Inhibition of TGF-β Enhances the *In Vivo* Antitumor Efficacy of EGF Receptor–Targeted Therapy

Atul Bedi[1,2,3], **Xiaofei Chang**[1], **Kimberly Noonan**[2], **Vui Pham**[1], **Rishi Bedi**[1], **Elana J. Fertig**[2], **Michael Considine**[2], **Joseph A. Califano**[1,3], **Ivan Borrello**[2,3], **Christine H. Chung**[2,3], **David Sidransky**[1,2,3], and **Rajani Ravi**[1,2]

[1]Division of Head and Neck Cancer Research, Department of Otolaryngology-Head and Neck Surgery [2]Department of Oncology, The Johns Hopkins University School of Medicine, Baltimore, Maryland [3]Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, The Johns Hopkins University School of Medicine, Baltimore, Maryland

Abstract

EGF receptor (EGFR)–targeted monoclonal antibodies (mAb), such as cetuximab, execute their antitumor effect *in vivo* via blockade of receptor–ligand interactions and engagement of Fcγ receptors on immune effector cells that trigger antibody-dependent cell-mediated cytotoxicity (ADCC). We show that tumors counteract the *in vivo* antitumor activity of anti-EGFR mAbs by increasing tumor cell-autonomous expression of TGF-β. We show that TGF-β suppresses the expression of key molecular effectors of immune cell–mediated cytotoxicity, including Apo2L/TRAIL, CD95L/FasL, granzyme B, and IFN-γ. In addition to exerting an extrinsic inhibition of the cytotoxic function of immune effectors, TGF-β–mediated activation of AKT provides an intrinsic EGFR-independent survival signal that protects tumor cells from immune cell–mediated apoptosis. Treatment of mice-bearing xenografts of human head and neck squamous cell carcinoma with cetuximab resulted in emergence of resistant tumor cells that expressed relatively higher levels of TGF-β compared with untreated tumor-bearing mice. Although treatment with cetuximab alone forced the natural selection of TGF-β–overexpressing tumor cells in nonregressing tumors, combinatorial treatment with cetuximab and a TGF-β–blocking antibody

prevented the emergence of such resistant tumor cells and induced complete tumor regression. Therefore, elevated levels of TGF-β in the tumor microenvironment enable tumor cells to evade ADCC and resist the antitumor activity of cetuximab *in vivo*. Our results show that TGF-β is a key molecular determinant of the *de novo* and acquired resistance of cancers to EGFR-targeted mAbs, and provide a rationale for combinatorial targeting of TGF-β to improve anti-EGFR–specific antibody therapy of EGFR-expressing cancers.

Introduction

Concurrent chemoradiation for locally advanced head and neck squamous cell carcinoma (HNSCC) is limited by its toxicity and the development of recurrent disease in 30% to 40% of patients (1, 2). Efforts to improve the treatment of HNSCC have targeted the EGF receptor (EGFR), a receptor tyrosine kinase that is overexpressed and aberrantly activated in almost all such neoplasms (3–5). Activation of EGFR signaling promotes tumor cell proliferation and survival, and facilitates tumor angiogenesis (6, 7). Strategies to target EGFR have focused on either EGFR tyrosine kinase inhibitors (TKI) or monoclonal antibodies (mAb) that specifically bind the extracellular domain of the receptor, such as the human–mouse chimeric IgG1 mAb, cetuximab (8, 9). The direct effect of EGFR-targeted mAbs on tumor cells involves specific blockade of EGFR signaling via interference with binding of EGFR ligands to the extracellular domain of the receptor (10–12). In addition, the interaction of the Fc region of an antibody to Fcγ receptors on immune effector cells also induces antibody-dependent cellular cytotoxicity (ADCC; refs. 12–16).

Treatment of patients with locoregionally advanced HNSCC with a combination of cetuximab and radiation improved overall survival compared with radiation alone (17). With a median follow-up of 54.0 months, the median duration of overall survival was 49.0 months among patients treated with combined therapy and 29.3 months among those treated with radiotherapy alone. However, the survival benefit from cetuximab was not uniformly observed across all patients. The beneficial effect of cetuximab seemed to be preferentially evident in a subset of patients with the typical characteristics of human papillomavirus (HPV)-positive head and neck cancer (those with oropharyngeal cancer who were males and less than 65 years). After cetuximab and radiation therapy, patients with HPV-positive tumors showed a 60% 2-year progression-free survival (PFS) compared with only 23% PFS for patients with HPV-negative tumors. Identification of the molecular determinants of resistance to EGFR-targeted mAbs is crucial for improving their clinical benefit against HNSCC.

In this study, we find that patients with HPV-negative HNSCC exhibit an abnormal elevation of serum levels of TGF-β, a multifunctional cytokine that regulates cell growth and differentiation (18, 19). We show that TGF-β exerts an extrinsic inhibition of the cytotoxic function of immune effectors while simultaneously providing an intrinsic EGFR-independent survival signal that protects tumor cells from immune cell–mediated ADCC. Although the autonomous expression of TGF-β enables tumor cells to evade ADCC and resist the antitumor activity of cetuximab *in vivo*, combinatorial treatment with cetuximab and a TGF-β–blocking antibody prevents the emergence of such resistant tumor cells and improves the regression of HNSCC tumor xenografts. These results show that tumor cell expression of TGF-β is a key determinant of resistance to EGFR-targeted antibodies, and provide a rationale for combinatorial targeting of TGF-β to improve the response of HNSCC and other EGFR-expressing cancers to cetuximab-based therapy.

Materials and Methods

Measurement of serum levels of cytokines in patients with HNSCC

Plasma samples were obtained from 47 patients with HNSCC and 10 patients with pleomorphic adenoma (as non-HNSCC controls) who consented for the Institutional Research Board-approved tissue banking protocol at Johns Hopkins University (Baltimore, MD). The serum levels of TGF-β1, TGF-β2, TGF-β3, IL-10, IL-6, VEGF, IFN-α, IFN-γ, IL-2, IL-12p70, IL-12p40/p70, IL-15, TNF-α, eotaxin, osteopontin, GCSF, SDF-1α, IL-4, GRO-α, and IL-8 were measured by multiplex bead assays using reagents from Millipore and the Luminex 100 system in accordance with the manufacturer recommended protocols (20). The HPV status was determined by in situ hybridization using high-risk HPV-specific probes and p16 immunohistochemical staining as described (21).

Cell lines and cell cultures

The human HNSCC cell line UM-SCC-1 (designated SCC-1 or SCC1) and its isogenic derivative 1CC8 (designated SCC-1CC8 or 1CC8) were a kind gift from the University of Michigan and maintained in 10% FBS in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 1% hydrocortisone. The erlotinib-resistant isogenic derivative of SCC-1 cells (designated SCC-1T) was generated via continuous exposure of SCC-1 cells to escalating concentrations of erlotinib over 6 months (22), and maintained in culture medium supplemented with erlotinib. The human HNSCC cell lines (HACAT, HO1u1, 93VU147T, CAL27, SKN3, SCC25, HN5, SCC47, UNC7, SQ20B, HSC3, SQ9G, SCC6, HMS001, SCC61, FADU, SCC9, JHU22, SCC090, HSC2, JSQ3, SCC15, UNC10) and H358 lung carcinoma cells were cultured in their respective recommended media. Cells were maintained in a humidified atmosphere containing 5% CO_2 at 37°C. Each cell line was authenticated using fingerprinting at the Johns Hopkins Genetic Resources Core Facility, and used within 6 months of authentication.

