Eradication of Established Tumors by a Fully Human Monoclonal Antibody to the Epidermal Growth Factor Receptor without Concomitant Chemotherapy

Xiao-Dong Yang, Xiao-Chi Jia, Jose R. F. Corvalan, Ping Wang, C. Geoffrey Davis, and Aya Jakobovits

Abgenix, Inc., Fremont, California 94535

ABSTRACT

A fully human IgG2κ monoclonal antibody (MAb), E7.6.3, specific to the human epidermal growth factor (EGF) receptor (EGFr) was generated from a human antibody-producing XenoMouse strains engineered to be deficient in mouse antibody production and to contain the majority of the human antibody gene repertoire on megabase-sized fragments from the human heavy and κ light chain loci. The E7.6.3 MAb exhibits high affinity ($K_d = 5 \times 10^{-11} \text{M}$) to the receptor, blocks completely the binding of both EGF and transforming growth factor α (TGF-α) to various EGFr-expressing human carcinoma cell lines, and abolishes EGFr-dependent cell activation, including EGFr tyrosine phosphorylation, increased extracellular acidification rate, and cell proliferation. The antibody (0.2 mg i.p. twice a week for 3 weeks) prevents completely the formation of human epidermoid carcinoma A431 xenografts in athymic mice. More importantly, the administration of E7.6.3 without concomitant chemotherapeutics results in complete eradication of established tumors as large as 1.2 cm$^3$. Tumor eradication of A431 xenografts was achieved in nearly all of the mice treated with total E7.6.3 doses as low as 3 mg, administered over the course of 3 weeks, and a total dose of 0.6 mg led to tumor elimination in 65% of the mice. No tumor recurrence was observed for more than 8 months after the last antibody injection, which further indicates complete tumor cell elimination by the antibody. The potency of E7.6.3 in eradicating well-established tumors without concomitant chemotherapy indicates its potential as a monotherapeutic agent for the treatment of multiple EGFr-expressing human solid tumors, including those for which no effective chemotherapy is available. Being a fully human antibody, E7.6.3 is expected to exhibit minimal immunogenicity and a longer half-life as compared with mouse or mouse-derivative MAbs, thus allowing repeated antibody administration, including in immunocompetent patients. These results suggest E7.6.3 as a good candidate for assessing the full therapeutic potential of anti-EGFr antibody in the therapy of multiple patient populations with EGFr-expressing solid tumors.

INTRODUCTION

Most applications of MAb2s in cancer therapy rely on the ability of the antibody to specifically deliver to the cancerous tissues cytotoxic effector functions such as immune-enhancing isotopes, toxins, or drugs. An alternative approach is to use MAb to directly affect the survival of tumor cells by depriving them of essential extracellular proliferation signals such as those mediated by growth factors through their cell receptors. One of the attractive targets in this approach is EGFr, which binds EGF and TGF-α (1–4). The binding of EGF or TGF-α to EGFr—a $M_r$ 170,000 transmembrane cell surface glycoprotein—triggers a cascade of cellular biochemical events including EGFr autophosphorylation and internalization, which culminates in cell proliferation (1).

Several observations implicate EGFr in supporting the development and progression of human solid tumors. Overexpression of EGFr has been shown to induce transformed properties in recipient cells (5).

EGFr expression has been found to be up-regulated on many human tumors including lung, colon, breast, prostate, brain, head and neck, ovarian, and renal carcinoma, and the increase in receptor levels has been reported to be associated with a poor clinical prognosis (2, 3, 6–8). In many cases, the increased surface EGFr expression was accompanied by the production of TGF-α or EGF by the tumor cells, suggesting the involvement of an autocrine growth control in the progression of these tumors (2, 3, 6, 8). These observations suggested that blocking the interaction between the growth factors and EGFr could result in the arrest of tumor growth and could possibly affect tumor survival (2–6).

