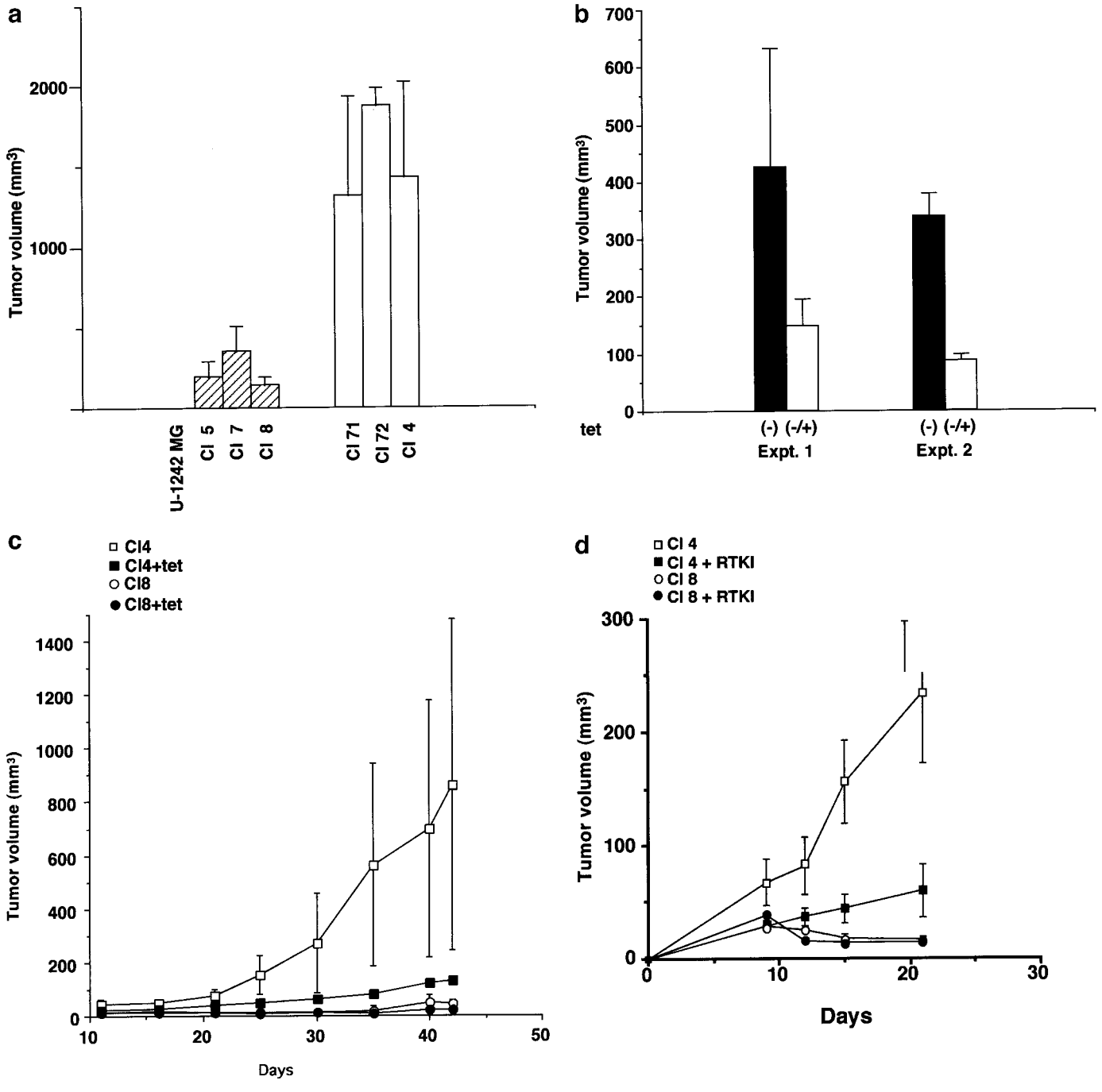

FIG. 6. (a) Tumor volumes observed in *in vivo* experiments with different TGF-α transfected clones. The figure shows mean sizes of tumors that developed in nude mice from subcutaneous injection of different U-1242 MG-derived cell clones. The cells injected were the TGF-α expressing clones (4, 71, and 72), control clones (5, 7, and 8), and the nontransfected original cell line (U-1242 MG). Mice were not given tetracycline in drinking water. (b) Tumor volumes regulated by tetracycline in the second set of *in vivo* experiments. Clone 4 cells were grown in the absence of tetracycline for 5 days before injected subcutaneously in nude mice. Half of the mice were given tetracycline in drinking water (2.2 mg/ml) starting from the second week. Mice that were exposed to tetracycline developed only small tumors. Two series of separate experiments were performed. (c) Tumor volumes regulated by tetracycline in the third set of *in vivo* experiments. The TGF-α expressing clone 4 and control clone 8 were grown in the absence of tetracycline for 5 days before the cells were injected subcutaneously in nude mice. Beginning immediately after injection, half of the mice were given tetracycline in drinking water (2.2 mg/ml). Significant increase in tumor volume was observed in mice injected with clone 4 and not exposed to tetracycline compared to in mice given tetracycline. (d) Tumor volumes observed after administration of an EGFR tyrosine kinase inhibitor. Mice were injected subcutaneously with TGF-α transfected clone 4 and control clone 8 cells that had been grown for 5 *in vitro* days in the absence of tetracycline. The RTKI was daily injected intraperitoneally as described under Methods to half of the mice, while DMSO was injected to the other half in a similar way. The RTKI had a clear inhibitory effect on tumors that developed from clone 4 but very little effect on tumors that developed from clone 8.

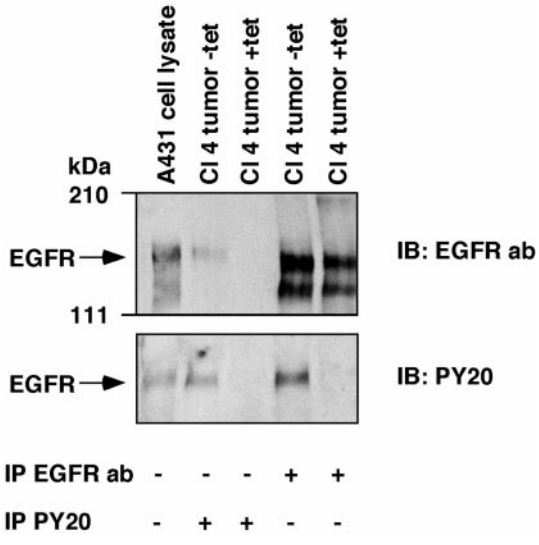