Tumor xenografts and treatment of mice

Tumor xenografts were generated by implanting 3×10^6 tumor cells into the right flank of 5- to 6-week-old female athymic mice (*nu/nu*; National Cancer Institute, Bethesda, MD). Once the tumors reached a size of approximately 100 mm^3, the mice were randomized (8–10 mice per group), and treated with cetuximab [Imclone; 5 mg/kg intraperitoneally (i.p.) twice weekly for 4 weeks) and/or TGF-β antibody (Bioxcell; 5mg/kg i.p., once weekly for 4 weeks). Tumor size was measured weekly and tumor volume was calculated using the formula (length × width × height). The animals were maintained in accordance with guidelines of the American Association of Laboratory Animal Care and a research protocol approved by the Johns Hopkins University Animal Use and Care Committee.

[18F]FDG-PET/CT imaging of mice

The night before imaging, mice were fasted for 12 hours. Water was provided *ad libitum*. On the day of imaging, each mouse was injected with 250 μCi of [18F]FDG via the tail vein and imaged 45 minutes post injection, using the Mosaic HP (Philips) small animal PET imagers with 15-minute static acquisition. A CT scan was done at the same time using the CT component NanoSPECT/CT (Bioscan) *in vivo* animal imagers. The standard uptake values were computed by normalizing the PET activity for each mouse to the injected dose and animal weight and coregistered with CT images using Amira 5.2.2 (Visage Imaging, Inc.).

Measurement of TGF-β in serum and tumor cell supernatants

Serum was collected from mice by tail bleeding for measurement of TGF-β using ELISA (R&D Systems). Tumor cells were extracted from xenografts using collagenase digestion followed by RBC lysis, and cultured for 48 hours in DMEM containing 0.1% FBS. Tumor cell supernatants were evaluated by ELISA to determine the amount of TGF-β expressed by 1×10^6 cells per 24 hours.

Antibody-dependent cellular cytotoxicity assay

Human peripheral blood mononuclear cells (PBMC) from normal donors were stimulated with recombinant human interleukin-2 (rh IL-2; 200 IU/mL; Chiron) in the presence or absence of rhTGFβ1 (5 ng/mL; R&D Systems) in Adoptive Immunotherapy Media V (AIM V) containing low Ig FBS (Invitrogen) for 48 hours and used as effectors. Tumor cells (5,000 cells/well) were cultured in a 96-well U-bottomed plate in growth medium containing low Ig serum for 24 hours before being used as targets. Target cells were washed and coated with cetuximab 2 μg/mL and incubated for an hour in a CO_2 incubator. PBMCs were washed, counted, and plated on target cells in a range of effector:target (E:T) ratios. Plates were incubated for 3 to 4 hours and analyzed for ADCC using an aCella-Tox kit (Cell Technology Inc.).

Reverse transcription and quantitative real-time PCR—PBMCs were stimulated as described earlier (ADCC assay) for 48 hours and RNA was extracted using TRIzol (Invitrogen) followed by RNAeasy kit cleanup (Qiagen). RNA was reverse transcribed to cDNA using Superscript III (Invitrogen) that then used as a template for real-time PCR. PCR primers were designed to amplify cDNA fragments approximately 150 to 250 bp in length using primer3 software. Gene products were amplified using iTaq SYBR green Supermix with Rox dye (Bio-Rad Laboratories) with the following amplification program for 40 cycles: 95°C for 15 minutes, 60°C for 1 minute. All reactions were conducted in triplicate, with water controls, and relative quantity was calculated after normalizing to GAPDH expression. The following primer sequences were used: (i) NKG2D: 5′-GGCTCCATTCTCTCACCCA-3′ (forward) and 5′-TAAAGCTCGAGGCATAGAGTGC-3′ (reverse); (ii) granzyme A: 5′-TCCTATAGATTTCTGGCATCCTCTC-3′ (forward) and 5′-TTCCTCCAATAATTTTTTCACAGACA-3′ (reverse); (iii) granzyme B: 5′-TCCTAAGAACTTCTCCAACGACATC-3′ (forward) and 5′-GCACAGCTCTGGTCCGCT-3′ (reverse); (iv) Apo2L/TRAIL: 5′-CCCCTGCTGGCAAGTCAA-3′ (forward) and 5′-TGAACTGTAGAAATGGTTTCCTCAGA-3′ (reverse); (v) IFN-γ: 5′-GAAAAGCTGACTAATTATTCGGTAACTG-3′ (forward) and 5′-GTTCAGCCATCACTTGGATGAG-3′ (reverse); and (vi) GAPDH: 5′-CAACTACATGGTTTACATGTTC-3′ (forward) and 5′-GCCAGTGGACTCCACGAC (reverse). Expression of each specific mRNA (gene) relative to GAPDH was calculated based on the threshold cycle (C_t) as $2^{-\Delta(\Delta Ct)}$, where $\Delta(\Delta C_t = \Delta C_{t\text{gene}} - \Delta C_{t\text{GAPDH}}$.

Flow cytometric analysis

PBMCs were stimulated as described earlier (ADCC assay) for 3 days and stained extracellularly with anti-CD56 APC (BD Biosciences), anti-Apo2L/TRAIL PE, or anti-FAS-L PE (eBioscience) for 10 to 15 minutes at 4°C. The cells were washed twice and then run on the FACS Calibur (BD Biosciences) and evaluated with Cell Quest software.

Cell viability and drug sensitivity assay

Tumor cells were plated at a density of 3,000 per well in 96-well plates. The following day, cells were treated with 0 to 10 μmol/L erlotinib for an additional 72 hours. Cell viability was subsequently assayed using the MTT colorimetric assay. The plates were read using a SpectraMax plate reader (Molecular Devices Corp.) at 570 nm with a reference wavelength of 650 nm. A minimum of 6 wells were tested for each erlotinib dose. In some experiments, tumor cells were pretreated with either rhTGFβ1 or rhTGFβ3 (5 ng/mL; R&D Systems) to evaluate the effect of TGF-β on their sensitivity to erlotinib. The effect of TGF-β on the response of SCC47 cells to erlotinib was evaluated after transfection with either 100 nmol/L AKT siRNA or Control siRNA (Cell Signaling Technology) for 48 to 72 hours.