MAb specific to the human EGFr, capable of neutralizing the EGF and TGF-α binding to tumor cells and of inhibiting ligand-mediated cell proliferation in vitro, have been generated from mice and rats (2, 4, 6). Some of these antibodies, such as the mouse 108 (9), 225, and 528 (2, 3) or the rat ICR16, ICR62, and ICR64 (6, 10, 11) MAb were evaluated extensively for their ability to affect tumor growth in mouse xenograft models. Most of the anti-EGFr MAb were efficacious in preventing tumor formation in athymic mice when administered together with the human tumor cells (2, 11). When injected into mice bearing established human tumor xenografts, the mouse MAb 225 and 528 caused partial tumor regression and required the coadministration of chemotherapeutic agents such as doxorubicin or cisplatin for the eradication of the tumors (2, 3, 12, 13). A chimeric version of the 225 MAb (C225), in which the mouse antibody variable regions are linked to human constant regions, exhibited an improved in vivo antitumor activity but only at high doses (14, 15). The rat ICR16, ICR62, and ICR64 antibodies caused regression of established tumors but not their complete eradication (11). These results established EGFr as a promising target for antibody therapy against EGFr-expressing solid tumors and led to human clinical trials with the C225 MAb in multiple human solid cancers (2, 3, 6). However, the limited efficacy of these MAb as monotherapeutic agents required their assessment in combination with chemotherapy (16, 17). This requirement can limit the utilization of anti-EGFr antibodies in patients for whom chemotherapy is not available. Therefore, the identification of an anti-EGFr antibody capable of eradicating established human tumors by itself can expand the patient populations and cancer indications to which EGFr antibody therapy can be applied successfully. In addition, the MAb currently pursued in human clinical trials, being murine chimeric antibodies (2, 4), are likely to induce immune or allergic responses to the mouse components in immunocompetent patients, which leads to reduction in the antibody efficacy and safety. Therefore, anti-EGFr antibody therapy can be fully evaluated with the availability of a fully human anti-EGFr antibody that exhibits therapeutic efficacy on EGFr-expressing tumors and that can be administered repeatedly to all appropriate patient populations.

To this end, we used our human antibody-producing XenoMouse strains to generate potent fully human anti-EGFr MAb. As described previously (18), these mouse strains were engineered to be deficient in mouse antibody production and to contain integrated megabase-sized fragments from the human heavy and κ light chain loci with the majority of the human antibody gene repertoire. The human immunoglobulin loci provided the XenoMouse strains with the ability to produce high-affinity human MAb to a broad spectrum of antigens including human antigens.
MATERIALS AND METHODS

**XenoMouse Strains.** Generation and characterization of the XenoMouse-G2 strains, engineered to produce fully human IgG2x MAbs, are described by Mendez et al. (18).

**Human MAB Production and Purification.** XenoMouse strains (8–10 weeks old) were immunized i.p. with 2 × 10^6 A431 cells (American Type Culture Collection, CRL-7907) in incomplete Freund's adjuvant. Mice were boosted with the same number of A431 cells in incomplete Freund's adjuvant three times before fusion. The fusion of splenocytes from immunized mice and selection of hybridomas were carried out as described previously (18). EGFr-specific hybridomas were identified by ELISA using purified A431 cell membrane-derived EGFr (Sigma). Large quantities of antibodies were purified from ascites that were derived from severe combined immunodeficient mice inoculated with antibody-producing hybridomas, using protein-A affinity chromatography.

**Measurement of Antibody Affinity to EGFR.** Affinities of purified anti-EGFr antibodies were determined by plasmon resonance technology using BIAcore 2000 (Pharmacia). On the basis of the general procedures outlined by the manufacturer, kinetic analyses of the antibodies were performed using either purified membrane-derived EGFr or recombinant extracellular domain of EGFr (20) immobilized onto the sensor surface at a low density (303 RU). The association (kₐ) and dissociation (k₅) rates were determined using the BIAevaluation2.1 software provided by the manufacturer. Affinity measurements of antibody in solution were carried out as described previously (18).

**EGFr Binding Assays.** EGFr binding assays were conducted using human recombinant 125I-EGFr or 125I-TGF-α (Amersham Life Science, Arlington Heights, IL) as described previously (18). Briefly, human carcinoma cells growing in DMEM containing 10% FCS were detached with trypsin, washed with PBS, and resuspended in binding buffer (PBS containing 0.1% BSA (Sigma) and 0.02% NaN₃), and distributed in 96-well Multiscreen filter plates (Millipore) at 4 × 10^³ cells/well in 50 μl of medium. Fully human anti-EGFr or control anti-keyhole limpet hemocyanin MAbs, control human myeloma IgG2x MAb (Calbiochem, Cambridge, MA), or mouse anti-EGFr 225 or 528 MAbs (Calbiochem) diluted in binding buffer, were added in 50 μl aliquots per well. Plates were incubated for 30 min at 4°C. 125I-EGFr or 125I-TGF-α (0.1 nM) was added and the plates were further incubated for 90 min at 4°C. After incubation, the plates were washed five times with binding buffer, air-dried, and counted in a scintillation counter. The percentage of specifically bound antibody was calculated as follows:

\[
\text{Specific binding} = \frac{\text{cpm detected in the presence of antibody}}{\text{cpm detected in the presence of buffer only}} \times 100
\]

The binding data obtained was fitted using GraphPad Prism (GraphPad Software, Inc., San Diego, CA).

