neu Antigen-Negative Variants Can Be Generated after neu-Specific Antibody Therapy in neu Transgenic Mice

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ABSTRACT

Prolonged administration of HER-2/neu-specific monoclonal antibody therapy is now widely used for the treatment of HER-2/neu-overexpressing tumors in advanced-stage breast cancer patients. Monoclonal antibody therapy has the potential to promote reduced tumor expression of HER-2/neu by receptor down-modulation and/or the generation of antigen-negative variants. Loss of antigen by either mechanism could potentially impact subsequent therapeutic strategies targeting HER-2/neu. In this study, the effects of chronic neu-specific monoclonal antibody therapy on tumor growth and neu protein expression were examined in a murine model of neu-overexpressing breast cancer. Treatment of neu-overexpressing tumors with neu-specific antibody, in vitro or in vivo, resulted in significant tumor growth inhibition. When neu antibody was used to treat neu-overexpressing tumor cells both in vitro and in vivo in tumor-bearing mice, neu receptor expression was not diminished after cessation of therapy. However, in the setting of clinically undetectable disease in a fraction of animals, antigen-negative variants were generated. An understanding of the effects of monoclonal antibodies on target antigen expression is critical for the future design and testing of novel HER-2/neu-targeted therapies administered in combination with or after HER-2/neu-specific monoclonal antibody therapy.

INTRODUCTION

HER-2/neu is an oncoprotein that is commonly overexpressed on a variety of different types of tumors. Approximately 20–30% of human breast cancers overexpress HER-2/neu. Patients with HER-2/neu-overexpressing breast cancer have poorer overall survival rates and shorter times to disease progression compared with patients whose tumors do not overexpress HER-2/neu (1). HER-2/neu overexpression is also associated with increased resistance to chemotherapy (2). Because of these clinical characteristics of HER-2/neu protein expression in tumors, great effort is being applied to developing therapies that specifically target HER-2/neu.

HER-2/neu-specific monoclonal antibody (i.e., trastuzumab) therapy is now a standard of care for the treatment of advanced breast cancers that overexpress the HER-2/neu oncoprotein (3). Other emerging HER-2/neu-targeted therapies therefore will most likely be tested in combination with anti-HER-2/neu monoclonal antibody therapy. In general, antibody therapy can alter the cell surface expression of the target antigen by one of two mechanisms. The first mechanism is receptor down-modulation, a process by which antibody induces internalization and degradation resulting in reduced or non-existent expression of the receptor at the cell surface. This mechanism is rapid and reversible on removal of antibody (4). The second mechanism is antigen-negative variant (ANV) selection, which results in the outgrowth of a cell population that permanently expresses little or no receptor. The development of neu ANVs after monoclonal antibody therapy would have a great impact on the clinical efficacy of subsequent neu-directed therapies.

In this study, we evaluated the effects of multiple doses of neu-specific antibody on tumor growth and assessed the impact of the therapy on neu protein expression. The growth-inhibitory effects of the anti-neu antibody 7.16.4 were evaluated in a transgenic (neu-transgenic) mouse model of neu-overexpressing breast cancer in various disease settings from minimal disease to advanced, established malignancy.

MATERIALS AND METHODS

Animals. Neu-transgenic mice [strain name, FVB/N-TgN (MMTV-neu)-202Mul] were obtained from Charles River Laboratory (Bar Harbor, ME; Ref. 5). The mice harbor nonmutated, nonactivated rat neu under control of the mouse mammary tumor virus promoter. This model is parallel to human HER-2/neu-overexpressing cancer in a number of ways. First, expression of neu under the mouse mammary tumor virus promoter results in amplified expression in the breast epithelium. This is analogous to gene amplification in humans that results in overexpression of the nonmutated HER-2/neu with a significant proportion of cases expressing medium to high levels of the protein (6). In our studies, only female mice, 8–12 weeks old, were used for experimentation. Animal care and use were in accordance with institutional guidelines.