FIG. 7. Immunoblotting of tyrosine phosphorylated EGFR in tumors. Tumor lysates were immunoprecipitated with anti-EGFR-1005 antibody and PY20 agarose conjugated antibody, respectively, and the membranes were probed with the same antibodies in the way shown. A phosphorylated 170-kDa protein was observed in lysate from the tumor that developed from clone 4 in a mouse not exposed to tetracycline, but not in the tumor that developed from the same clone in a mouse which was given tetracycline (set number 3 of *in vivo* experiments).

were rounded and well circumscribed and we observed no diffuse invasion of the surrounding tissue. Although the administration of the RTKI caused clear decrease in the volumes of the clone 4-derived tumors it did not seem to have any effects on the histological appearance of these tumors (Fig. 9b).

DISCUSSION

In this study it was demonstrated that introduction of a TGF-α/EGFR autocrine loop into U-1242 MG human glioma cells leads to an increased growth potential both *in vitro* and *in vivo*. It was also shown that the TGF-α/EGFR autocrine loop is accessible and can be interrupted by an EGFR specific quinazoline tyrosine kinase inhibitor. The U-1242 MG cell line expresses EGFR but lacks endogenous expression of TGF-α (21). Previously, we have transfected this cell line with a human TGF-α cDNA using the tetracycline off system and shown that activation of a TGF-α/EGFR autocrine loop increases the motility of the glioma cells (22). We also showed previously that the expression of TGF-α mRNA and protein could be controlled *in vitro* by addition of tetracycline.

In the present study the generated glioma clones were used for testing the effect of the induced TGF-α on tumor cell proliferation *in vitro* and tumor growth *in vivo* by adding tetracycline to the cell culture media and to mice drinking water, respectively. We observed increased tumor cell proliferation *in vitro* and tumor growth *in vivo* strictly in the absence of tetracycline. Thus, the increased growth was likely to be mediated purely by the TGF-α. Northern blot analysis confirmed that TGF-α mRNA was expressed in the tumors. TGF-α protein was also generated since it could be detected in the media of explanted tumor cells.