**In Vitro Tumor Cell Proliferation Assay.** The effect of antibodies on the growth of human tumor cells was determined using the method described by Ishiyama et al. (21). Briefly, 2 × 10^³ cells in 100 μl of DMEM:F12 growth medium without serum were seeded into each well of a 96-well plate. Aliquots of each diluted antibody (100 μl/well) were added in triplicate to the wells and concentrations of antibody in solution were carried out as described previously (18). EGFr-specific hybridomas were identified by ELISA using purified A431 cell membrane-derived EGFr (Sigma). Large quantities of antibodies were purified from ascites that were derived from severe combined immunodeficient mice inoculated with antibody-producing hybridomas, using protein-A affinity chromatography.

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\[
\text{Growth inhibition} = \frac{\text{Mean A₅₉₅ measured in medium only} - \text{A₅₉₅ in the presence of antibody}}{\text{Mean A₅₉₅ in the presence of medium only}} \times 100
\]

**EGFR Phosphorylation.** Seventy % confluent A431 cells were preincubated overnight with 0.5% of fetal bovine serum at 37°C. The cells were then treated with 16 nM EGF in the absence or presence of different concentrations of E7.6.3 MAb for 30 min at 37°C. After the 30-min incubation, the cells were washed three times with cold PBS and scraped into 0.5 ml of lysis buffer (10 mM Tris, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.1 mg/ml PMSF, 1 μg/ml aprotonin, 1 μg/ml leupeptin, and 1 mM sodium orthovanadate). After 30 min of incubation on ice, the lysates was centrifuged at 10,000 rpm for 5 min in an Eppendorf microcentrifuge at 4°C. The protein concentration in the supernatant was measured using BCA protein assay reagents (Pierce). A small portion of the supernatant was mixed with sample buffer (Novex, San Diego, CA) and boiled for 3 min. The proteins in the supernatant were then separated by 12% SDS-PAGE. Equal amounts of total protein were loaded from each cell lysate. Mouse antiphosphotyrosine (Zymed Laboratories, South San Francisco, CA) was used for the detection of EGFr tyrosine phosphorylation on Western blots. Enhanced chemiluminescence Western blotting detection reagents (Amersham) and the Hyperfilm Enhanced chemiluminescence (Amersham) were used for visualizing the signal. The integrated densities of the bands of interest were analyzed using an AGFA Scanner and the Scanalytics OneDscan software (Hewlett Packard, Mountain View, CA).

**Measurement of Cell Activation by Cytosensor Microphysiometry.** To assess the effect of antibody on EGF-mediated signaling, Cytosensor Microphysiometry (Molecular Devices) was used. The Cytosensor detects early biochemical events in cell activation based on increases in the rate of acid release by the cells (22). Acid release was measured as described in the user’s manual provided by Molecular Devices, Inc. Briefly, A431 cells (5 × 10^⁶) were seeded in 1 ml of medium in a Cytosensor cell capsule and cultured at 37°C for 24 h. After incubation, the cell capsules were assembled and loaded in the Cytosensor sensing chamber maintained at 37°C. The chamber was perfused with low buffer RPMI 1640 containing 1 mM sodium phosphate and 1 mg/ml endotoxin-free BSA. Acid release rates were determined with 30-s potentiometric pH measurements after an 85-s pump cycle and 5-s delay (120-s total cycle time). Basal acid release rates ranged from 60 to 120 mV/s. Percent inhibition is calculated as follows:

\[
\text{Acid release in the presence of EGF only} - \text{acid release in the presence of EGF and antibody} \times 100
\]

**Tumor Xenograft Mouse Models.** Male BALB/c-nu/nu mice (6–8 weeks of age) were injected s.c. with 5 × 10^⁶ A431 or MDA-MB-468 (American Type Culture Collection, HTB-132) cells in 100 μl of PBS. Tumor sizes were measured in a blind fashion twice a week with a vernier caliper and tumor volume was calculated as the product of length × width × height ÷ 6. Mice with established tumors were randomly divided into treatment groups. Animals were treated with antibodies using different regimens. Typically, mice received antibody treatment twice a week over three consecutive weeks either concomitant with the tumor cell injection (prophylactic treatment) or after tumor establishment (therapeutic treatment). The mice were followed for tumor xenograft growth and survival for at least 60 days.

**Tumor Histopathological Evaluation.** Biopsies obtained from athymic mice carrying human xenografts were fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned. The sections were then stained with H&E as described previously (23).

