Acquired Resistance to the Antitumor Effect of Epidermal Growth Factor Receptor-blocking Antibodies in Vivo: A Role for Altered Tumor Angiogenesis

Alicia Viloria-Petit, Tania Crombet, Serge Jothy, Daniel Hicklin, Peter Bohlen, Jean Marc Schlaeppi, Janusz Rak, and Robert S. Kerbel

ABSTRACT

Inhibitors of epidermal growth factor receptor (EGFR) signaling are among the novel drugs showing great promise for cancer treatment in the clinic. However, the possibility of acquired resistance to such drugs because of tumor cell genetic instabilities has not yet been explored. Here we report the experimental derivation and properties of such cell variants obtained from recurrent tumor xenografts of the human A431 squamous cell carcinoma, after two consecutive cycles of therapy with one of three different anti-EGFR monoclonal antibodies: mR3, hR3, or C225. Initial response to a 2-week period of treatment was generally total tumor regression and was not significantly different among the three antibody groups. However, tumors often reappeared at the site of inoculation, generally after prolonged latency periods, and most of the tumors became refractory to a second round of therapy. Cell lines established from such resistant tumors retained high EGFR expression, normal sensitivity to anti-EGFR antibody or ligand, and an altered growth rate when compared with the parental line in vitro. In contrast, the A431 cell variants exhibited an accelerated growth rate and a significantly attenuated response to anti-EGFR antibodies in vivo relative to the parental line. Because of the reported suppressive effect of EGFR inhibitors on vascular endothelial growth factor (VEGF) expression, and the demonstrated role of VEGF in the angiogenesis and growth of A431 tumor xenografts, relative VEGF expression was examined. Five of six resistant variants expressed increased levels of VEGF, which paralleled an increase in both angiogenic potential in vitro and tumor angiogenesis in vivo. In addition, elevated expression of VEGF in variants of A431 cells obtained by gene transfection rendered the cells significantly resistant to anti-VEGF antibodies in vivo. Taken together, the results suggest that, at least in the A431 system, variants displaying acquired resistance to anti-EGFR antibodies can emerge in vivo and can do so, at least in part, by mechanisms involving the selection of tumor cell subpopulations with increased angiogenic potential.

INTRODUCTION

Among the most successful of the new molecular-targeted drugs for the treatment of cancer are the signal transduction inhibitors (1). These drugs, in general, target specific molecular alterations that are thought vital for such functions as cancer cell proliferation, survival, and ability to induce angiogenesis. Examples include humanized mAbs such as Herceptin, directed against the overexpressed EGFR family member erbB2/Her2/ neu (2, 3); chimeric mAbs such as C225; or small molecule antagonists such as ZD1839 (Iressa) directed to the EGFR (erbB1) receptor (2, 4), ST1571, which is highly specific for the bcr-abl kinase (5), and to Ras farnesyltransferase inhibitors (6). Some of these drugs are already clinically approved, e.g., Herceptin and ST1571, or have shown impressive effects in clinical trials, either on their own or in combination with standard treatments such as radiation or chemotherapy, e.g., C225 in several types of solid tumors (7). Indeed, a recurring theme of signal transduction inhibitors is the ability of such drugs to function as effective radiation or chemosensitizers, thereby rendering tumor cells more vulnerable to the cytotoxic effects of standard therapies (8–12). This may occur by various mechanisms such as lowering the threshold for apoptosis or impairing DNA repair processes (12, 13).

A surprising feature of the preclinical research undertaken on anticancer signal transduction therapy is the almost total lack of information dealing with the issue of acquired resistance to such drugs. This stands in conspicuous contrast to conventional therapeutics such as all classes of cytotoxic chemotherapeutic drugs. Because genetic instabilities of cancer cells are a major driving force of acquired drug resistance (14, 15), it would be expected that acquired resistance would eventually develop to signal transduction inhibitors that target exclusively a cancer cell-associated genetic or phenotypic alteration. An example of this possibility is the emergence of variant resistant cells to a Ras farnesyltransferase inhibitor after prolonged exposure of drug-sensitive tumor cells in vitro (16). Another is the development of resistance to ST1571 in vivo in bcr-abl-positive chronic myelogenous leukemia cells grown in nude mice, as a result of the binding of α1 acid glycoprotein to the drug (17). Similar studies undertaken in an in vivo context with other signal transduction inhibitors, including those targeting EGFR, are lacking. With this in mind, we embarked on a long-term study to isolate and to begin characterizing tumor cell variants resistant to EGFR-targeting drugs such as monoclonal neutralizing Abs. Our approach was based on the observation that a small number of human tumor cell lines, e.g., the A431 squamous cell carcinoma or the 253J B-V bladder cell carcinoma, when grown as established xenografts, can be induced to substantially or even totally regress when treated with anti-EGFR Abs such as C225 (18, 19). We reasoned that if similarly treated tumors were then followed for long periods, variants might eventually emerge, and if so, such recurrent tumors could represent the outgrowth of the progeny of rare variants no longer fully responsive to the EGFR-targeting drug.

The rationale for undertaking such experiments was not only for the obvious intrinsic value of deriving such variants but also as a means of exploring in greater depth the hypothesis that EGFR-targeting agents function in vivo, at least in part, by blocking angiogenesis (19, 20). For example, C225 treatment can block the production of several proangiogenic growth factors in treated EGFR-positive tumor cells, including VEGF, interleukin-8, and basic fibroblast growth factor (19–22). We hypothesized that this effect could contribute to its therapeutic efficacy in vivo (20). If this assumption was correct, the experiments described herein were undertaken to address this question.
RESEARCH

MATERIALS AND METHODS

Abs. The neutralizing anti-EGFR mAb C225, a human-mouse chimeric form of the original mouse monoclonal 225, initially described by Kawamoto et al. (24) was manufactured by ImClone Systems, Inc. (New York, NY). The humanized anti-EGFR mAb R3 as well as its murine form mR3 (25, 26) were provided by York Medical (YM) Biosciences, Inc. (Mississauga, Ontario, Canada). The anti-hVEGF mAb 4301-42-35 was described previously (27).