The tetracycline system has been used previously *in vivo* (31, 32), and it has been used for controlling tumor cell growth such as human colon carcinoma cells and C6 rat glioblastoma cells (33, 34). It appears to be a tightly regulated system. In the present study we use human glioma cells and add a thorough analysis of the tumors *in vivo*, including effects on EGFR tyrosine phosphorylation to confirm the specificity of the tetracycline effect. We showed that controlled TGF-α production led to activation of the EGFR tyrosine kinase, i.e., the receptor was phosphorylated on tyrosine also in tumors. Thus, the effect on tumor cell growth was most likely mediated by an activated TGF-α/EGFR autocrine loop. This was further confirmed by adding EGFR antibodies *in vitro* and an EGFR specific tyrosine kinase inhibitor both *in vitro* and *in vivo*.

Several EGFR protein tyrosine kinase inhibitors have previously been tested and found to suppress TGF-α/EGFR or EGF/EGFR autocrine loops in tumor cells both *in vitro* and *in vivo* (35). In this study we have used an EGFR tyrosine kinase specific inhibitor, 4-(3-chloroanilino)-6,7-dimethoxy-quinazoline, to inhibit TGF-α induced glioma cell growth both *in vitro* and *in vivo*. Our *in vitro* results showed that the inhibitor was highly efficient and stable. Also *in vivo* the inhibitor seemed to efficiently block receptor phosphorylation as monitored after 3 hours, but the growth of TGF-α producing tumors was not reduced completely

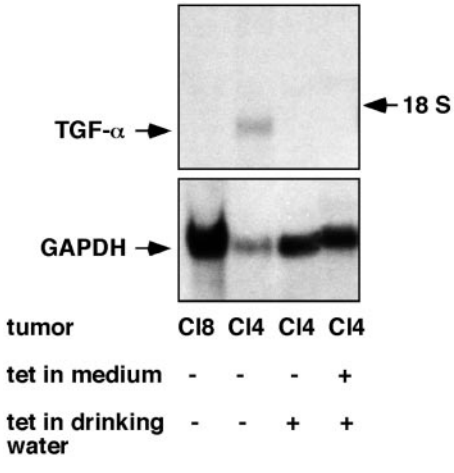

FIG. 8. Expression of TGF-α mRNA in tumors. Two parallel sets of clones 4 and 8 were grown *in vitro* in the presence of 1 μg/ml tetracycline and in tetracycline-free medium, respectively, for 5 days prior to sc injection in nude mice**.** The mice in turn were either given tetracycline or not in the drinking water. TGF-α mRNA was only detected in the tumor that developed from clone 4 in the absence of tetracycline.