**RESULTS**

**High-Affinity Neutralizing Fully Human Anti-EGFr MAbs from XenoMouse Strains.** XenoMouse-G2 strains that produce fully human IgG2x antibodies were immunized with the human vulgar
epidermoid carcinoma A431 cells. These cells express approximately 1 x 10^6 EGFr/cell (Refs. 2, 3 and data not shown). Fusion of B cells from immunized Xenomouse strains with mouse myeloma cells yielded a panel of 30 hybridomas that secrete human IgG2κ MAbs specific to the extracellular domain of human EGFr, as determined by ELISA, BIAcore, Western blots, and flow cytometry analysis (data not shown). The human γ2 was chosen as the preferred isotype with minimal immune-associated cytotoxicity against EGFr-expressing normal tissues.

To identify human MAbs with neutralization activity, purified antibodies were evaluated for their ability to block the binding of EGF and TGF-α to human tumor cell lines that express low (colon carcinoma SW948, 5 x 10^3/cell) or high (A431, or breast adenocarcinoma MDA-MB-468, 1 x 10^5/cell) levels of EGFr. As positive controls, the commercially available murine MAbs, 225 and 528, were tested in parallel. A Xenomouse-derived IgG2κ antibody PK16.3.1, specific for keyhole limpet hemocyanin, or a nonspecific human myeloma IgG2κ antibody were used as a negative control. Fig. 1A represents the results obtained with a subset of the fully human anti-EGFr MAbs tested in these assays. Three of the five human anti-EGFr antibodies shown, E7.6.3, E2.5.1, and E2.3.1, and the mouse anti-EGFr 225 and 528 MAbs blocked the binding of [125I]-EGF (0.1 nm) to A431 in a concentration-dependent manner. In contrast, E7.5.2 and E7.8.2 did not have any effect on EGF binding. The calculated IC_{50} values (3.0 nm for E7.6.3, 5.6 nm for E2.5.1, 9.1 nm for E2.3.1, 8.8 nm for 225, and 15.2 nm for 528) suggested E7.6.3 as a potent neutralizing antibody. Furthermore, EGF binding to SW948 cells was also blocked by the human E7.6.3 and E2.3.1 and by the mouse 225 MAbs (Fig. 1B). The IC_{50} values detected in studies with SW948 cells were 0.9 nm for E7.6.3, 0.24 nm for E2.3.1, and 0.17 nm for 225. The efficacy of E7.6.3 in neutralizing ligand binding was also demonstrated in blocking TGF-α binding to A431 cells (data not shown). These results indicated that XenoMouse strains are capable of producing fully human anti-EGFr antibodies that recognize different epitopes on the receptor, including those involved in ligand binding.

The affinity of the purified E7.6.3 MAb to EGFr was determined to be 5 x 10^{-11} M by both solid phase and solution measurements (K_{d} = 1.97 x 10^{-6}; K_{eff} = 1.13 x 10^{-4}). E7.6.3 exhibits cross-reactivity with African Green monkey EGFr but not with the mouse EGFr (data not shown). The E7.6.3 hybridoma exhibited significant levels of antibody production that reached a specific productivity rate of 12 pg/ cell/day in serum-free medium growth conditions. On the basis of its high affinity to EGFr and its potency in blocking EGF/TGF-α binding, E7.6.3 MAb was selected for further evaluation of its efficacy in affecting tumor cell growth in vitro and in vivo.

**E7.6.3 MAb Inhibits EGF-mediated Tumor Cell Activation.**

The ability of E7.6.3 to inhibit tumor cell activation was evaluated by examining its effects on EGF-activated cellular responses such as the tyrosine kinase activity of EGFr, the extracellular acidification rate, and cell proliferation.

One of the first events after EGF binding to its receptor is the induction of EGFr tyrosine kinase activity, which results in the auto-phosphorylation of the receptor (1). As shown in Fig. 2, incubation of human EGF (16 nm) with A431 cells induced the tyrosine phosphorylation of the M_{r} 170,000 EGFr. While E7.6.3 did not activate the receptor tyrosine kinase activity, the antibody blocked EGF-activated EGFr tyrosine phosphorylation in a dose-dependent manner, with a nearly complete inhibition at a concentration of 133 nm (antibody: EGF molar ratio, 8:1; Fig. 2).