Cell Lines and Culture Conditions. The human epidermoid carcinoma cell line A431 was obtained from the American Type Culture Collection (ATCC, Rockville, Maryland). Independent variants of A431 cells resistant to EGFR-blocking Abs (R cells) were derived during this study (see "Derivation of EGFR Ab-resistant Variants" below). Both the parental and A431 cell variants were cultured in DMEM supplemented with 5% FBS (Life Technologies, Inc., Grand Island, NY). HUVECs were cultured on gelatin-coated dishes as described previously (28). Cultures were maintained at 37°C and 5% CO2 in a humidified incubator.

Determination of in Vitro Cell Growth. The effect of different conditions on anchorage-dependent growth of parental and variant A431 cells was measured by a [3H]thymidine incorporation assay in monolayer cultures. Briefly, recently trypsinized cells were resuspended at a density of 3 × 10^5 per 100 μl of DMEM (Life Technologies, Inc., Gaithersburg, MD) containing 1% FBS, and plated in a 96-well plate (Nalge Nunc Inc., Naperville, IL). Plates were incubated under standard conditions for 24 h, at which point, 100 μl of the given treatment (2× concentrated C225, hR3, or TGFRα in 1% FBS/DMEM) was added. Cells were incubated for an additional 48 h, and 2 μCi/well of [3H]thymidine were added for the last 4–6 h. The incorporated radioactivity was determined using a Betaplate liquid scintillation counter (Pharmacia). The same assay was performed to test the effect of CM from A431 or R cells on the growth of HUVECs. In this case, cells were plated on gelatin-coated wells in complete growth medium, which was replaced with CM 24 h later. To compare the growth properties of the Ab-resistant variants and A431 parental cells, 10^4 cells/0.5 ml/well were plated on a 24-well plate in regular growth medium (DMEM + 5% FBS). Cells were incubated under standard conditions and counted every day to day 6 using a Coulter counter. Growth medium was replaced at day 3. Cell number after 24-h incubation was taken as baseline. For the clonogenic survival assay, cells were plated under the same conditions, except for the lower density (10^3/well). The colonies formed after 1 week were stained with Crystal Violet and counted by naked eye.

Evaluation of the in Vivo Antitumor Activity of Three Anti-EGFR mAbs. A431 cells (5 × 10^7/200 μl of PBS) were injected s.c. into the right flank of 6–8-week-old SCID mice (average weight, 23 g). After 10 days (tumor average size, 300 mm3) the animals were randomly separated into seven groups (four to five mice per group). “High-dose” groups were treated with 1 mg of C225, hR3, or mR3 administered i.p. every 48 h. “Low-dose” groups were treated with one-fourth of this dose, i.e., 0.25 mg of the given Ab following the same schedule. Control group was treated with PBS. Treatment was stopped after a total of eight injections.

Derivation of EGFR Ab-resistant Variants of A431 Cells. After 2 weeks of continuous treatment with either the higher dose (1 mg per injection) or a lower dose (0.25 mg per injection) of the Abs C225, hR3, or mR3, most tumors regressed, with only a few exceptions. Mice were then maintained and monitored for tumor recurrences, which were first observed in a proportion of mice in the 0.25-mg-dose-per-injection groups, 2 months after the beginning of treatment (see “Results” for details). As soon as the recurrent tumors reached a size similar to that of the initially treated tumors (i.e., 200–600 mm3), a second round of treatment was initiated using the same Ab, dose, and schedule initially applied to treat that tumor-bearing mouse (e.g., 0.25 mg of mR3 every 48 h for 2 weeks, if the recurrent tumor was in a mouse from the mR3 lower-dose treatment group). Tumors that did not respond during the 1st week of treatment with the 0.25-mg dose were then treated with 1 mg per injection according to the same schedule (i.e., every 48 h) during the next 2 weeks or less, depending on tumor response. Tumors initially treated with a dose of 1 mg per injection were treated only with the same dose and schedule. Tumors that did not progress or remain dormant (as in the first round of treatment) after the second treatment were considered resistant. The corresponding mouse was then euthenized, and the tumor cells were recovered in vitro by enzymatic treatment (29) under aseptic conditions and were grown in tissue culture. Cells were passaged in culture at least three times before any further testing.

DNA Fingerprinting. Genomic DNA (10 μg) was digested with Hinfl (New England Biolabs Inc., Mississauga, Ontario, Canada), resolved in a 0.6% agarose gel, and transferred onto a nylon membrane. Banding pattern was evaluated after hybridization with a NICE-labeled 33.15 multilocus probe (Cellmark Diagnostic, United Kingdom) used in combination with CDP-Start chemiluminescent substrate (Tropi Inc., Bedford, MA) following the manufacturer’s instructions.

Western Blotting. To detect the expression of EGFR and HER-2, cell lysates were obtained as described previously (28). Proteins were resolved by SDS-PAGE and transferred onto an Immobilon-P membrane (Millipore, Bedford, MA). After blocking in 1% casein/TBST, the membrane was incubated with either the rabbit polyclonal anti-EGFR Ab SC-003 or the anti-HER-2/Neu Ab SC-284 (Santa Cruz Biotechnologies, Santa Francisco, CA) at 0.2 μg/ml, then washed and incubated with a peroxidase-conjugated goat antirabbit Ab (Jackson Immunoresearch Laboratories Inc., West Grove, PA). The loading was verified by α-actin probing with A5441 (Sigma Chemical Co.) at 0.22 μg/ml. Phosphorylated EGFR was determined in lysates from TGF-α-stimulated cells using the mouse anti-pEGFR no. 324864 (Calbiochem) at 0.1 μg/ml. The peroxidase-conjugated antimouse W402B (Promega) at 0.2 μg/ml was used as secondary to detect mouse-derived primary Abs. Blots were visualized using the ECL chemiluminescence kit (Amersham Corp., Arlington Heights, IL).