Immunoblot analysis

Xenograft samples were homogenized in radioimmunoprecipitation assay (RIPA) buffer followed by sonication. In some experiments, cells were plated at a density of 500 per well in 6-well plates and cultured with either rhTGFβ1 (5 ng/mL; R&D Systems) or TGFβRII-Fc (500 ng/mL; refs. 23, 24) for 1 to 7 days before immunoblot analysis. Cells were lysed in RIPA lysis buffer containing protease inhibitors (Roche Diagnostic Systems) and phosphatase inhibitor cocktail (Sigma-Aldrich). Lysate protein concentrations were determined by the Lowry protein assay (Bio-Rad Laboratories). Equal amounts of protein were mixed with Laemmli sample buffer (62.5 mmol/L Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.1 mol/L DTT, and 0.01% bromophenol blue), run on 4% to 12% NuPAGE gels and transferred to nitrocellulose membrane (Bio-Rad Laboratories). The membrane was blocked with PBS supplemented with 0.1% Tween 20 and 5% nonfat milk for 1 hour at room temperature, and probed with primary antibody for 1 hour at room temperature or overnight at 4°C followed by HRP-conjugated appropriate secondary antibodies (Santa Cruz Biotechnology). The following primary antibodies were used: antibodies against EGFR, phospho-EGFR, AKT, phospho-AKT (Ser473), E-cadherin, vimentin (Cell Signaling Technology), and anti–β-actin antibody (Sigma-Aldrich). Signals from immunoreactive bands were detected by enhanced chemiluminescence (GE Healthcare).

Statistical analysis

Differentially expressed cytokines were determined comparing non-HNSCC controls versus HNSCC, HPV-positive versus HPV-negative, and collection at the time of new diagnosis versus recurrence diagnosis. Student unpaired t tests were used for statistical analysis to compare each of the 2 groups. When comparing across cytokines, results with Benjamini–Hotchberg adjusted P values (25) below 0.05 were considered significant. When making comparisons between classes for single cytokines, results with unadjusted $P < 0.05$ were considered significant. All statistical analyses of cytokines were conducted using R according to the script in Supplementary Information (Supplementary Methods). Student unpaired t test was used for all other statistical analyses between 2 groups. Results with $P < 0.05$ were considered significant. Statistical analyses were conducted using GraphPad InStat version 3.0a for Macintosh (GraphPad Software).

Results

Patients with HPV-negative HNSCC exhibit elevated levels of serum TGF-β

Plasma samples were procured from 47 patients with HNSCC (Supplementary Table S1) and 10 patients with pleomorphic adenoma (as benign non-HNSCC controls). Thirty-six samples were obtained at the time of new diagnosis of HNSCC and 11 samples were obtained at the time of recurrence. Measurement of serum levels of 20 cytokines showed that TGF-β1 was significantly elevated in patients with HNSCC compared with non-

HNSCC controls ($P = 0.0085$; Fig. 1A and Supplementary Fig. S1). A striking variation in serum levels of TGF-β1 was also noted among patients with HNSCC (Fig. 1A and Supplementary Table S2). Univariate analysis of samples from newly diagnosed patients showed that TGF-β1 levels were significantly higher in HPV-negative HNSCC compared with non-HNSCC controls ($P < 0.0003$; Fig. 1B). Whereas patients with HPV-negative tumors showed an abnormal elevation of serum TGF-β1, patients with HPV-positive tumors exhibited relatively lower levels of TGF-β1 that were not significantly higher than non-HNSCC controls (Fig. 1B).

Tumor cell expression of TGF-β inhibits the expression of cytotoxic effector molecules in immune cells and suppresses their ability to induce cetuximab-mediated ADCC of tumor cells

To determine whether HNSCC cells express and secrete TGF-β into the tumor microenvironment, the amount of TGF-β produced by 25 different human HNSCC cell lines in supernatants of tumor cells was measured by ELISA. Akin to the variable serum levels of TGF-β observed in patients with HNSCC, HNSCC cell lines produced differing amounts of TGF-β *in vitro* (Fig. 2A). Significantly, isogenic variants of the same cell line (SCC-1) expressed strikingly different levels of TGF-β. Although parental SCC-1 cells expressed low levels of TGF-β ($37.8 \text{ pg}/10^6$ cells/24 h), derivatives of SCC-1 cells selected for resistance to cetuximab (SCC-1CC8) or the EGFR TKI, erlotinib (SCC-1T), expressed significantly higher levels of TGF-β [SCC-1CC8, $124.5 \text{ pg}/10^6$ cells/24 h ($P = 0.004$); SCC-1T, $550 \text{ pg}/10^6$ cells/24 h ($P < 0.001$); Fig. 2A]. To determine whether tumor cell production of TGF-β influences the ability of immune effector cells to induce cetuximab-mediated ADCC, cetuximab-coated tumor cells were exposed to normal human PBMCs that were pretreated with rhIL-2 in the presence or absence of rhTGF-β1 for 48 hours. HNSCC cell lines exhibited variable susceptibility to cetuximab-mediated ADCC (Fig. 2B). Exposure of PBMCs to rhTGF-β1 resulted in a marked decline in their ability to induce cetuximab-mediated ADCC of all HNSCC cell lines, including HPV-positive cell lines (SCC47, SCC90) and HPV-negative cell lines (SCC1, 1CC8, SCC-1T, SCC6, UNC10; Fig. 2B). Exposing PBMCs to rhTGF-β resulted in a significant diminution in their expression of several cytotoxic effector molecules, including granzyme B, Apo2L/TRAIL, CD95L/FasL, and IFN-γ (Fig. 2C and D). Real-time PCR showed that exposure to rhTGF-β resulted in approximately 6-fold reduction in the mRNA levels of granzyme B and Apo2L/TRAIL, as well as approximately 4-fold decrease in levels of IFN-γ mRNA (Fig. 2C). Similarly, flow cytometry analysis showed a significant reduction of surface expression of Apo2L/TRAIL and CD95L/FasL on CD56+ NK cells after exposure to rhTGF-β (Fig. 2D). These data indicate that the autonomous production of TGF-β by tumor cells can exert a tumor cell-extrinsic inhibition of the cytotoxic function of immune effectors that induce cetuximab-mediated ADCC.

Autonomous expression of TGF-β activates AKT and enables EGFR-independent survival of tumor cells

The SCC-1T cell line was derived by culturing SCC-1 cells in the presence of escalating concentrations of the EGFR TKI, erlotinib. Although SCC-1 cells exhibited a dose-dependent reduction of viability in response to erlotinib, SCC-1T cells were relatively resistant to treatment (Fig. 3A). SCC-1T cells expressed more than 10-fold higher levels of TGF-β compared with SCC-1 cells (Fig. 3B). The elevation in autonomous expression of TGF-β in SCC-1T cells was associated with increased phosphorylation of AKT and epithelial–mesenchymal transition (EMT), as showed by the loss of expression of E-cadherin and appearance of vimentin (Fig. 3C). Because tumor cell acquisition of an erlotinib-resistant phenotype was coselected with an increase in autonomous expression of TGF-β, we investigated whether exposure to TGF-β directly enables EGFR-independent

survival of tumor cells. Immunoblot analyses showed that treatment of EGFR-expressing H358 tumor cells with either rhTGF-β1 or rhTGF-β3 resulted in increased expression of phosphorylated AKT (p-AKT) that was apparent after 7 days of exposure (Fig. 3D). The constitutive activation of AKT was associated with TGF-β—induced EMT, as evidenced by the appearance of vimentin and concurrent reduction in the expression of E-cadherin (Fig. 3D). The acquisition of an EMT phenotype by tumor cells after exposure to TGF-β was attended with a concurrent decrease in the expression of phosphorylated EGFR (p-EGFR; Fig. 3D). These effects of TGF-β were also confirmed in 3 HNSCC cell lines [SCC6 (HPV-negative), SCC47 (HPV-positive), and UNC10 (HPV-negative)]. Exposure of HNSCC cell lines to TGF-β resulted in increased expression of p-AKT, EMT, and decreased p-EGFR expression (Fig. 3E).