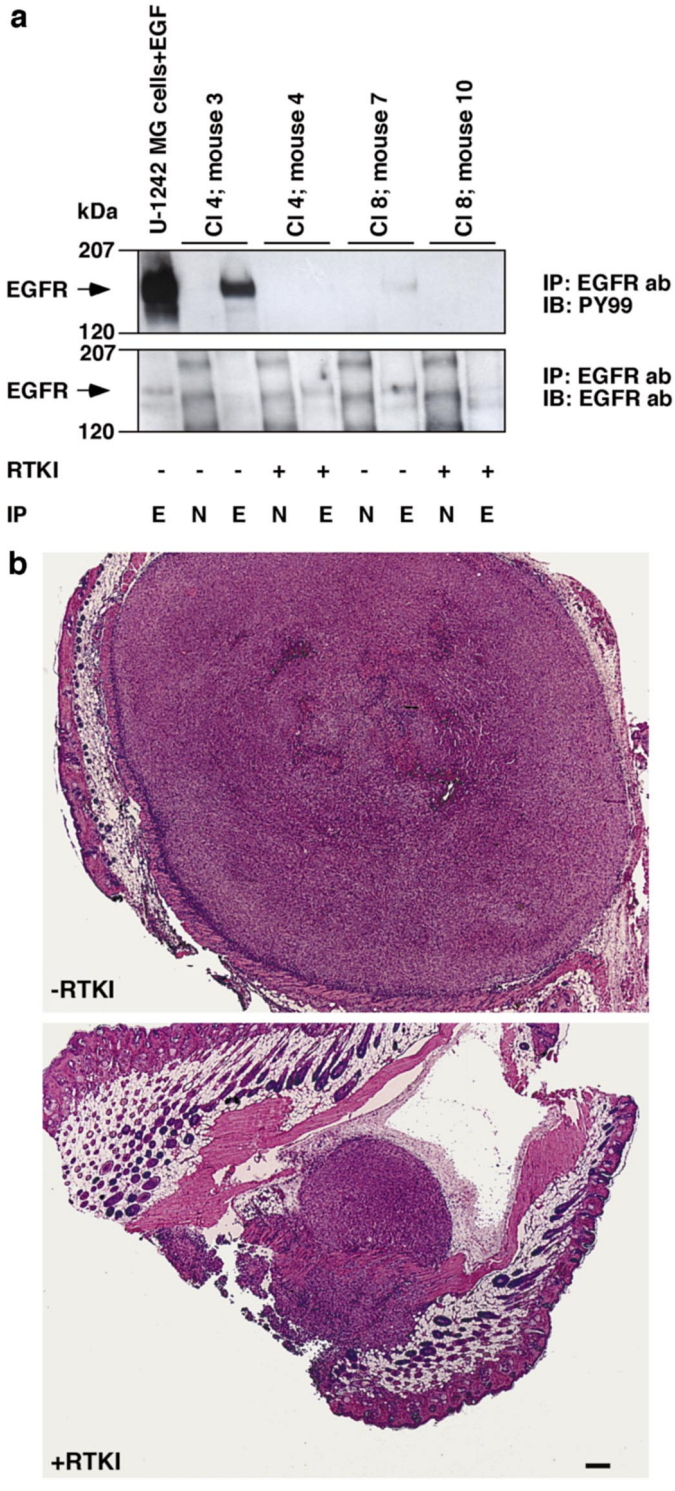

FIG. 9. Effect of an EGFR tyrosine kinase inhibitor on TGF-α driven tumors *in vivo*. (a) Inhibition of the EGFR tyrosine phosphorylation in tumors *in vivo*. Tumor lysates were preimmunoprecipitated with normal rabbit serum (N) followed by precipitation with an anti-EGFR-1005 antibody (E), and membranes were probed with the PY99 antibody and with the EGFR antibody. Intraperitoneal injection of the RTKI resulted in a clear inhibitory effect on the EG/FR tyrosine phosphorylation in tumors derived from the clone 4. There was also an effect of the RTKI on the slight phosphorylation in control clone 8-derived tumors. U-1242 MG cells were stimulated with EGF (50 ng/ml) for 2 h on ice and used as a positive control EGFR phosphorylation. (b) Tumor sections showing the effect of the RTKI on morphology. The tumors were derived from TGF-α expressing clone 4 cells grown in a mouse treated with RTKI or in a mouse given only DMSO. Though no difference in morphology could be noted, a clear inhibition of tumor size was observed in the mouse injected with the RTKI.

to that of control tumors. The inhibitory effect on phosphorylation may therefore have been incomplete at later time-points, as the compound was only administered once daily, or there may have been a true clonal variation in the ability of the U-1242 MG derived cells to grow in an *in vivo* situation. Quinazolines are also known to act as reversible inhibitors of the EGFR kinase, and this might be an alternative explanation for the incomplete effect. Recently, irreversible and hopefully even more efficient EGFR inhibitors have been developed (25). Initial experiments showed toxic effects in the form of weight-loss and death in doses over 50 mg/kg (Hesselager, unpublished data). No serious adverse effects were noted in the dosage range of 10–40 mg/kg used in this study.

The disruption of a TGF-α/EGFR autocrine loop by introducing an anti-sense TGF-α cDNA construct into TGF-α expressing cells, can significantly decrease the growth of several types of tumor cells. Tang *et al.* (36) showed for example that an anti-sense oligonucleotide directed to TGF-α led to dose dependent inhibition of the growth of a glioma cell line. Hirsch *et al.* (37) evaluated TGF-α mediated autonomous growth and constitutive EGFR activation in the human adenocarcinoma cell line SW403. Their findings suggest that the proliferation of these cells is regulated by an autocrine TGF-α/EGFR loop and that this regulatory pathway can be interrupted by using an anti-EGFR mAb. These and other previous results show that many types of human tumors may be driven by a TGF-α/EGFR autocrine loop. Also, many human malignant gliomas coexpress TGF-α and EGFR (24, 38) and in addition TGF-α might be produced in normal cells of the adjacent brain tissue leading to paracrine stimulation of EGFR expressing tumor cells (39). It is therefore possible that an activated EGFR drives a majority of gliomas even in the absence of EGFR amplification or genetically aberrant EGFRs. Therefore, EGFR-specific tyrosine kinase inhibitors can be regarded as very promising future therapeutic agents for gliomas (40, 41) as well as for many other types of tumors.

In conclusion, the observed increase in tumor growth in mice injected with TGF-α-producing glioma cells suggested that a TGF-α/EGFR autocrine loop can play an important role in these tumors. This work also visualized the feasibility of using specific receptor tyrosine kinase inhibitors to inhibit tumor growth *in vivo*.

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