Measurement of Human and Mouse VEGF Protein Levels in CM. A commercially available human or mouse VEGF ELISA kit (R&D Systems, Inc., Minneapolis, MN) was used to determine VEGF protein levels in CM obtained from A431, Ab-resistant variants, or mVEGF-transfected clones. Briefly, cells were plated at a density of 10^4 cells/0.5 ml/well in a 24-well plate under normal serum conditions and allowed to reach 80% confluency, at which point, the medium was replaced by DMEM/1% FBS with or without C225. Medium was collected after 24 h and cells were counted as described previously (20).

Northern Blot Analysis. Approximately 10^7 cells were used for the extraction of total RNA using Trizol (Life Technologies, Inc., Gaithersburg, MD) following the manufacturer’s protocol. VEGF and TSP-1 Northern blots were performed as described previously (30). Equal RNA loading and the efficiency of the transfer were visualized by ethidium bromide staining of both the gel and the membrane.

Derivation of A431 Variants Overexpressing VEGF. An expression plasmid encoding mVEGF.S3 (981 bp) driven by a CMV promoter, a generous gift from Dr. Kevin Claffey (Beth Israel Deaconess Medical Center-Research North, Boston, MA), was transfected into A431 cells using SuperFect reagent (Qiagen Inc., Valencia, CA) following the manufacturer’s instructions. Control clones resulted from transfection with an empty vector. Positive clones were selected with increasing concentrations (500–800 μg/ml) of Geneticin (Life Technologies, Inc., Grand Island, NY) and were maintained in the presence of 800 μg/ml of the drug. Three of the clones (mVEGF.S1, mVEGF.S2, and mVEGF.S3) were chosen for an EGFR Ab resistance test in vivo, which was performed after the low-dose-treatment regimen described in a previous section.

VEGF-neutralizing Treatment in Vivo. VEGF-induced angiogenesis was examined in both R and A431 parental cells after a short VEGF-neutralizing in

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vivo experiment. Briefly, SCID mice (five per group) bearing A431, R1, and R5 s.c. tumor xenografts (average tumor volume, 250 mm³) were treated with two injections of either PBS (control) or 200 μg of anti-VEGF Ab 4201–42-35 (Novartis). Tumors were recovered for immunohistochemical analysis after 1 week (average tumor volume: 321 mm³, 452 mm³, and 423 mm³ for A431, R1, and R5, respectively).

Immunohistochemistry. VEGF staining was performed on formalin-fixed and paraffin-embedded specimens as described previously (20). Blood vessels were visualized by CD31 staining, which was performed on 7-μm-thick cryosections using the rat antimonoclonal CD31 n° 01951D (PharMingen) at a concentration of 1 μg/ml 1:500. Biotinylated rabbit anti-rat (Jackson Immunoresearch) and Histostain kit (Zymed Laboratories, San Francisco, CA) reagents were used to label antigen as a red signal. Contrast was provided with Harris Hematoxylin (Suripath Canada, Inc., Winnipeg, Manitoba, Canada).

Statistics. Statistical analysis was performed using the GraphPAD InStat software, version 1.14 (GraphPAD Inc., San Diego, CA). In vivo experiments were analyzed by a one-way ANOVA test coupled to a Bonferroni test for multiple comparisons. In vitro data were compared by a t test.

RESULTS

Antitumor Activity of hR3 and C225 mAbs. One of the two main aims of this study was to evaluate the possibility that the use of EGFR-blocking Abs in vivo might result in a selection of drug-resistant variants. We decided to explore this subject using at least two different EGFR-neutralizing Abs, C225 and hR3, which possess similar binding affinities but different specificities (24, 26). We chose the human squamous cell carcinoma A431 based on its extensive use as a model over the past 15 years in the study of the efficacy of many different antagonists of EGFR, including C225 (18, 31, 32). We have previously found (i.e., by using [3H]Thymidine incorporation assays) that the maximum growth inhibitory effect of either C225 or hR3 in vitro is 40%. This was achieved on monolayer cultures after 48-h exposure to doses ranging between 50 and 100 μg/ml (4). To investigate the relative efficacy of the Abs (C225, mR3, and hR3) in vivo, we tested their antitumor properties on A431 s.c. tumor xenografts, thus resembling the orthotopic location for this tumor, implanted into SCID mice. Under such conditions, the three Abs seemed equally efficient in causing total regression of well-established tumors after 2 weeks of treatment (eight injections) with a low- or a high-dose regimen (Fig. 1), at least during the first 3 weeks of follow-up. No significant difference was observed among the groups when compared by dose or by Ab type (P > 0.05 in all cases).

Sporadic Tumor Recurrence after Treatment with Anti-EGFR Abs. After the tumor regressions became evident in the first responders, treatment was terminated, and the mice were monitored for tumor recurrence. The first such recurrences occurred 1.5 months after termination of the first round of treatment in the 0.25-mg-dose-per-injection groups injected with mR3 or hR3 (Fig. 1, A and C). In each of these groups, one tumor did not regress completely after the eight injections, which perhaps suggests a low level of intrinsic resistance to EGFR inhibition. These tumors were the first to regrow (as expected) over a period as short as 18 days after the maximum antitumor effect was achieved (Fig. 1, A and C). In the 0.25-mg-dose-per-injection C225 treatment group, one tumor recurrence was observed after a latency period of 2 months (Fig. 1E). Recurrence of tumors in the higher-dose groups took longer and was first evident after a 4.5-month latency period in the mR3-treatment group and after 1.5 months in the hR3-treatment group (Fig. 1, B and D). Recurrence also took a long period to occur in the higher-dose C225 group, e.g., after 3.5 months (Fig. 1F). The frequencies of tumor recurrence for C225, hR3, and mR3, respectively, were 20, 40, and 80% for the 0.25-mg-dose treatment groups and 25, 25, and 80% for the 1-mg-dose groups. Mice with recurrent tumors in all of the groups subsequently received a second round of treatment (Table 1).