To determine whether the TGF-β–induced switch of tumor cells from an active EGFR/low p-AKT phenotype to an inactive EGFR/high p-AKT phenotype renders them independent of EGFR-activated survival signals, HNSCC cell lines (SCC6, SCC47, and UNC10) or H358 cells that were pretreated with either TGF-β1 or TGF-β3 and their untreated counterparts (control) were exposed to graded concentrations of erlotinib (26). Although SCC6, SCC47, and H358 cells exhibited a dose-dependent reduction of viability after treatment with erlotinib, pretreatment with TGF-β rendered all 3 lines relatively resistant to erlotinib (Fig. 3F). We further established the importance of AKT activation in TGF-β–mediated resistance to EGFR-targeted therapy using siRNA-mediated knockdown of AKT in SCC47 cells (Fig. 3F and G). Treatment of SCC47 cells with siRNA against AKT inhibited TGF-β–induced elevation of p-AKT (Fig. 3G). siRNA-mediated loss of AKT sensitized SCC47 cells to erlotinib and counteracted the ability of TGF-β to confer resistance to erlotinib (Fig. 3F).

Treatment of tumor-bearing animals with cetuximab results in the *in vivo* selection of resistant tumor cells with elevated expression of TGF-β and TGF-β–dependent activation of AKT

To investigate whether autonomous expression of TGF-β modulates the response of tumor cells to EGFR-targeted mAbs *in vivo*, we examined the effect of cetuximab on tumor xenografts comprising either SCC-1 cells or its derivative, SCC-1CC8 (1CC8). Although the *in vivo* growth of SCC-1 tumors was arrested by cetuximab (untreated vs. treated, $P < 0.05$), 1CC8 tumors were relatively less responsive to the same treatment (untreated vs. treated, $P = 0.09$; Fig. 4A). However, cetuximab alone was not sufficient to induce regression of either SCC-1 or 1CC8 tumor xenografts. Because TGF-β inhibited cetuximab-mediated ADCC of tumor cells *in vitro*, we investigated whether treatment of tumor-bearing mice with cetuximab results in the *in vivo* selection of resistant tumor cells that express higher levels of TGF-β. Tumor cells extracted from residual tumors in untreated or cetuximab-treated mice were cultured *ex vivo*, and the production of TGF-β in tumor cell supernatants was measured by ELISA. Explanted SCC-1 tumor cells derived from residual nonregressed tumors after cetuximab treatment expressed significantly higher levels of TGF-β (135 pg/10^6 cells/24 h) compared with either SCC-1 cells derived from untreated tumor-bearing animals (40 pg/10^6 cells/24 h; $P = 0.01$) or SCC-1 cells before inoculation into mice (37.8 pg/10^6 cells/24 h; $P = 0.01$; Fig. 4B). Indeed, the elevated level of expression of TGF-β in residual SCC-1 tumor cells derived from cetuximab-treated mice approximated that of residual 1CC8 cells after cetuximab therapy ($P = 0.6$; Fig. 4B). Consistent with the increase in the autonomous expression of TGF-β, tumor-derived SCC-1 cells from cetuximab-treated animals exhibited increased phosphorylation of AKT and reduced expression of E-cadherin, a marker of EMT (Fig. 4C). Treatment of residual SCC1 or 1CC8 tumor cells with a TGF-β antagonist (TGFβRII-Fc) reversed the phosphorylation of AKT, indicating that tumor cell–derived TGF-β was responsible for EGFR-independent activation of AKT (Fig. 4D). In accordance with the *in vivo* selection of TGF-β–overexpressing resistant tumor cells in

cetuximab-treated animals, serum levels of TGF-β were equally elevated in SCC-1- and 1CC8–tumor-bearing mice after cetuximab therapy, but were restored to that of naïve mice after treatment with an antagonist TGF-β antibody (TGF-β antibody; Fig. 4E).

Inhibition of TGF-β improves the *in vivo* antitumor efficacy of cetuximab against tumor xenografts of HNSCC cells

Because treatment with cetuximab resulted in the selection of TGF-β–overexpressing resistant tumor cells, we investigated whether inhibition of TGF-β with TGF-β antibody can improve the *in vivo* antitumor efficacy of cetuximab against tumor xenografts of HNSCC cells. Consistent with the selection of TGF-β–overexpressing variants of SCC-1 tumor cells in cetuximab-treated animals, cetuximab alone was not sufficient to induce regression of either SCC-1 or 1CC8 tumor xenografts (Fig. 5A and B). However, combinatorial treatment of mice with TGF-β antibody improved the antitumor efficacy of cetuximab against tumor xenografts of SCC-1 cells as well as 1CC8 cells (Fig. 5A and B). Unlike cetuximab alone, combined treatment with cetuximab and TGF-β antibody resulted in regression of both SCC-1 and 1CC8 tumor xenografts (Fig. 5A and B). In animals bearing 1CC8 tumor xenografts, treatment for 30 days with the combination of cetuximab and TGF-β antibody resulted in significantly smaller tumors (mean $\pm$ SEM = 15 ± 6 mm^3) compared with cetuximab alone (mean $\pm$ SEM = 109 ± 11 mm^3; $P < 0.0001$). Analyses of tumor-free survival by *in vivo* PET-CT imaging at day 50 showed that cetuximab alone failed to induce complete tumor regression in any animal bearing either SCC-1 tumors (0/8) or 1CC8 tumors (0/10; Fig. 5C). In contrast, combined treatment with cetuximab and TGF-β antibody resulted in complete regression of SCC-1 tumors in 7 of 8 mice (87.5%) and 1CC8 tumors in 9 of 10 mice (90%; Fig. 5C). Treatment with TGF-β antibody alone did not have any appreciable effect on the growth of either SCC-1 or 1CC8 tumor xenografts. These data showed that combinatorial treatment with cetuximab and TGF-β antibody significantly improved tumor-free survival in comparison to either TGF-β alone or cetuximab alone in mice bearing either SCC1 tumors ($P < 0.0001$) or 1CC8 tumors ($P < 0.0001$). Therefore, inhibition of TGF-β not only negated the relative *de novo* resistance of 1CC8 tumors to cetuximab, but also augmented the antitumor efficacy of cetuximab against SCC-1 tumors. Moreover, there was no evidence of toxicity in animals treated with either cetuximab alone or the combination of cetuximab and TGF-β.