Engagement of EGFr with its receptors results in cell activation, which is reflected by changes in the extracellular acidification rate. These changes can be detected by the Cytosensor Microphysiometer, a pH-sensitive silicon sensor that measures real-time changes in the acidification of the microenvironment surrounding a population of stimulated cells (22). Using this assay, we examined the effect of E7.6.3 on EGF-mediated A431 cell activation. As shown in Fig. 3A, the addition of 1.67 nm EGF to A431 cells induced an immediate increase in the extracellular acidification rate. No effect was observed when the cells were incubated only with E7.6.3 antibody at concentrations up to 100 nm (not shown). The concurrent addition of E7.6.3 resulted in a dose-dependent inhibition of EGF-mediated extracellular acidification (Fig. 3A and B), whereas no effect was detected with the isotype-matched control antibody PK16.3.1 (Fig. 3B).

Lastly, we examined the effect of E7.6.3 on the in vitro proliferation of the EGFr-expressing tumor cell lines A431 and MDA-MB-468, again in comparison with the mouse anti-EGFr antibodies. Both cell lines, expressing high levels of EGFr, have been shown to secrete TGF-α and to be growth-inhibited by the addition of exogenous EGF at nm concentrations (24, 25). Therefore, the experiments using these two cell lines were carried out in the absence of exogenous EGF, E7.6.3 inhibited the growth of A431 cells in a dose-dependent manner, with a maximal inhibition of 60%, a level similar to that obtained with the mouse antibody 225 and higher than that observed for the 528 antibody (Fig. 4A). The control antibody did not have any effect on the cell proliferation (Fig. 4A).

The calculated IC_{50} values for E7.6.3 (0.125 nm), 225 (0.48 nm), or 528 (0.66 nm)
EGF/TGF-β-human antibody to block autocrine growth stimulation and thus to inhibit EGF was added to the culture, these results indicate the ability of the observed with MDA-MB-468 cells (Fig. 4). A431 tumor xenografts that reached a size of 0.13–1.2 cm³ (calculated from the different cell lysates were loaded in each lane (not shown). EGFr tyrosine phosphorylation in cell lysates was visualized (A) and quantitated (B) using Western blot analysis and an antiphosphotyrosine antibody as described in “Materials and Methods.” EGF-induced EGFr tyrosine phosphorylation in the absence of antibodies was designated as 100%.

0.25-cm³-sized tumors were treated i.p. with 1 mg of either E7.6.3 MAb or the human myeloma IgG2 k Mab or the human myeloma IgG2 k Mab. A431 cells were incubated with or without 16 nM EGF, in the absence or presence of increasing concentrations of E7.6.3 MAb (0.2–133 nM) for 30 min. Cell lysates were separated by PAGE as described in “Materials and Methods.” Equal amounts of total protein from the different cell lysates were loaded in each lane (not shown). EGFr tyrosine phosphorylation in cell lysates was visualized (A) and quantitated (B) using Western blot analysis and an antiphosphotyrosine antibody as described in “Materials and Methods.” EGF-induced EGFr tyrosine phosphorylation in the absence of antibodies was designated as 100%.

To compare the antitumor activity of E7.6.3 to that of the mouse 225 antibody, which was reported to affect the growth of established A431 tumors but not cause their elimination (12, 13), we used suboptimal E7.6.3 doses (0.05 mg and 0.2 mg, given twice a week for 3 weeks) that also caused primarily tumor regression in A431 xenografts. At these antibody doses, there was a significant difference between the ability of E7.6.3 and 225 to arrest the growth of A431 xenografts (Fig. 5C).

E7.6.3 MAB Prevents Human Tumor Formation in Mice. To examine the effect of E7.6.3 on tumor cell growth in vivo, the antibody was first evaluated for its ability to prevent the formation of A431 tumor xenografts in athymic mice. A431 cells (5 × 10⁶/mouse) were injected s.c. into mice in conjunction with i.p. administration of PBS (group 1), 1 mg of the control antibody PK16.3.1 (group 2), or 0.2 mg or 1 mg of E7.6.3 (groups 3 and 4). The antibody administration was repeated twice a week for 3 weeks, for a total dose of 1.2 mg (group 3) or 6 mg (groups 2 and 4). As shown in Table 1, all of the mice treated with either PBS or the control antibody developed tumors by day 10 after inoculation and were killed at day 30 because of the large size of the tumors. In contrast, none of the mice treated with E7.6.3 antibody developed tumors for more than 8 months after the last antibody injection. The data indicated that E7.6.3 prevented the formation of A431 xenografts, probably by exerting its neutralization activity at the initial phase of the tumor cell proliferation.