A431 Recurrent Tumors Escape a Second Round of Anti-EGFR Treatment: Derivation of Variants. While administering the second round of treatment, we observed that in most cases the tumors did not respond to the same extent as they did originally. Tumors regrew rapidly during, or immediately after, this cycle of therapy. Cell lines established from these tumors were designated as follows: R1 and R2 [two morphologically different subpopulations of a resistant tumor from the mR3, 0.25-mg-dose group (Fig. 1A)]; R3 and R4, from two individual resistant tumors of the hR3, 0.25-mg-dose group (Fig. 1C); R5, recovered from a tumor in the hR3, 1-mg-dose group (Fig. 1D); and R6, which was recovered from a resistant tumor in the mR3, 1-mg-dose-group (Fig. 1B). More detailed data on the derivation of the resistant variants are summarized in Table 1.

A common cellular origin for the A431 R variants was demonstrated using a DNA fingerprinting analysis (Fig. 2A). Identical banding patterns suggest that all of the cell lines are A-431-derived and are not comprised of contaminating human or mouse tumor cells. Having established this, two resistant variants (R1 and R5) were chosen for a final in vitro resistance test based on a pilot screening. R1 and R5 were both representative of the similar in vivo growth properties found in the majority of the resistant cells. For the final test, 10⁶ cells (A431, R1, and R5) were inoculated s.c. into 6-week-old nude mice (five per treatment group), and tumor growth was monitored every 4 days to compare tumor take rates. Treatment was initiated when tumors reached an average size of 200–300 mm³ and was administered as described for the 0.25-mg/injection group. Both R1 and R5 manifested an accelerated growth in vivo (Fig. 2B), which became evident 18 days after tumor cell injection and which became significant by day 22. The increased growth rate of these variants was even more significant after a 30-day period of growth, as observed in an independent tumor-take experiment (data not shown). These in vitro results stand in marked contrast to the in vitro data, in which similar growth rates between the R variants and the A431P cells were observed (Table 2). When the variants were tested for their response to EGFR-blocking Abs (e.g., C225) in vivo, both R1 and R5 showed a similar and significant delayed response. (Fig. 2C). By day 45, all of the five tumors included in the A431 group had totally regressed, whereas in the R1 and R5 groups, only one and two such total regressions were observed, respectively. Maximum average responses for R tumors were registered at day 45. The remaining R1 and R5 tumors did not regress by day 86, at which point at least one of the tumors in each group (R1 or R5) grew to the maximum allowed size (1700 mm³) according to the guidelines of the Canadian Council on Animal Care (CCAC), and the experiment was terminated. No sign of tumor was seen by day 86 in any of the animals included in the A431 group treated with C225. Similar results were obtained after hR3 treatment (data not shown).

Absence of Changes in EGFR Status in A431 Variants Resistant to EGFR-blocking Abs in Vivo. As a second step in the characterization of the A431-resistant variants, we examined the level of EGFR expression, because resistance to treatment with EGFR-blocking agents could have obviously resulted from loss or reduction in the level of expression of the receptor itself. However, we did not detect evidence to implicate such a mechanism, because five of six variants showed levels of EGFR similar to those expressed by A431 parental cells (Fig. 3A). To demonstrate that EGFR was equally functional in A431 and the variants, we stimulated the different cell lines with increasing concentrations of TGF-α and measured the level of phospho-active EGFR. Activation of EGFR was found to be equivalent...
between A431 and all of the R variant cells, with the exception of R6, which showed less phosho-active receptor (data not shown) in proportion to a lower amount of EGFR expressed by these cells. Only the results obtained with R1 and R5 are shown (Fig. 3A). The similar pattern in expression level and ligand-induced activation of EGFR between A431 and the R variants suggests that major changes in the EGFR status are unlikely to be responsible for the resistant phenotype. This conclusion is reinforced by the similar growth responses of A431 cells and the R variants to the inhibitory effects of C225 (50 μg/ml) and TGF-α (100 ng/ml) treatment in vitro (Table 2). High concentrations of EGFR-specific ligands, i.e., EGF and TGF-α, have been reported to cause a potent growth inhibition of A431 cells, a phenomenon associated with their considerably elevated expression of EGFR (33, 34). The virtually identical pattern of growth inhibition of A431...
and the R variants R1-R5 by TGF-α is consistent with their equivalent levels of EGFR expression. Similarly, we did not find significant changes in the levels of expression of HER-2 (Fig. 3A), the second member of the EGFR family also known to be involved in cell transformation and tumor progression (35). HER-2 has been shown to heterodimerize with EGFR, altering the patterns of downstream cellular signaling (36, 37). With the exception of R6, which appears to express lower levels of HER-2, none of the variants expressed levels of HER-2 that were significantly different from that of the parental cells, which suggests that changes in HER-2 expression are not a common feature of the resistant variants. In agreement with the decreased expression of EGFR and HER-2 found in R6, this cell line showed an unusually slow growth rate (data not shown).