Discussion

The mechanisms by which anti-EGFR antibodies, such as cetuximab, execute their antitumor effect *in vivo* include blockade of receptor–ligand interactions that stimulate tumor cell survival and growth and engagement of Fcγ receptors on immune effector cells that trigger ADCC of EGFR-expressing tumor cells (11, 12, 15, 27). The importance of both these mechanisms to the *in vivo* efficacy of anti-EGFR mAbs is supported by previous studies that showed that the F(ab')$_2$ fragment of the anti-EGFR antibody 225 (on which cetuximab is based) was able to reduce the *in vivo* growth of A431 tumor cells with only 50% of the activity shown by the intact antibody comprising a functional Fc domain (11). The crucial contribution of ADCC in mediating the *in vivo* antitumor activity of therapeutic mAbs is also evident from studies showing that the ability of these agents to arrest growth of tumors in normal mice is impaired in FcRγ$^{-/-}$ mice that are deficient in activating Fc receptors (27). Although the antibody Fc region triggers the activation of immune effector cells, the concomitant blockade of EGFR-mediated survival signals in tumor cells by the EGFR-specific antibody may also serve to render tumor cells more susceptible to immune effector cell-mediated death (28). The data presented here indicate that tumors play an active role in counteracting both these actions of anti-EGFR mAbs by increasing tumor cell-autonomous expression of TGF-β (29).

Our data show that tumor cell expression of TGF-β can exert a 2-pronged inhibitory effect on cetuximab-mediated ADCC of tumor cells. We find that TGF-β suppresses the expression of several key molecular effectors of immune cell–mediated cytotoxicity, including Apo2L/TRAIL, CD95L/FasL, granzyme B, and IFN-γ. In addition to exerting a tumor cell extrinsic inhibition of the cytotoxic function of immune effectors, TGF-β also promotes the activation of AKT (30), thereby providing an intrinsic EGFR-independent survival signal that protects tumor cells from the direct- and immune cell–mediated cytotoxic effects of cetuximab or erlotinib. Our data indicate that treatment with cetuximab results in the selection of tumor cells that express higher levels of TGF-β, thereby counteracting ADCC and limiting the efficacy of treatment. Conversely, combinatorial treatment with TGF-β–blocking antibody counteracts the selection of TGF-β–overexpressing tumor cells and immune suppression in tumor-bearing animals treated with cetuximab, thereby restoring ADCC and enhancing the antitumor efficacy of cetuximab *in vivo*. These results identify TGF-β as a key molecular determinant of the *de novo* or acquired resistance of cancers to EGFR-targeted mAbs, and provide a rationale for combinatorial targeting of TGF-β to improve the antitumor efficacy of EGFR-specific antibody therapy.

Although TGF-β exerts a tumor-suppressive effect on normal epithelial cells, tumor cells frequently become refractory to the growth inhibitory effect of TGF-β and acquire an ability to increase expression and secretion of TGF-β (31–34). This switch enables tumor cells to leverage the tumor promoting effects of TGF-β in the tumor microenvironment to facilitate tumor progression, invasion, and metastasis via promotion of EMT in carcinoma cells and suppression of immune responses (31–39). Many human cancers, including a majority of HNSCC, overexpress TGF-β, and the elevation of TGF-β is correlated with tumor progression, invasion, metastases, and poor prognosis. As such, the production TGF-β by tumor cells may be a frequent mechanism by which cancers induce immune tolerance in the tumor microenvironment and evade elimination by cetuximab. TGF-β–mediated resistance to cetuximab is distinct from previously described mechanisms associated with reduced therapeutic efficacy of cetuximab, such as mutated EGFR variants, redundant autocrine, or paracrine signaling by other EGFR receptors or insulin-like growth factor receptor (IGF-1R), or constitutive activation of either PI3K/AKT or Ras (40–45). The abnormal elevation of serum TGF-β in patients with HPV-negative HNSCC may underlie the relatively lower benefit of cetuximab-based therapy in these patients compared with that observed in patients with HPV-positive HNSCC who exhibit relatively lower levels of TGF-β. Our studies suggest that the clinical efficacy of anti-EGFR antibodies against HNSCC and other EGFR-expressing cancers could be enhanced by strategies to simultaneously sequester and counteract TGF-β in the tumor microenvironment (46–48).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

References

1. Forastiere AA, Goepfert H, Maor M, Pajak TF, Weber R, Morrison W, et al. Concurrent chemotherapy and radiotherapy for organ preservation in advanced laryngeal cancer. N Engl J Med. 2003; 349:2091–8. [PubMed: 14645636]

2. Cooper JS, Pajak TF, Forastiere AA, Jacobs J, Campbell BH, Saxman SB, et al. Postoperative concurrent radiotherapy and chemotherapy for high-risk squamous-cell carcinoma of the head and neck. N Engl J Med. 2004; 350:1937–44. [PubMed: 15128893]

3. Arteaga CL. Overview of epidermal growth factor receptor biology and its role as a therapeutic target in human neoplasia. Semin Oncol. 2002; 29:3–9. [PubMed: 12422308]

4. Mendelsohn J. Targeting the epidermal growth factor receptor for cancer therapy. J Clin Oncol. 2002; 20:1S–13S. [PubMed: 12235219]

5. Brashears J, Clyburn V, Shirai K. Introduction to molecular agents in the treatment of head and neck cancer: focus on Cetuximab. Cancer Therapy. 2008; 6:997–1004.

6. Huang SM, Bock JM, Harari PM. Epidermal growth factor receptor blockade with C225 modulates proliferation, apoptosis, and radio-sensitivity in squamous cell carcinomas of the head and neck. Cancer Res. 1999; 59:1935–40. [PubMed: 10213503]

7. Huang SM, Harari PM. Modulation of radiation response after epidermal growth factor receptor blockade in squamous cell carcinomas: inhibition of damage repair, cell cycle kinetics, and tumor angiogenesis. Clin Cancer Res. 2000; 6:2166–74. [PubMed: 10873065]

8. Mendelsohn J, Baselga J. Status of epidermal growth factor receptor antagonists in the biology and treatment of cancer. J Clin Oncol. 2003; 21:2787–99. [PubMed: 12860957]

9. Graham J, Muhsin M, Kirkpatrick P. Cetuximab. Nat Rev Drug Discov. 2004; 3:549–50. [PubMed: 15272498]

10. Masui H, Moroyama T, Mendelsohn J. Mechanism of antitumor activity in mice for anti-epidermal growth factor receptor monoclonal antibodies with different isotypes. Cancer Res. 1986; 46:5592–8. [PubMed: 3756906]

11. Fan Z, Masui H, Altas I, Mendelsohn J. Blockade of epidermal growth factor receptor function by bivalent and monovalent fragments of 225 anti-epidermal growth factor receptor monoclonal antibodies. Cancer Res. 1993; 53:4322–8. [PubMed: 8364927]