E7.6.3 MAB Eradicates Large Human Tumor Xenografts. The effect of E7.6.3 on the growth of established tumors was examined on A431 tumor xenografts that reached a size of 0.13–1.2 cm³ (calculated as length × width × height × π/6). Initially, mice bearing 0.13- to 0.25-cm³-sized tumors were treated i.p. with 1 mg of either E7.6.3 Mab or the human myeloma IgG2κ control antibody, twice a week for 3 weeks. As shown in Fig. 5A and in Table 2, the tumors in untreated mice or in mice treated with the control antibody continued their aggressive growth to reach the size of 3 cm³ by day 30, at which point the mice were killed. In contrast, treatment with E7.6.3 not only arrested further growth of the tumors but also caused continuous tumor regression that resulted in a complete tumor eradication in all of the mice treated (Fig. 5A, Table 2). No recurring tumors were detected for more than 250 days in any of the mice that were monitored, demonstrating a long-lasting effect of the antibody and its ability to completely eliminate all of the tumor cells.

We next evaluated the potency of E7.6.3 antibody to treat large established tumor xenografts. Mice bearing 0.13-, 0.56-, 0.73-, or 1.2-cm³-sized A431 tumors were treated i.p. with 1 mg of E7.6.3 twice a week for 3 weeks, initiated on day 7, 11, 15, or 18, respectively. As demonstrated in Fig. 5B, E7.6.3 caused a profound tumor regression in all of the treated mice regardless of their initial tumor size, even with tumors as large as 1.2 cm³. Furthermore, this treatment led to a complete disappearance of the tumors (Fig. 5B) and no tumor recurrence in any of the mouse groups for 210 days after the last antibody injection (data not shown). As summarized in Table 2, the antibody effect appears to be dose-dependent with a total dose of 3 mg leading nearly to a complete tumor eradication and a total dose of 0.6 mg eliminating 65% of the established A431 xenografts.

Fig. 2. The inhibition of EGF-induced tyrosine phosphorylation of EGFr by E7.6.3 MAb. A431 cells were incubated with or without 16 nM EGF, in the absence or presence of increasing concentrations of E7.6.3 MAb (0.2–133 nM) for 30 min. Cell lysates were separated by PAGE as described in Materials and Methods. Equal amounts of total protein from the different cell lysates were loaded in each lane (not shown). EGFr tyrosine phosphorylation in cell lysates was visualized (A) and quantitated (B) using Western blot analysis and an antiphosphotyrosine antibody as described in Materials and Methods. EGF-induced EGFr tyrosine phosphorylation in the absence of antibodies was designated as 100%.

Fig. 3. The inhibition of EGF-mediated cell activation by anti-EGFβ antibodies. A, activation of A431 cells by 1.67 nM EGF, in the absence or presence of different concentrations (0.1–1000 nM) of E7.6.3, was measured by Cytosensor as changes in extracellular acidification rate. Arrow, the time when EGF and/or E7.6.3 were added to the cells. The response is presented as % of baseline acidification rate (designated as 100%). B, the effect of increasing concentrations of E7.6.3 and control PK16.3.1 antibodies on A431 cell activation induced by 1.67 nM EGF as determined by Cytosensor. The response to EGF was measured at the peak acidification rate shown in A. The response in the absence of antibodies was designated as 100%. The data shown are representative of two different experiments.
HUMAN ANTI-EGFr MAb ERADICATES LARGE TUMOR XENOGRAFTS

DISCUSSION

Utilization of XenoMouse strains for the production of human antibodies specific to the human EGFr yielded the fully human IgG2κ Mab, E7.6.3, characterized by high affinity and strong neutralization activity. Its demonstrated efficacy in eradicating large established human tumor xenografts without concomitant chemotherapy strongly suggests that it is a suitable candidate for antibody monotherapy in patients with EGFr-expressing tumors.

E7.6.3 exhibited strong efficacy in blocking the binding of EGF and TGF-α to EGFr on the surface of different human carcinoma cell lines, including those that express high levels of receptors (Fig. 1). The complete inhibition of ligand binding to the receptors on A431 and SW948 cells resulted in an abolishment of the signaling events triggered by EGF or TGF-α, including EGFr autophosphorylation, increased extracellular acidification rate, and cell proliferation. Our results indicate that E7.6.3 can block ligand-induced cell activation and that E7.6.3 does not act as an agonist to trigger cellular responses in EGFr-expressing tumors (Figs. 2 and 3).