Reagents. FCS was obtained from Gemini Bioproducts (Woodland, CA). RPMI 1640, PBS, penicillin-streptomycin, and L-glutamine were obtained from Life Technologies Inc. (Grand Island, NY). Monoclonal antibody 7.16.4, a mouse IgG2a antibody reactive with the rat neu oncogene-encoded p185 molecule, was generously provided by Dr. Mark Greene and has been described previously (7). Rat anti-mouse IgG-FITC antibody was obtained from Pharmingen (San Diego, CA), and 4G10 antibody was obtained from Upstate Biotechnology Inc. (Lake Placid, NY). Rabbit anti-neu antibody (Ab-1) was obtained from Oncogene Research Products (Cambridge, MA). Enhanced chemiluminescence reagents and enhanced chemiluminescence film were from Amersham International (Oakville, Ontario, Canada). EDTA was obtained from Ambion (Austin, TX). The protein quantification kit ProteiniNE was obtained from Bio-Rad (Hercules, CA).

Cell Lines. Mouse mammary carcinoma (MMC) cell line was established from a spontaneous tumor harvested from the neu-transgenic mice. MMC cells were grown and maintained in RPMI 1640 supplemented with 20% FCS as well as penicillin/streptomycin and L-glutamine. ANV is a cell line derived from a neu-loss variant tumor and is maintained in culture identical to MMC.

Tumor Growth in Vitro and in Vivo. For in vitro experiments, 1.0 × 10^5 MMC or ANV cells were plated in 6-well plates with media alone, mouse IgG2a (Sigma Aldrich, St. Louis, MO), or 7.16.4. Cells were harvested with a NaCl solution (0.8%) with 2 mM EDTA and washed before injection. Mice were inoculated with 6 × 10^5 MMC cells s.c. on the mid-dorsum with a 23-gauge needle, which is a dose of tumor cells that results in the development of tumors in 100% of neu-transgenic mice. Tumors were measured every other day with Vernier calipers, and tumor size was calculated as the product of length × width. In vivo data are presented as mean ± SE. For in vivo studies, significance (P < 0.05) was determined using Student’s t test by comparing the means of different treatment groups (GraphPad InStat for Windows 95/NT; GraphPad Software, San Diego, CA). Mice were treated every other day with tail vein dosing of either 100 μl of PBS as control or 30 μg of 7.16.4 in PBS. Tumor measurements were taken every 2–3 days during tumor growth studies.
Flow Cytometry. Tumor cells were removed from plates using PBS with 2 mM EDTA. Tumor cells grown in vivo were harvested from mice by careful dissection under sterile conditions and then passed through a fine wire mesh and washed. Both in vitro- and in vivo-harvested cells were washed in PBS containing 1% FCS before labeling. Cells (0.5–1.0 × 10^5) were incubated with 2–5 µl of primary antibody (control IgG2a or anti-neu 7.16.4) added at 4°C for 30 min and washed three times, followed by secondary labeling with FITC-conjugated goat antirabbit antibody at 4°C for 30 min, followed by three washes. Samples were run on a FACScan II and analyzed using Cell Quest software (Becton Dickinson, San Diego, CA). For intracellular staining, the cells were permeabilized before applying the antibodies using CytoFix/Cytoperm (BD Biosciences, San Diego, CA).

Western Blotting and Immunoprecipitation. Whole cell lysates, prepared as described previously (8), were analyzed by SDS-PAGE and immunoblotting with anti-phosphotyrosine monoclonal antibody (4G10) and anti-neu antibody (Ab-1) using methods described previously (9). Protein concentrations were determined using Bio-Rad Protein Assay. Rat neu was immunoprecipitated from tumor cells by incubating 0.5–1.5 mg of lysate with anti-neu antibody 7.16.4 for 2 h at 4°C followed by the addition of 50 µl of protein G-Sepharose for 2 h. To assess the amount of neu protein, membranes were probed with 5 µg/ml anti-neu polyclonal antibody Ab-1 and a 1:2000 dilution of antiserum horseradish peroxidase and developed by enhanced chemiluminescence as described previously (9).