Constitutive VEGF Up-Regulation in Anti-EGFR-resistant Variants of A431. Because of the clear discrepancy between the in vitro findings (in which resistance was not seen) and in vivo findings (in which resistance was detected), we considered the possibility that the R variants possess increased survival properties under restricted growth conditions or, alternatively, an increased angiogenic ability, which could translate into both superior growth and prolonged survival in vivo. Because neither C225 nor hr3 is able to induce cell death of A431 cells under restricted culture conditions, e.g., three-dimensional/low serum cultures, we could not compare differences in survival in response to Ab treatment under such conditions. An alternative experiment, a clonogenic survival analysis, failed to show a survival advantage in the resistant variants (Table 2), which suggests that an improved basal cellular survival is unlikely to explain the in vivo growth advantage. To evaluate the possibility of enhanced proangiogenic capacity in the R variants, we measured the levels of the proangiogenic factor VEGF, at both the RNA and protein levels in all of the cell lines. The importance of this growth factor for tumor angiogenesis has been established for A431 cells (38), and, furthermore, we have reported previously that the EGFR-neutralizing Ab C225 is able to down-regulate VEGF protein up to 50% in vitro and to an even greater extent in vivo in these cells (20), which we hypothesized could account for the reduced vessel counts found in this and other tumor models after treatment with EGFR-blocking agents (19, 20). We found that five of the six resistant variants, R6 being the exception, expressed at least 2-fold more VEGF mRNA and protein than the A431P cells (Fig. 3, B and C). Although VEGF levels were still down-regulated in vitro by 50% as a result of treatment with C225 (50 μg/ml), it should be noted that, even under such conditions, variant cells still produce 2- to 4-fold more VEGF than does A431 (Fig. 3C). We discarded the possibility that the elevated VEGF expression was merely the result of passaging of A431 cells, by comparing the expression of VEGF with that in A431 parental cells passaged in vivo for 14 days. These cells expressed levels of VEGF that were similar to those of the parental cells and also showed an equivalent growth rate in vivo (data not shown). In addition, we also observed an increase in the growth response of HUVECs to the CM of R variants 1 to 5 (Fig. 3D), but not to that from variant R6 (data not shown), suggesting an increased angiogenesis-inducing capacity in the VEGF-overexpressing cells. The absence of an increase in VEGF expression in addition to the reduced levels of EGFR, HER-2, and the delayed growth rate found in R6 cells suggests that this variant escaped the antitumor effect of anti-EGFR Ab treatment by a different mechanism from that in the other variants.

In contrast to the results with VEGF, no common pattern was observed in the levels of TSP-1 (Fig. 3B), a naturally occurring angiogenesis inhibitor previously shown to be down-regulated by activation of Ras, one of the downstream effectors of EGFR (39). Whereas A431 cells appear to express very low amounts of TSP-1, two variants, R2 and R5, exhibit a paradoxical increase in TSP-1 transcript levels. We have no explanation for this increased TSP-1 and its biological impact because it is obvious from the in vivo data that increased TSP-1 does not confer a growth disadvantage to R5 (Fig. 2B).

In Vivo Resistance to EGFR-neutralizing Ab of A431 Cell Lines Engineered to Overexpress VEGF. Because of the apparent increase in VEGF in the resistant versus A431P cells, we decided to test whether or not such increased VEGF levels could account, at least in part, for the resistant phenotype to EGFR-blocking agents in vivo. We reasoned that the resistance of these variants could result from the inability of EGFR-blocking Abs to down-regulate VEGF levels to the same extent that they do in the parental cells. This would limit the ability of these agents to achieve the same degree of antiangiogenic effect as in the case of A431 parental tumors. Consequently, an endogenous EGFR-independent increase in VEGF expression could act as a de facto resistance mechanism for EGFR inhibitors. To test this hypothesis, we decided to boost VEGF production in A431 parental cells using a gene transfection procedure. An expression vector was used that encoded the murine form of VEGF₁₆₅ (equivalent to human isoform VEGF₁₆₅), a secretable form of VEGF that appears to be overexpressed in the R variants of A431 cells. After

<table>
<thead>
<tr>
<th>Group</th>
<th>mAb (dosing)*</th>
<th>Cells per group</th>
<th>Recurrent tumors (%)</th>
<th>Tumor 1.D.</th>
<th>Initial size first cycle</th>
<th>Initial size second cycle</th>
<th>Second cycle&lt;sup&gt;a&lt;/sup&gt; (no. of injections × dose)</th>
<th>Recurrent variant obtained</th>
<th>Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>mR3 (0.25 mg)</td>
<td>5</td>
<td>4 (80)</td>
<td></td>
<td></td>
<td></td>
<td>5 × 0.25 mg</td>
<td>no</td>
<td>Mice 2 and 5 euthanized</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>8 × 0.25 mg</td>
<td>R1, R2</td>
<td>Cells from T4 did not plate.</td>
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<tr>
<td>II</td>
<td>mR3 (1 mg)</td>
<td>5</td>
<td>4 (80)</td>
<td></td>
<td></td>
<td></td>
<td>7 × 0.25 mg</td>
<td>no</td>
<td>Mouse 1 euthanized (open tumor)</td>
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<td></td>
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<td></td>
<td></td>
<td>7 × 1 mg</td>
<td>R6</td>
<td>Cells from T4 did not plate.</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>8 × 1 mg</td>
<td>no</td>
<td>Mouse 5 was found dead after eighth injection of second cycle.</td>
</tr>
<tr>
<td>III</td>
<td>hr3 (0.25 mg)</td>
<td>5</td>
<td>2 (40)</td>
<td></td>
<td></td>
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<td>8 × 0.25 mg + 8 × 1 mg</td>
<td>R3</td>
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<td></td>
<td>same as T2</td>
<td>R4</td>
<td></td>
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<tr>
<td>IV</td>
<td>hr3 (1 mg)</td>
<td>4</td>
<td>1 (25)</td>
<td></td>
<td>T3 352 446</td>
<td></td>
<td>8 × 1 mg</td>
<td>R5</td>
<td>Mouse was euthanized</td>
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<td></td>
<td></td>
<td>T5 298 368</td>
<td></td>
<td>8 × 1 mg-break- 8 × 1 mg</td>
<td>no</td>
<td>Tumor did not regress or grow back after second cycle. It began to regrow during a third cycle. Cells could not be recovered.</td>
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<tr>
<td>VI</td>
<td>C225 (0.25 mg)</td>
<td>5</td>
<td>1 (20)</td>
<td></td>
<td>T1 225 361</td>
<td></td>
<td>2 × 0.25 mg</td>
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<td>8 × 0.25 mg</td>
<td>R6</td>
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<td>2 × 0.25 mg</td>
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*This dose of the given Ab was injected every other day for 2 weeks during the first cycle of treatment.