12. Bleeker WK, Lammerts van Bueren JJ, van Ojik HH, Gerritsen AF, Pluyter M, Houtkamp M, et al. Dual mode of action of a human anti-epidermal growth factor receptor monoclonal antibody for cancer therapy. J Immunol. 2004; 173:4699–707. [PubMed: 15383606]

13. Clynes R, Takechi Y, Moroi Y, Houghton A, Ravetch JV. Fc receptors are required in passive and active immunity to melanoma. Proc Natl Acad Sci U S A. 1998; 95:652–6. [PubMed: 9435247]

14. Ravetch JV, Bolland S. IgG Fc receptors. Annu Rev Immunol. 2001; 19:275–90. [PubMed: 11244038]

15. Lopez-Albaitero A, Ferris RL. Immune activation by epidermal growth factor receptor specific monoclonal antibody therapy for head and neck cancer. Arch Otolaryngol Head Neck Surg. 2007; 133:1277–81. [PubMed: 18086972]

16. Takai T, Li M, Sylvestre D, Clynes R, Ravetch JV. FcR gamma chain deletion results in pleiotrophic effector cell defects. Cell. 1994; 76:519–29. [PubMed: 8313472]

17. Bonner JA, Harari PM, Giralt J, Azarnia N, Shin DM, Cohen RB, et al. Radiotherapy plus cetuximab for squamous-cell carcinoma of the head and neck. N Engl J Med. 2006; 354:567–78. [PubMed: 16467544]

18. Massague J. A very private TGF-beta receptor embrace. Mol Cell. 2008; 29:149–50. [PubMed: 18243107]

19. Groppe J, Hinck CS, Samavarchi-Tehrani P, Zubieta C, Schuermann JP, Taylor AB, et al. Cooperative assembly of TGF-beta superfamily signaling complexes is mediated by two disparate mechanisms and distinct modes of receptor binding. Mol Cell. 2008; 29:157–68. [PubMed: 18243111]

20. Byers LA, Holsinger FC, Kies MS, William WN, El-Naggar AK, Lee JJ, et al. Serum signature of hypoxia-regulated factors is associated with progression after induction therapy in head and neck squamous cell cancer. Mol Cancer Ther. 2010; 9:1755–63. [PubMed: 20530716]

21. Ang KK, Harris J, Wheeler R, Weber R, Rosenthal DI, Nguyen-Tan PF, et al. Human papillomavirus and survival of patients with oropharyngeal cancer. N Engl J Med. 2010; 363:24–35. [PubMed: 20530316]

22. Benavente S, Huang S, Armstrong EA, Chi A, Hsu KT, Wheeler DL, et al. Establishment and characterization of a model of acquired resistance to epidermal growth factor receptor targeting agents in human cancer cells. Clin Cancer Res. 2009; 15:1585–92. [PubMed: 19190133]

23. Komesli S, Vivien D, Dutartre P. Chimeric extracellular domain type II transforming growth factor (TGF)-beta receptor fused to the Fc region of human immunoglobulin as a TGF-beta antagonist. Eur J Biochem. 1998; 254:505–13. [PubMed: 9688260]

24. Muraoka RS, Dumont N, Ritter CA, Dugger TC, Brantley DM, Chen J, et al. Blockade of TGF-beta inhibits mammary tumor cell viability, migration, and metastases. J Clin Invest. 2002; 109:1551–9. [PubMed: 12070302]

25. Benjamini Y, Yektueli D. The control of the false discovery rate in multiple testing under dependency. Ann Stat. 2001; 29:1165–88.

26. Chen G, Kronenberger P, Teugels E, Umelo IA, De Greve J. Targeting the epidermal growth factor receptor in non-small cell lung cancer cells: the effect of combining RNA interference with tyrosine kinase inhibitors or cetuximab. BMC Med. 2012; 10:28. [PubMed: 22436374]

27. Clynes RA, Towers TL, Presta LG, Ravetch JV. Inhibitory Fc receptors modulate *in vivo* cytoxicity against tumor targets. Nat Med. 2000; 6:443–6. [PubMed: 10742152]

28. Ravi R, Fuchs EJ, Jain A, Pham V, Yoshimura K, Prouser T, et al. Resistance of cancers to immunologic cytotoxicity and adoptive immunotherapy via X-linked inhibitor of apoptosis protein expression and coexisting defects in mitochondrial death signaling. Cancer Res. 2006; 66:1730–9. [PubMed: 16452233]

29. Zou W. Immunosuppressive networks in the tumour environment and their therapeutic relevance. Nat Rev Cancer. 2005; 5:263–74. [PubMed: 15776005]

30. Yi JY, Shin I, Arteaga CL. Type I transforming growth factor beta receptor binds to and activates phosphatidylinositol 3-kinase. J Biol Chem. 2005; 280:10870–6. [PubMed: 15657037]

31. Bierie B, Moses HL. Tumour microenvironment: TGFbeta: the molecular Jekyll and Hyde of cancer. Nat Rev Cancer. 2006; 6:506–20. [PubMed: 16794634]

32. Lu SL, Herrington H, Reh D, Weber S, Bornstein S, Wang D, et al. Loss of transforming growth factor-beta type II receptor promotes metastatic head-and-neck squamous cell carcinoma. Genes Dev. 2006; 20:1331–42. [PubMed: 16702406]

33. Koli KM, Arteaga CL. Complex role of tumor cell transforming growth factor (TGF)-beta s on breast carcinoma progression. J Mammary Gland Biol Neoplasia. 1996; 1:373–80. [PubMed: 10887511]

34. Wang SE, Yu Y, Criswell TL, Debusk LM, Lin PC, Zent R, et al. Oncogenic mutations regulate tumor microenvironment through induction of growth factors and angiogenic mediators. Oncogene. 2010; 29:3335–48. [PubMed: 20383197]

35. Li MO, Wan YY, Sanjabi S, Robertson AK, Flavell RA. Transforming growth factor-beta regulation of immune responses. Annu Rev Immunol. 2006; 24:99–146. [PubMed: 16551245]

36. Thomas DA, Massague J. TGF-beta directly targets cytotoxic T cell functions during tumor of immune surveillance. Cancer Cell. 2005; 8:369–80. [PubMed: 16286245]

37. Trotta R, Col JD, Yu J, Ciarlariello D, Thomas B, Zhang X, et al. TGF-beta utilizes SMAD3 to inhibit CD16-mediated IFN-gamma production and antibody-dependent cellular cytotoxicity in human NK cells. J Immunol. 2008; 181:3784–92. [PubMed: 18768831]

38. Chow A, Arteaga CL, Wang SE. When tumor suppressor TGFbeta meets the HER2 (ERBB2) oncogene. J Mammary Gland Biol Neoplasia. 2011; 16:81–8. [PubMed: 21590373]

39. Muraoka-Cook RS, Kurokawa H, Koh Y, Forbes JT, Roebuck LR, Barcellos-Hoff MH, et al. Conditional overexpression of active transforming growth factor beta1 *in vivo* accelerates metastases of transgenic mammary tumors. Cancer Res. 2004; 64:9002–11. [PubMed: 15604265]