RESULTS

neu-Specific Monoclonal Antibody Treatment Inhibits neu-Mediated Tumor Growth in Vitro without Loss of Cell Surface neu Expression. Neu-specific monoclonal antibody 7.16.4 was evaluated at several concentrations for growth inhibition, in vitro, against neu⁺ MMC and neu⁻ ANV cells as shown in Fig. 1. Antibody was added only once to culture on day 0, and cell numbers were evaluated 5 days later. Antibody 7.16.4 inhibited the growth of MMC but not ANV (Fig. 1). The response of MMC was dose dependent. Growth at 100 µg/ml 7.16.4 antibody resulted in a cell yield of 15 ± 4% (mean ± SD) compared with control cells that had been incubated without 7.16.4. In contrast, ANV cells were unaffected by the inclusion of 7.16.4, and at 100 µg/ml 7.16.4, the cell yield of ANV was 111 ± 2% of control ANV cells. Growth of MMC cells in the presence of 50 µg/ml isotype-matched, nonspecific mouse IgG resulted in only a minor incremental decrease in cell yield (87 ± 7% versus control; data not depicted in graph).

The cell surface expression of neu on MMC was examined in cells that were treated with 7.16.4 or isotype-matched IgG in vitro. As shown in Fig. 2A, compared with either control media (PBS) or nonspecific IgG, the addition of 7.16.4 antibody did not result in changes in the mean levels of neu expression at the cell surface of MMC over the range of doses examined in Fig. 1. Furthermore, changes in the total cellular levels of neu expression were not altered, as assessed by immunoblotting of cellular lysates (data not shown). Representative histograms are shown in Fig. 2. B–E, staining of 7.16.4-treated MMC cells with 7.16.4 and FITC-conjugated antiserum IgG antibody revealed no significant down-modulation of cell surface neu. FITC-α-IgG, when used alone, also stained the cells (open histograms), reflecting the presence of the initial dose of 7.16.4. Untreated control cells (Fig. 2B) did not demonstrate binding of FITC-α-IgG. The effects of nonspecific IgG treatment of MMC are shown in Fig. 2C.

neu-Specific Monoclonal Antibody Inhibits neu-Mediated Tumor Growth in Vivo in an Established Tumor Setting without Neu Receptor Down-Modulation. The effect of 7.16.4 treatment on in vivo growth of tumors was examined (Fig. 3). Continuous treatment
every other day with 7.16.4, starting on either day 10 or day 14 after tumor implant, did not result in complete inhibition or regression but did reduce the rate of tumor growth. The earlier therapy was started, the greater the impact on overall tumor growth. At 20–24 days after tumor challenge, tumors of mice that were treated beginning on day 10 after challenge were approximately 90% smaller than controls, whereas sizes of tumors in mice treated beginning on day 14 were 60% smaller. At day 24, the mean tumor sizes differed significantly from each other (control versus day 10, \( P = 0.01 \); control versus day 14, \( P = 0.004 \); day 14 versus day 10, \( P = 0.01 \)). The cell surface expression of neu was also examined in tumors harvested from antibody-treated animals (Fig. 4). There was no measurable difference in the relative mean fluorescent intensity of cell surface neu expression in antibody-treated animals as compared with PBS-treated animals (Fig. 4A). Fig. 4, B–D, show that antibody-treated tumors still maintain high levels of neu expression. The levels of neu expression in tumors from treated animals were similar to controls. Isotype control antibody was also tested and did not result in inhibition of tumor growth or down-regulation of neu expression (data not shown).

**neu ANVs Can Develop in Animals after Treatment with a neu-Specific Monoclonal Antibody.** When therapy with 7.16.4 monoclonal antibody is initiated at the time of tumor injection, tumor growth was either completely inhibited or significantly delayed (Fig. 5). The antibody-treated group received antibody injections every other day for a total of 30 days. Whereas all of the mice that received control infusions of PBS developed tumor, only 2 of 16 (13%) mice that were treated with 7.16.4 developed measurable tumors during the course of therapy after day 18. The treated mice were followed for an additional 45 days (75 days, total) after therapy to evaluate potential recurrence for relapse. The mice were not treated with antibody therapy during the follow-up period. Ten of the mice developed tumors after the cessation of treatment. A representative tumor growth curve is shown in Fig. 5A. The survival of all of the mice is shown in Fig. 5B. A total of 10 of 16 (63%) mice relapsed with tumors at the tumor injection site during follow-up. The remaining mice did not develop tumors up to day 75. The tumors were examined in the relapsing mice for cell surface expression of neu (Fig. 6). Compared with the MMC cell line or tumors from PBS-treated mice, relapse tumors demonstrated little or no neu expression. The relative mean fluorescence intensity \( \pm SE \) for neu expression of tumors from was \( 1.6 \pm 0.5 \) (\( n = 11 \)) for the 7.16.4-treated mice and \( 7.4 \pm 0.6 \) (\( n = 3 \)) for the PBS-treated mice. Treatment of animals with isotype-matched antibody did not result in either tumor inhibition (data not shown) or loss of antigen expression (Fig. 6A; mean fluorescence intensity, \( 8.3 \pm 3.3 \); \( n = 2 \)).