The antitumor activity of E7.6.3 was examined in multiple xenograft mouse experiments in which the effects of various antibody doses on different sizes of tumors were established (Figs. 5 and 6). The results obtained from these studies demonstrated the unique antitumor properties of E7.6.3 MAb as compared with the other reported anti-EGFr antibodies. E7.6.3 not only arrested the growth of human tumor xenografts but also completely eradicated established tumors by itself, without the need for concomitant chemotherapy. Tumor eradication of A431 xenografts was achieved in nearly all of the mice treated with total doses as low as 3 mg administered over the course of 3 weeks, and a total dose of 0.6 mg led to tumor elimination in 65% of the mice (Fig. 5 and Fig. 6B, Table 2). In comparison, 8 mg of 225 and 10 mg of 528 antibodies, given over 4 and 10 weeks, respectively, had only a limited effect on A431 tumors and required the coadministration of chemotherapeutic drugs to achieve the elimination of the tumors (12, 13). A direct comparison between E7.6.3 and 225 MAbs at low doses demonstrated E7.6.3 as a more potent antibody in regressing established A431 tumors and arresting their growth (Fig. 5C). The chimeric C225 MAb, which was reported to acquire higher affinity to EGFr and enhanced in vivo antitumor activities, achieved complete A431 tumor eradication at a total dose of 10 mg given for 5 weeks, whereas total doses of 2.5 and 5 mg led to only 14% and 57% of tumor-free mice (14). The potent antitumor activity of E7.6.3 was

The complete inhibition of ligand binding to the receptors on A431 xenograft-bearing mice that were treated with the lower antibody doses. Biopsies taken from these nodules at day 79 were shown to contain a thin fibrovascular capsule lined by necrotic cells with its center filled with keratinoic and calcified debris (Fig. 7A). There was no evidence of neoplastic cells, which were readily detected in tumor taken from mice treated with PBS or control antibody (Fig. 7B). A mild inflammatory infiltration of neutrophils, lymphocytes, plasma cells, macrophages, and multinucleated giant cells surrounded the capsule. Taken together with the long-lasting inhibitory effect, the data strongly indicate complete tumor cell eradication by E7.6.3 antibody.

Table 1

Prevention of tumor formation by E7.6.3 MAb

<table>
<thead>
<tr>
<th>Time (day)</th>
<th>PBS</th>
<th>PK16.3.1 (1 mg)</th>
<th>E7.6.3 (0.2 mg)</th>
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<td>0/5</td>
<td>0/5</td>
<td>0/10</td>
<td>0/10</td>
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<td>0/5</td>
<td>0/5</td>
<td>0/10</td>
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<tr>
<td>100</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>250</td>
<td>ND</td>
<td>ND</td>
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</tr>
</tbody>
</table>

*a* ND, not determined.
further validated by its ability at a 6 mg total dose to completely eliminate established tumors as large as 1.2 cm³ in all of the mice treated.

This antitumor potency of E7.6.3 is likely to originate primarily from the intrinsic activity of the antibody inasmuch as its human γ2 isotype was shown to minimally engage the immune system-derived effector functions such as cell-mediated cytotoxicity or complement-dependent cytolysis. In comparison, the antitumor activities of the rat ICR62, mouse

Table 2 The effect of E7.6.3 MAb on established A431 xenograft tumors

<table>
<thead>
<tr>
<th>Treatment (dose/injection)</th>
<th>Total dose (mg)</th>
<th>Total no. of mice</th>
<th>Tumor-free mice on day 60</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>71</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Control IgG2κ (1 mg)</td>
<td>16</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>E7.6.3 (1 mg)</td>
<td>6</td>
<td>50</td>
<td>50 100</td>
</tr>
<tr>
<td>E7.6.3 (0.5 mg)</td>
<td>3</td>
<td>20</td>
<td>19 95</td>
</tr>
<tr>
<td>E7.6.3 (0.25 mg)</td>
<td>1.5</td>
<td>3</td>
<td>3 60</td>
</tr>
<tr>
<td>E7.6.3 (0.2 mg)</td>
<td>1.2</td>
<td>19</td>
<td>5 26</td>
</tr>
<tr>
<td>E7.6.3 (0.1 mg)</td>
<td>0.6</td>
<td>20</td>
<td>13 65</td>
</tr>
<tr>
<td>E7.6.3 (0.05 mg)</td>
<td>0.3</td>
<td>15</td>
<td>1 7</td>
</tr>
</tbody>
</table>

Fig. 5. The eradication of established A431 tumor xenografts by E7.6.3 MAb. A431 (5 x 10⁶) cells were injected s.c. into nude mice on day 0. A, at day 7, when tumor size reached an average volume of 0.13–0.25 cm³, mice were randomly divided into treatment groups (n = 5) and were injected i.p. with PBS (○) or with 1 mg of either E7.6.3 (●) or the control human myeloma IgG2κ (□) antibody twice a week for 3 weeks. B, when the mean tumor sizes reached 0.13 (▲), 0.56 (▼), 0.73 (●), or 1.2 (○) cm³, mice (n = 10) were treated with 1 mg E7.6.3 twice a week for 3 weeks. Control mice (○, n = 10) received no treatment. C, at day 10, when tumor sizes reached 0.15 cm³, mice (n = 8) were injected i.p. with 200 μg (▲) or 50 μg (●) doses of E7.6.3, or 200 μg (▲) or 50 μg (□) doses of 225 MAbs, twice a week for 3 weeks. Control mice (○) received no treatment. Tumor volumes were measured twice a week as described in “Materials and Methods.” The data are presented as the mean tumor size ± SE.