40. Montagut C, Dalmases A, Bellosillo B, Crespo M, Pairet S, Iglesias M, et al. Identification of a mutation in the extracellular domain of the epidermal growth factor receptor conferring cetuximab resistance in colorectal cancer. Nat Med. 2012; 18:221–3. [PubMed: 22270724]

41. Karapetis CS, Khambata-Ford S, Jonker DJ, O'Callaghan CJ, Tu D, Tebbutt NC, et al. K-ras mutations and benefit from cetuximab in advanced colorectal cancer. N Engl J Med. 2008; 359:1757–65. [PubMed: 18946061]

42. Viloria-Petit A, Crombet T, Jothy S, Hicklin D, Bohlen P, Schlaeppi JM, et al. Acquired resistance to the antitumor effect of epidermal growth factor receptor-blocking antibodies *in vivo*: a role for altered tumor angiogenesis. Cancer Res. 2001; 61:5090–101. [PubMed: 11431346]

43. Prenzel N, Fischer OM, Streit S, Hart S, Ullrich A. The epidermal growth factor receptor family as a central element for cellular signal transduction and diversification. Endocr Relat Cancer. 2001; 8:11–31. [PubMed: 11350724]

44. Perera AD, Kleymenova EV, Walker CL. Requirement for the von Hippel-Lindau tumor suppressor gene for functional epidermal growth factor receptor blockade by monoclonal antibody C225 in renal cell carcinoma. Clin Cancer Res. 2000; 6:1518–23. [PubMed: 10778984]

45. Chandarlapaty S, Sawai A, Scaltriti M, Rodrik-Outmezguine V, Grbovic-Huezo O, Serra V, et al. AKT inhibition relieves feedback suppression of receptor tyrosine kinase expression and activity. Cancer Cell. 2011; 19:58–71. [PubMed: 21215704]

46. Yingling JM, Blanchard KL, Sawyer JS. Development of TGF-beta signalling inhibitors for cancer therapy. Nat Rev Drug Discov. 2004; 3:1011–22. [PubMed: 15573100]

47. Akhurst RJ. Large- and small-molecule inhibitors of transforming growth factor-beta signaling. Curr Opin Investig Drugs. 2006; 7:513–21.