<sup>a</sup>Tumor I.D., the number assigned to a particular tumor in a group of treatment.

<sup>b</sup>Initial size, the size of the tumor (in mm<sup>3</sup>) at time 0 in either first or second cycle of treatment.

<sup>c</sup>Injections in second cycle of treatment were given every other day.

**Table 1 Data summary of the in vivo generation of A431 recurrent variants displaying a resistant phenotype to a second cycle of treatment with anti-EGFR Abs**
preliminary screening, three clones (mVEGF.S1, mVEGF.S2, and mVEGF.S3), expressing varying amounts of VEGF ranging between 800 and 12,000 pg/ml, were selected for further analysis. The levels of mVEGF produced by these clones were completely refractory to treatment with C225 (Fig. 4A). This was expected because the expression of exogenous mVEGF164 is under the control of the constitutively active and strong CMV promoter. In addition, the clones were found to maintain an intact in vitro response to C225 in terms of growth inhibition (data not shown) and expression of endogenous hVEGF (Fig. 4A). We compared the in vivo responsiveness of VEGF-overexpressing (mVEGF.S) and A431P cells to the C225 Ab. As previously found, a low-dose regimen was sufficient to cause total tumor regression of well-established A431 parental and control (vector-transfected) tumors but was unable to do so in the case of the VEGF transfectants (Fig. 4, B and C), which showed significant (P < 0.05) resistance compared with the control groups. No significant difference was found (P > 0.05) among the three VEGF-overexpressing clones, although a tendency of a direct correlation between the level of VEGF produced and the degree of resistance was observed. In this regard, it is important to mention that the clone expressing the lowest amount of exogenous VEGF (mVEGF.S1) secretes an overall quantity of this protein that is in close similarity to that secreted by some of the resistant variants themselves. The fact that this clone still exhibits a significant resistance to EGFR-blocking treatment in vivo reinforces the idea of an involvement of VEGF in the resistance mechanism.

Histopathological analysis of 3-week-old s.c. tumors of parental and mVEGF164-transfected A431 cells revealed no differences in cellular morphology but did reveal a slightly larger central area of necrosis in the mVEGF164 tumors, probably reflecting their more accelerated growth rate. In addition, tumors derived from clones S2 and S3 exhibited focal areas of hemorrhage that were particularly large in the S2 tumors (data not shown), which suggests that the extremely nonphysiological amount of VEGF164 produced by this clone could be responsible for this characteristic. Staining of blood vessels with the endothelial-specific marker CD31 did not show an increase in the microvessel density of mVEGF-overexpressing tumors but showed a striking difference in the size of the vascular channels found in mVEGF164 transfectants compared with those found in A431 parental tumors (Fig. 5). Vessels in mVEGF-expressing tumors were highly enlarged, consisting in many cases of a complex structures suggestive of splitting of the vessel lumen by endothelial intravessel walls (Fig. 5, B and C). The overall appearance of these vessels seem to resemble that found in the previously described “mother vessels” (40), which result from increased mVEGF164-dependent angiogenesis. The functionality and impact of these enlarged vessels on tumor biology is still unclear. However, in the context of the s.c. A431 model, these vessels appear to be a hallmark of VEGF164-dependent angiogenesis, which did not result, in this case, in an increase in microvessel density. This altered angiogenesis could account for the reduced response of A431 tumors to EGFR-blocking Abs, such as C225 (Fig. 4, B and C). The general appearance of tumor tissue from mVEGF164-transfected and parental tumors after C225 treatment would appear to support this hypothesis. A431 tumors appear almost totally necrotic, with only a rim of viable tissue in the periphery (Fig. 5E), which virtually lacks blood vessels (Fig. 5F). In contrast, C225-treated mVEGF164 transfectant tumors are comprised of a mixture of...

**Table 2. Growth properties of in vivo-derived recurrent variants of A431**

<table>
<thead>
<tr>
<th>Cell type</th>
<th>C225-dependent growth inhibition (% ± SD)</th>
<th>TGF-α-dependent growth inhibition (% ± SD)</th>
<th>Doubling time (h ± SD)</th>
<th>Clonogenic survival (colony, n ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A431</td>
<td>33 ± 5.6</td>
<td>91 ± 5.2</td>
<td>30.2 ± 2.3</td>
<td>186 ± 4.5</td>
</tr>
<tr>
<td>R1</td>
<td>41 ± 10.0</td>
<td>80 ± 3.2</td>
<td>28.7 ± 0.6</td>
<td>189 ± 1.5</td>
</tr>
<tr>
<td>R3</td>
<td>43 ± 5.3</td>
<td>88 ± 7.9</td>
<td>30.6 ± 1.1</td>
<td>111 ± 6.0</td>
</tr>
<tr>
<td>R4</td>
<td>33 ± 11.4</td>
<td>85 ± 7.5</td>
<td>32.4 ± 1.1</td>
<td>176 ± 13.2</td>
</tr>
<tr>
<td>R5</td>
<td>35 ± 8.1</td>
<td>84 ± 9.4</td>
<td>35.4 ± 1.6</td>
<td>58 ± 7.1</td>
</tr>
</tbody>
</table>

| Notes: | Values represent the average for two independent experiments, each one including triplicates. |
| Notes: | Calculated from triplicate cultures. |
| Notes: | The ability of cells to form clonal colonies was analyzed as described previously (58). See “Materials and Methods” for details. |
necrotic and viable tissue (Fig. 5G), with highly enlarged vascular channels supporting the growth of tumor cell “cuffs” (Fig. 5H). Taken together, the results suggest that increased VEGF expression, which is refractory to the effects of C225, could be responsible, at least in part, for the resistance of the variant A431 tumors to EGFR-blocking Abs.