In addition to evaluating neu expression in freshly excised tumors, expression was also monitored after *in vitro* culture to determine whether the loss or reduction in neu expression was due to reversible down-regulation. As shown in Fig. 7, an analysis of neu expression in cell lines derived from tumors from 7.16.4-treated mice did not show up-regulation of neu expression over the course of 3 weeks in culture, suggesting that the loss of neu expression was a stable event (Fig. 7). A representative example of a cell line carried in culture for 21 days is shown in Fig. 7, B–D, and is compared with a typical flow cytometry profile of the neu-positive MMC (Fig. 7A). These findings were corroborated with neu-specific intracellular staining, which also shows relative mean neu-specific fluorescence intensity \( \pm SE \) for the 7.16.4-treated mice and 7.4 \( \pm 0.6 \) \( \pm SE \) of \( 3 \).

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**Fig. 3.** neu-specific monoclonal antibody inhibits neu-mediated tumor growth *in vivo* in an established tumor setting. Shown are tumor measurements from tumor-bearing control mice (PBS; ●) and tumor-bearing mice treated with 7.16.4 antibody starting on either day 10 (●) or day 14 (●) after tumor cell injection. Each data point is the mean tumor measurement \( \pm SE \) from 15 or 16 mice.

**Fig. 4.** neu-specific monoclonal antibody treatment, *in vivo*, does not result in the loss of cell surface neu receptor expression. A shows relative mean neu-specific fluorescence intensity (rMFI) of tumors from control (PBS) and 7.16.4-treated (days 10 and 14) mice. Each bar represents the neu-specific mean relative mean fluorescence intensity \( \pm SE \) of three different tumors using 5000 gated events. Representative histograms are shown for PBS-treated control (B) and 7.16.4-treated mice with treatment started 10 days (C) and 14 days (D) after tumor cell injection. Staining was performed using 7.16.4 anti-neu followed by FITC-conjugated rat antimouse IgG (filled histograms) or with FITC-conjugated antimouse IgG alone (open histograms).
did not reveal expression of the neu protein, suggesting immunoselection of variants rather than intracellular sequestration of neu protein (data not shown).

DISCUSSION

In this study, we investigated the impact of chronic neu-specific monoclonal antibody therapy on the expression of the target antigen, neu. The data demonstrated that neu monoclonal antibody therapy of neu-overexpressing tumors can inhibit tumor growth when therapy is begun during large disease burden but does not result in the down-modulation of neu expression. When therapy was begun at the time of minimal disease burden, tumor growth could be prevented. Relapses after monoclonal antibody therapy, initiated at the time of minimal disease burden, consisted of true antigen loss variants.