48. Arteaga CL. Inhibition of TGFbeta signaling in cancer therapy. Curr Opin Genet Dev. 2006; 16:30–7. [PubMed: 16377175]
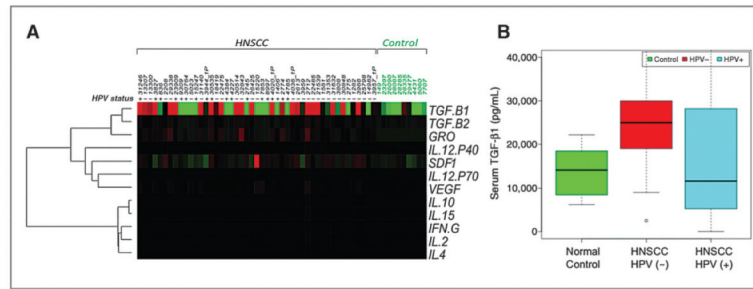
Figure 1.
Patients with HPV-negative HNSCC exhibit elevated levels of serum TGF-β. A, heatmap showing differential serum levels of the indicated cytokines in patients with HNSCC and pleomorphic adenoma (noncancer control). B, differential levels of serum TGF-β1 in patients with HPV-negative HNSCC, HPV-positive HNSCC, and pleomorphic adenoma (noncancer control).
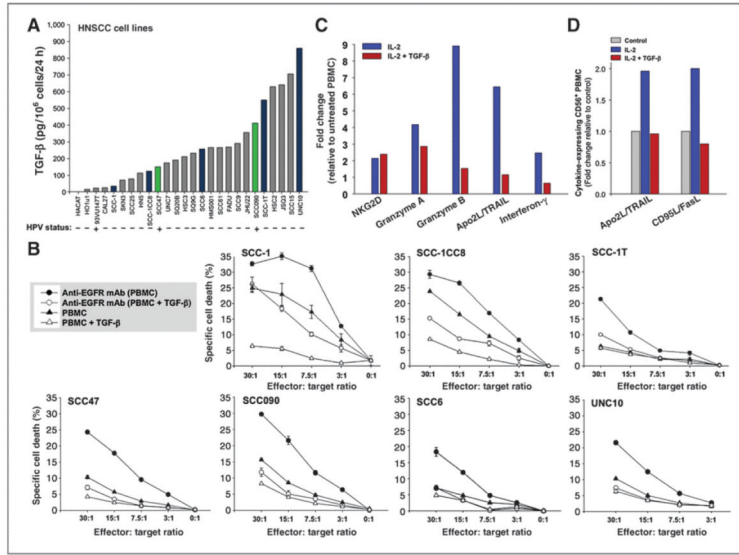
Figure 2.
Tumor cell expression of TGF-β inhibits the expression of cytotoxic effector molecules in immune cells and suppresses their ability to induce cetuximab-mediated ADCC of tumor cells. A, differential levels of TGF-β produced by human HNSCC cell lines in tumor cell supernatants analyzed by ELISA. B, comparative susceptibility of the indicated HNSCC cell lines to anti-EGFR mAb (cetuximab)-mediated ADCC induced by normal PBMC stimulated with rhIL-2 in the presence or absence of rhTGFβ1 for 48 hours. C, TGF-β inhibits the expression of cytotoxic effector molecules in immune effector cells. Normal PBMCs were stimulated with rhIL-2 in the presence or absence of rhTGFβ1 for 48 hours, and expression of the specific mRNA (granzyme B, Apo2L/TRAIL, and IFN-γ) was quantified by real-time PCR. D, TGF-β inhibits surface expression of Apo2L/TRAIL and CD95L/FasL on immune effector cells. Normal PBMCs were stimulated with rhIL-2 in the presence or absence of rhTGFβ1 for 72 hours, and surface expression of Apo2L/TRAIL and CD95L/FasL on CD56^{+} NK cells were analyzed by flow cytometry.
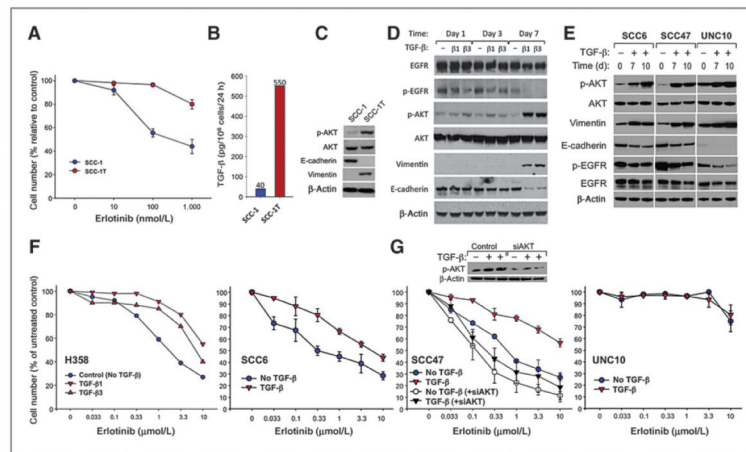
Figure 3.
Autonomous expression of TGF-β activates AKT and enables EGFR-independent survival of tumor cells. A, sensitivity of SCC-1 and SCC-1T cells to the EGFR TKI, erlotinib. Cells were exposed to erlotinib for 72 hours, and cell viability was assayed using the MTT assay. B, differential expression of TGF-β by SCC-1 cells (blue) and its isogenic variant, SCC-1T cells (red), in tumor cell supernatants analyzed by ELISA. C, immunoblot analysis of phospho-AKT, AKT, E-cadherin, and vimentin in SCC-1 and SCC-1T cells showing increased phosphorylation of AKT and EMT in SCC-1T cells. D and E, *in vitro* treatment of tumor cells with rhTGF-β results in activation of AKT, induction of EMT, and loss of EGFR activity. H358 cells (D) or the indicated HNSCC cell lines (E) treated with either rhTGF-β1 or rhTGF-β3 for the indicated time and their untreated controls were subjected to immunoblot analyses for expression of AKT, phospho-Akt (Ser473), vimentin, E-cadherin, EGFR, and phospho-EGFR. F, TGF-β activates EGFR-independent AKT-mediated survival signals that render tumor cells relatively resistant to erlotinib. HNSCC cell lines (UNC10, SCC6, AKT siRNA- or control siRNA-transfected SCC47) or H358 cells that were pretreated with either TGF-β1 or TGF-β3 and their untreated counterparts (control) were exposed to the indicated concentrations of erlotinib for 48 hours and subsequently assessed for cell viability. G, treatment of SCC47 cells with siRNA against AKT inhibits TGF-β–induced elevation of p-AKT. Immunoblot analysis of p-AKT in SCC47 cells (with or without TGF-β for 48 or 72 hours) after transfection with either siRNA against AKT (siAKT) or control siRNA.
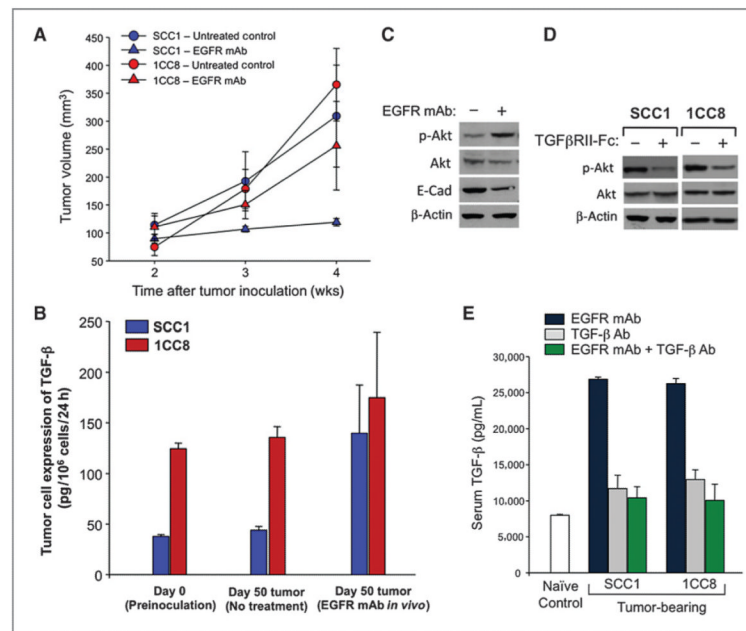
Figure 4.
Treatment of tumor-bearing animals with cetuximab results in the *in vivo* selection of resistant tumor cells with elevated expression of TGF-β and TGF-β–dependent activation of AKT. A, *in vivo* response of tumor xenografts of SCC-1 cells or its isogenic variant 1CC8 to treatment with EGFR mAb (cetuximab). Athymic (nu/nu) mice injected subcutaneously with SCC-1 or 1CC8 tumor cells were treated with cetuximab (5 mg/kg i.p. twice weekly × 3 weeks) or PBS (untreated control). Tumor volume was measured weekly. B, treatment with EGFR mAb (cetuximab) results in selection of tumor cells with higher autonomous expression of TGF-β. Explanted tumor cells derived from tumors in untreated mice or from residual tumors after cetuximab treatment were cultured *ex vivo*, and the amount of TGF-β in cell supernatants was measured by ELISA. C, *in vivo* treatment with cetuximab forces selection of SCC1 tumor cells that exhibit autonomous TGF-β–dependent activation of AKT and EMT. Explanted tumor cells derived from tumors in untreated mice or from residual tumors after cetuximab treatment were analyzed for expression of AKT, phospho-Akt (Ser473), and E-cadherin by immunoblot assays. D, TGF-β mediates EGFR-independent activation of AKT in tumor cells from cetuximab-treated animals. Explanted tumor cells derived from residual SCC1 and 1CC8 tumors after cetuximab treatment were cultured for 24 hours in the presence or absence of a TGF-β antagonist (TGFβRII-Fc, 500 ng/mL), and analyzed for expression of AKT and phospho-Akt (Ser473) by immunoblot assays. E, serum levels of TGF-β in naïve mice (nontumor bearing) and in SCC1- or 1CC8–tumor-bearing mice treated with EGFR mAb (cetuximab), TGF-β antibody (TGF-β Ab) or a combination of EGFR mAb with TGF-β antibody.
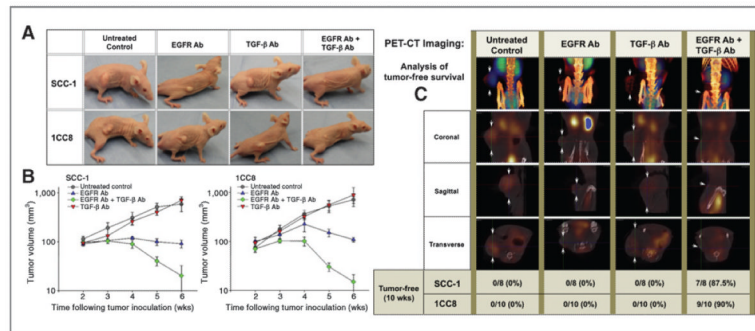
Figure 5.
Inhibition of TGF-β improves the *in vivo* antitumor efficacy of cetuximab against tumor xenografts of HNSCC cells. The 3×10^6 tumor cells were implanted into the right flank of female athymic mice (nu/nu) to generate tumor xenografts of SCC1 or 1CC8 cells. Once the tumors reached a size of approximately 100 mm^3, the mice were treated with cetuximab (5 mg/kg i.p. twice weekly for 4 weeks) and/or TGF-β antibody (5 mg/kg i.p., once weekly for 4 weeks). A and B, tumor volume was evaluated weekly. C, *in vivo* PET-CT imaging analyses showing the comparative responses and tumor-free survival of mice bearing HNSCC xenografts to treatment with either cetuximab alone, TGF-β antibody alone, or a combination of cetuximab and TGF-β antibody.