Evidence of a Causal Role of VEGF in the Altered Vascularity of A431 Variants. To determine whether the elevated VEGF expression of the resistant variants could result in an altered VEGF-dependent angiogenesis phenotype, we analyzed tumor sections obtained from a short in vivo experiment, in which mice bearing A431, R1, and R5 tumors were treated with either PBS (control) or anti-hVEGF Ab for 1 week, after which a moderate antitumor effect was observed in each group (treated:control ratio: 0.66, 0.61, and 0.81 for A431, R1, and R5, respectively; see “Materials and Methods” for details). Both VEGF expression and vascularity were evaluated by immunostaining. We observed that in both A431P and R tumor sections, VEGF staining was mostly localized at the tumor periphery. In the A431 tumors, VEGF expression was usually seen in large patches or “islands” (Fig. 6A) composed mainly of large cells with a moderately- to well-differentiated squamous morphology. Slightly smaller cells with higher nuclear/cytoplasmic ratio surrounding these islands were negative for VEGF staining (Fig. 6A). In contrast, VEGF staining in the R1 and R5 variants was more diffuse and present in almost all of the viable cells, which showed a relatively high nuclear:cytoplasmic ratio and a moderately differentiated morphology (Fig. 6, D and G). Staining for CD31 showed no obvious increase in the vessel density of R1 and R5 tumors compared with those of A431P cells. Instead, the size of the vessels appeared significantly larger in the R variant tumors (Fig. 6, B, E, and H). A majority of blood vessels in the R1 tumors were more than 2-fold larger than those detected in A431, whereas R5 tumors appeared to possess even more expanded and complex vascular channels, consisting in some cases of several compartments (Fig. 6H) resembling those found in mVEGF164 A431 transfectants (Fig. 5). These large vessels (40) in the R variant tumors appeared to be VEGF-dependent, because they became less common or smaller after 1 week of treatment with the anti-hVEGF Ab 4301-42-35 (Ref. 27; Fig. 6, F and I). In contrast, the same treatment caused a reduction in the microvascular density in A431 P tumors (Fig. 6, C versus B) but no apparent change in vessel size.

DISCUSSION

The clinical approval of drugs that block receptor tyrosine kinases, such as Herceptin, for the treatment of cancer, and the advanced clinical development status of others, such as IMC-225 (7, 41) and Iressa/ZD1839, both of which block EGFR function, highlight the
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surprising fact that no preclinical studies have been undertaken to evaluate whether, and how, acquired resistance to such drugs may develop. Our results show that acquired resistance can indeed develop in vivo to EGFR blocking Abs. However, it is encouraging that the efficiency with which such resistance develops appears to be rather modest. First, in our study, recurrent tumors did not develop in all of the mice, and when they did occur, it was usually after prolonged latency periods. Indeed, it was only as a result of undertaking such long-term-observation experiments that we detected the emergence of resistant variants. Second, it should be noted that the only possible target of the Abs in our model was the EGFR expressed by the implanted, genetically unstable human A431 tumor cells growing in the SCID mice. In contrast, in the clinical situation, such Abs would have the potential opportunity to block EGFRs expressed by normal (genetically stable) host endothelial cells of newly formed tumor blood vessels (42, 43) particularly in TGF-α-expressing tumors, in which TGF-α itself may up-regulate EGFR expression in activated endothelial cells (44, 45). As a result, EGFR-blocking agents might conceivably block angiogenesis not only indirectly but also directly, especially when such drugs are combined with chemotherapy. This was shown recently by Bruns et al. (22, 46), who reported significant induction of endothelial cell apoptosis in human pancreatic or prostate carcinoma xenograft microvessels by combination treatment with either a small molecule EGFR antagonist or C225 and gemcitabine. Third, acquired resistance may also be delayed by more protracted treatment with the Abs, i.e., continuing the treatment in tumors that do not regress after eight injections and even after tumors have regressed, or by combining the Ab treatment with other therapeutic modalities such as chemotherapy or radiation, which is currently the way EGFR-blocking drugs are used clinically (7, 41). In this regard, another interesting feature of our results is the necessity of undertaking very-long-term (e.g., up to one year) in vivo experiments to detect the emergence of resistant variants, an approach lacking in virtually all previous studies using such therapeutic agents.

We must also acknowledge the possible influence on the results as a consequence of using exclusively the A431 cell line for our studies, a cell line that expresses a very high number of EGFRs and that is known for its hypersensitivity to EGFR-blocking drugs in vivo. Analysis of other cell lines that have fewer numbers of EGFR may not reveal evidence of acquired resistance or, alternatively, may reveal evidence for such resistance but involving different mechanisms.

Possible Impact of Altered VEGF Expression and Tumor Angiogenesis in the Resistant Phenotype. One of the most interesting (and unexpected) findings of this study was the absence of an obvious alteration in the EGFR status in the R variants, which we established by different means, including Western blot analysis of EGFR expression and evaluation of the growth response of the variants to EGFR-blocking Abs (C225 and hR3), as well as the determination of levels of phospho-active EGFR after stimulation with increasing concentrations of TGF-α. We reasoned that major changes in the level of expression of EGFR and/or its ligands could translate into a different biological response to ligand-induced stimulation and/or phosphorylation (i.e., activation). However, we did not find any evidence for such changes. Of considerable importance in this regard was the virtually identical in vitro growth characteristics and inhibitory responses to treatment with the anti-EGFR blocking Abs of the variants, compared with the parental cell line. Thus, interestingly, the resistant phenotype manifested itself only in vivo. One possible explanation for this would be a reduced sensitivity to undergoing Fc receptor-mediated ADCC, a host process that can take place only in vivo and that has been implicated as a major determinant of response to certain therapeutic Abs such as Herceptin and Rituxin (47). However, ADCC does not appear to be similarly important for the antitumor activity of C225, because F(ab′)2 fragments of this Ab, which cannot activate an ADCC response, still retain a significant growth inhibitory activity against A431 tumor xenografts (48). Hence, we reasoned that an alternative host-dependent mechanism, namely angiogenesis (a process required in vivo but not in vitro for tumor growth), could conceivably contribute, at least in part, to the resistant phenotype.