Antibody-induced down-modulation of the target antigen has been reported for a number of cell surface receptors and is a relatively rapid event occurring immediately after antibody exposure (10). Vesicles internalizing the receptors fuse with lysosomes, which results in increased degradation and reduced steady-state levels of the receptor (10). Whereas not all monoclonal antibodies induce internalization, prior in vitro studies have demonstrated that 7.16.4 results in HER-2/neu down-modulation of neu-transformed NIH 3T3 cells (4, 11, 12). The loss of HER-2/neu expression by down-modulation significantly inhibits growth and causes reversion of the transformed phenotype (4). Receptor internalization induced by 7.16.4 in the NIH 3T3 cells is a rapid event occurring in the first hour of antibody exposure and is sustained as long as antibody is delivered (4). These findings have led to the conclusion that a major mechanism of the growth-inhibitory action of 7.16.4 is through antibody-induced internalization. In vitro studies with trastuzumab (Herceptin) have also led to similar conclusions (12). In the current study, in an established disease model, neu surface expression was retained. A potential explanation for why we did not observe receptor down-modulation in our studies is that receptor internalization is rapidly reversed with cessation of antibody therapy. Down-modulation of the receptor may have been reversed during the time necessary for harvesting and analysis of the tumor cells. Alternatively, up-regulation of neu expression induced by ther-
Fig. 7. Loss of neu antigen expression is a stable phenotype in immunoselected antigen-negative tumor cells derived from animals treated with neu-specific monoclonal antibody. A shows the mean relative mean fluorescence intensity for neu expression over a course of 3 weeks for several cell lines established from relapsed tumors (n = 5–9). The asterisk indicates the level of expression of neu in the neu-negative mouse mammary carcinoma cell line. B shows a representative flow cytometry histogram for neu expression (filled curve) in cultured neu-negative mouse mammary carcinoma tumor cells. C–E show representative histograms for neu expression in a representative antigen loss variant tumor carried in culture for the indicated number of days. The open histograms represent nonspecific staining using FITC-conjugated antimouse IgG alone.

The generation of ANVs resulting from immunoselection is a significant clinical issue in the development of immune-based therapies targeting a single antigen and has been best described for clinical studies focusing on T-cell-directed immunotherapy. For example, in a study by Jager et al. (16), melanoma patients treated with a MART-1 and tyrosinase peptide-based vaccine showed gradual losses in expression of both MART-1 and tyrosinase protein, suggesting immunoselection. Only recently has there been some indication that monoclonal antibody therapy may have occurred as reported previously, resulting in no change in neu protein levels (13). It is also possible that neu-positive tumor cells arising in the neu-transgenic mouse are endocytosis defective, as has been reported previously (14). Recently, loss of antigen expression has been observed in stage II and III breast cancer patients undergoing preoperative trastuzumab therapy. In that study, patients with HER-2/neu-overexpressing tumors were treated with trastuzumab and paclitaxel before surgery and adjuvant therapy (15). HER-2/neu status was determined before any treatment and at the time of surgery (i.e., after trastuzumab/paclitaxel therapy). Seventy-five percent of the patients had objective clinical responses to preoperative trastuzumab and paclitaxel therapy. Twenty-seven (68%) of the patients had assessable tumors after preoperative therapy in which it was observed that seven had significantly reduced levels of HER-2/neu staining. Whereas the authors suggest that down-modulation may have occurred, the analyses did not appear to be extensive enough to rule out the possibility that ANVs were observed.

In conclusion, many strategies are being tested that specifically target HER-2/neu, including monoclonal antibody therapy, gene therapy, vaccines, tyrosine kinase inhibition, adoptive T-cell therapy, and antisense therapy (18). HER-2/neu-specific monoclonal antibody therapy (i.e., trastuzumab) is now a standard of care for the treatment of HER-2/neu-overexpressing breast cancer (3). Current routine clinical use of anti-HER-2/neu monoclonal antibody therapy, for extended periods of time in advanced cancer patients, mandates that newer HER-2/neu-targeting therapeutics be tested in combination with trastuzumab. The mechanism of loss of antigen expression is important.

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If tumors lost surface antigen expression due mainly to receptor down-modulation, then monoclonal antibody therapy targeting HER-2/neu may improve other approaches such as HER-2/neu-specific adoptive T-cell therapy. For example, in an in vitro study, zum Buschenfelde et al. (19) observed that trastuzumab pretreatment enhanced the cytolytic activity of HER-2/neu-specific T cells against HER-2/neu-overexpressing tumors. In contrast, the generation of ANVs would render subsequent neu-directed therapies useless. An understanding of the effects of monoclonal antibody therapy on target antigen expression is critical for the future design and testing of novel HER-2/neu-directed therapies administered in patients undergoing HER-2/neu-specific monoclonal antibody therapy.

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