Fig. 6. The effect of E7.6.3 MAb on the growth of established human tumor xenografts. Five x 10⁶ MDA-MB-468 (A) or A431 (B) cells were injected s.c. into the nude mice on day 0. A, 7 days after injection of MDA-MB-468 cells, mice (n = 10) were injected i.p. with 2 mg of E7.6.3 MAb once a week for 2 weeks (▲). Control mice (n = 8) received no treatment (○). B, mice (n = 10) were given 0.5 mg E7.6.3 via i.p. (●), i.v. (▲), s.c. (▼), or i.m. (□) injections twice a week for 3 weeks. Control mice (○) received no treatment. The data represent the mean tumor size ± SE.
528, or chimeric C225 antibodies were suggested to reflect the participation of the host immune effector functions recruited by the respective rodent γ2b or human γ1 isotypes (2, 4, 6, 26).

The molecular mechanism(s) underlying the potent antitumor activity of E7.6.3 still remains elusive. Some hypotheses that can be proposed are based on the ability of the antibody to block ligand-triggered growth and survival signals, whereas others emphasize the possible effects that the antibody may exert upon the cell on its interaction with the receptor. The potency of E7.6.3 can be attributed, at least in part, to the high binding affinity ($K_D = 5 \times 10^{-11} \text{ M}$) to human EGFr, higher than the affinity reported for other anti-EGFr MAbs (12, 14). With its high affinity, E7.6.3 can inhibit or dissociate the ligand binding to the receptors very effec-

Fig. 7. Histopathology of E7.6.3-treated A431 xenografts. A, mice with established A431 xenografts were treated i.p. with 0.5 mg of E7.6.3 MAb twice a week for 3 weeks. On day 76 after tumor cell ($5 \times 10^6$) injection, tumor-like nodules were excised and examined histologically as described in “Materials and Methods.” B, histological analysis of A431 xenografts excised from an untreated mouse.
tively, thus depriving the cells completely from receiving essential growth and survival stimuli. Like other anti-EGF antibodies (2, 4, 6), E7.6.3 MAb does not act as an agonist and does not activate cells on binding to the receptor. The difference in efficacy between E7.6.3 and the other antibodies tested in xenograft mouse models can also be attributed to a unique E7.6.3 binding epitope on EGF that can mediate a stronger neutralization effect or induce cytotoxicity. The latter hypothesis is supported by the ability of E7.6.3 to eradicate well-established human xenografts as large as 1.2 cm³. The mechanism behind the in vivo cytotoxic effects of E7.6.3 is not yet clear and may involve the induction of programmed cell death, differentiation of the tumor cells, or modulation of expression of angiogenesis factors—mechanisms that were shown to be triggered by antibodies in cultured cells (27–31). Different mechanisms may account for the antibody effect on different tumors; and in some cases, probably more than one mechanism contributes to the anti-tumor activity.

The potency of E7.6.3 in eradicating well-established tumors indicates that this antibody can provide effective therapy to tumors that require EGF activation for their continuous progression and survival. Because E7.6.3 does not require the presence of chemotherapy to exert antitumor activity, the antibody could be applied to various EGF-expressing human solid tumors. Furthermore, being a fully human antibody, E7.6.3 is expected to have a long half-life and minimal immunogenicity with repeated administration, including in all immunocompetent patients. In addition, bearing a human γ2 constant region that interacts poorly with the effector arm of the immune system, E7.6.3 MAb may not induce cytotoxic effects on EGF-expressing normal tissues such as liver and skin.

Utilization of Mabs directed to growth factor receptors as cancer therapeutics has been validated recently by the tumor responses obtained from clinical trials with Herceptin, the humanized anti-HER2 antibody, in patients with HER2-overexpressing metastatic breast cancer (32, 33). The potent in vivo antitumor activity of E7.6.3, as demonstrated in this report, suggests that it is a good candidate for assessing the therapeutic potential of anti-EGF therapy in EGF-expressing human tumors.

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Xiao-Dong Yang, Xiao-Chi Jia, Jose R. F. Corvalan, et al.


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