Fig. 4. Exogenous expression of VEGF by mVEGF164 transfectants and its impact on the in vivo response to EGFR-neutralizing Ab C225. A, mVEGF164 (mVEGF164 isoform) protein produced by clones S1 to S3. The human A431P cells do not express the mouse protein, as expected. Horizontal black bar, the average level of hVEGF165 protein expressed by A431P cells as well as mVEGF164 transfectants. C225 down-regulates only the endogenous human protein (hVEGF165) but not the exogenous mVEGF164, as expected. The data represent the average from two independent determinations. B, antitumor effect of C225 Abs on the in vivo growth of s.c. tumor xenografts derived from A431P and vector or mVEGF164 transfectant clones S1–S3. C225 Ab treatment (arrow, day of injection) causes tumor regression only in A431P and vector control tumors but not in the mVEGF164 transfectants. C, tumor-bearing mice after treatment with C225. Tumor size represents the average for each treatment group.

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We, therefore, examined the R variants for changes in the expression of VEGF, not only because VEGF is a potent inducer of tumor angiogenesis in general but also because A431 cells have been shown to be highly dependent on VEGF for angiogenesis and, hence, in vivo growth (38). We found that five of six of the resistant variants express a significantly higher level of VEGF than does the parental line. Relative VEGF levels appeared to correlate with the in vivo growth of the variants as well as with their angiogenic profiles. Staining of VEGF on sections derived from R1 and R5 s.c. tumor xenografts suggests that these tumors express a higher overall amount of VEGF, mainly attributable to an increase in the ratio of VEGF expressing: nonexpressing cells. The mechanism responsible for this change is unknown, but it is likely that anti-EGFR Ab treatment selects for A431 subpopulations with an elevated degree of VEGF expression.

In addition to altered patterns of VEGF expression, we also found that variants R1 and R5 possessed conspicuously large blood vessels, which we suggest is the result of increased VEGF-dependent angiogenesis, because blocking VEGF signaling with a VEGF-neutralizing Ab reduces the size of these vessels (Fig. 6). The presence of such large vessels is a hallmark of pathological VEGF-dependent angiogenesis (40, 49). A recent study in which the properties of newly formed vessels induced by VEGF/VPF164 have been investigated in detail showed that large vessels are commonly found in situations of VEGF overexpression and are referred to as “mother vessels” (40). We found a striking resemblance between these mother vessels and the large vessels found in R variants, particularly R5. We also found similar, but even more enlarged, vascular channels in the tumors formed from A431 cells transfected with the mVEGF164 gene.

Our results raise the interesting possibility that, when applicable, assessment of changes in the number of such mother vessels (as opposed to changes in microvessel counts or density) could be a useful surrogate marker to evaluate and monitor the activity of certain anti-angiogenic drugs. However, measurement of vascular density seems inappropriate, as it is difficult to establish the margins of an individual vessel. Because of their compartmentalization, it is difficult to determine whether the structures that we detected correspond to several vessels or to just one vessel, subdivided into several compartments. For this reason, we did not undertake vessel counts in the present study.
Possible Mechanisms of Increased VEGF Production in the R Variants. An obvious and important question raised by our results concerns the mechanism responsible for the elevated VEGF levels detected in the R variants. At present, we do not know the answer, but there are a number of possibilities. For example, several different oncogenes, when activated, are known to induce or up-regulate VEGF (39). These include ras, src, and erbB2/neu (39, 50), among others. Likewise certain tumor suppressor genes, such as p53, VHL, or PTEN, when mutated/inactivated, can result in elevated VEGF (39, 51). Thus the variants may express elevated VEGF levels as a result of the selection of cells possessing one or more such genetic changes during the EGFR Ab-mediated therapy. Alternatively, aberrations in signaling pathways downstream of EGFR activation that are known to affect VEGF expression could conceivably be involved. Such changes, for example, could include phosphatidylinositol 3'-kinase (PI3 kinase; Refs. 51, 52), and/or src kinase (39, 53, 54) overactivation, and/or ras mutation (30, 39). Preliminary data suggest that neither increased src nor ras activity are obvious in the resistant variants of A431 (data not shown). Whatever the mechanism(s), the magnitude of the increase in VEGF that we detected in the variants could significantly increase tumor angiogenesis, based on results reported in several previous studies, using gene transfection approaches (55, 56).

Our results demonstrate that, in principle, acquired resistance to agents that block tumor cell EGFR function can develop in vivo. However, the extent of this resistance, and the rate at which it develops, appear encouraging for use of such drugs in the clinic, especially because they are frequently used as chemotherapy or radiation sensitizers, rather than single agents. The basis of acquired resistance in the A431 system appears, at least in part, to have a link with tumor angiogenesis, an observation that strengthens the putative linkage established between oncogene function, including that of oncogenic receptor tyrosine kinases, and tumor angiogenesis (39). Finally, we do not wish to infer that acquired resistance to EGFR inhibitors, when it occurs, will always involve the latter type of mechanism. Tumor cell resistance mechanisms with respect to chemotherapeutic drugs and other agents, e.g., ST1571 (17, 57), are usually highly pleiotropic, and the same is likely to be true for inhibitors of EGFR signaling. Indeed, the basis of acquired resistance of one of the six A431 variants that we analyzed, R6, is different from that of variants R1–R5 and may not involve altered angiogenesis. It
would be of interest, in this respect, to test other EGFR-positive cell lines for the possibility of additional mechanisms of acquired resistance, and to use different antagonists of EGFR function for their selection.

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REFERENCES


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Acquired Resistance to the Antitumor Effect of Epidermal Growth Factor Receptor-blocking Antibodies in Vivo: A Role for Altered Tumor Angiogenesis

Alicia Viloria-Petit, Tania Crombet, Serge Jothy, et al.


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