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DNA vaccination controls Her-2⁺ tumors that are refractory to targeted therapies

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Abstract

Her-2/neu⁺ tumor cells refractory to antibody or receptor tyrosine kinase inhibitors (RTKI) are emerging in treated patients. To investigate if drug resistant tumors can be controlled by active vaccination, gefitinib and antibody sensitivity of four neu⁺ BALB/c mouse mammary tumor (MMT) lines were compared. Significant differences in cell proliferation and Akt phosphorylation were observed. Treatment-induced drug resistance was associated with increased chromosomal aberrations as shown by spectral karyotyping analysis, suggesting changes beyond neu signaling pathways. When mice were immunized with pneuTM encoding the extracellular and transmembrane domains of neu, antibody and T cell responses were induced and both drug sensitive and resistant tumor cells were rejected. In T cell depleted mice, drug sensitive tumors were still rejected by vaccination, but drug- refractory tumors survived in some mice, indicating their resistance to anti-neu antibodies. To further test if T cells alone can mediate tumor rejection, mice were immunized with pcytneu encoding full-length cytoplasmic neu that is rapidly degraded by the proteasome to activate CD8 T cells without inducing antibody response. All test tumors were rejected in pcytneu immunized mice, regardless of their sensitivity to gefitinib or antibody. Therefore, CTL activated by the complete repertoire of neu epitopes were effective against all test tumors. These results warrant Her-2 vaccination whether tumor cells are sensitive or resistant to Her-2 targeted drugs or antibody therapy.

Keywords

Her-2/neu; DNA vaccine; mice

Introduction

ErbB-2/Her-2/neu, a member of the ErbB receptor tyrosine kinase family, is weakly to moderately expressed in normal adult tissues. Dysregulated signal transduction from overexpressed or mutated Her-2 leads to cellular immortalization, neoplastic transformation and tumor progression (1). Overexpression of Her-2/neu in 20-30% of human breast cancers is correlated with more aggressive disease and reduced survival (2-5). Upon dimer formation with other ErbB family members, transphosphorylation of tyrosine residues in the C-terminus provides docking sites for signal transduction and adaptor molecules, leading to cell

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proliferation, migration, adhesion and transformation (6-9). The activated signaling involves phospholipase C (PLC), mitogen activated protein kinase (MAPK), c-src, and the phosphatidylinositol-3 kinase (PI3 kinase)-Akt pathways.

Trastuzumab (Herceptin), a humanized murine mAb (4D5) which binds to Her-2 at the juxtamembrane region of domain IV, is used to treat metastatic Her-2⁺ breast cancer or in adjuvant settings for patients with less advanced disease (10). When tested as a monotherapy, trastuzumab demonstrated 15% response rate with 9.1 months median duration (11). The efficacy of trastuzumab can be increased by combining with paclitaxel with 59% response rate and 10.5 months duration (12). Several mechanisms may account for trastuzumab activity, such as down-modulation of Her-2, disruption of downstream signaling (13;14), or induction of antibody dependent cell mediated cytotoxicity (ADCC). Cardiac dysfunction is the most significant toxicity, particularly when combined with selected chemotherapy. A common failure site in patients treated with trastuzumab is the brain. In a retrospective review, 25% of metastatic breast cancer patients with prolonged survival from trastuzumab treatment developed brain metastases (15;16). Taken together, these results indicate the need for intervention before the stage of metastatic disease using multiple treatment modalities.

Tyrosine kinase inhibitors lapatinib and gefitinib are candidate therapeutics for Her-2⁺ breast cancer. Lapatinib is a dual receptor tyrosine kinase inhibitor (RTKI) with selective inhibitory activity for EGFR and Her-2 (17-19). Another RTKI, gefitinib, selective for EGFR, exerts inhibitory activity on Her-2 signaling at higher concentrations (20;21). In treating Her-2⁺ tumors, mAb, RTKI, and Her-2 vaccine may have overlapping or complementary activities.

Because Her-2/neu⁺ tumor cells refractory to antibody or RTKI are emerging in treated patients, we tested whether Her-2 DNA vaccination would be effective against drug sensitive versus resistant tumors. We assembled a panel of rat neu expressing tumors that are differentially sensitive to anti-neu mAb and RTKI. The neu oncogene, identified in ethylnitrosourea-induced rat neuroglioblastomas, is a homologue of human ErbB-2 (22). Expression of constitutively activated neu in transgenic mice was associated with spontaneous tumorigenesis (23). Neu⁺ tumor cell lines with varying levels of drug sensitivity were established from NeuT spontaneous tumors or by transfection of hormone induced tumors to mimic breast cancer cells with varying levels of responsiveness to Her-2 targeted therapies.

Materials and Methods

Mice

All animal procedures were conducted in accordance with accredited institution guidelines and the US Public Health Service Policy on Humane Care and Use of Laboratory Animals³. BALB/c (age 6-8 weeks) female mice were purchased from Charles River Laboratory (Frederick, MD). BALB NeuT (NeuT) mice expressing a transforming neu under the control of MMTV promoter were provided by Dr. Guido Forni (University of Torino, Torino, Italy) (24). Female NeuT mice developed spontaneous mammary tumors around 17 weeks of age. Male NeuT mice developed salivary gland tumors when they were about 7 months old. Heterozygous NeuT mice were maintained by mating with BALB/c mice and transgene positive mice were identified by PCR.

Cell lines and reagents

All tissue culture reagents were purchased from Invitrogen (Carlsbad, CA) unless otherwise specified. Cell lines were cultured in 5% CO₂, and maintained *in vitro* in DMEM supplemented

³<http://grants.nih.gov/grants/olaw/olaw.htm#pol>

with 10% heat-inactivated FBS (Sigma, St. Louis, MO), 10% NCTC 109 medium, 2 mM L-glutamine, 0.1 mM MEM non-essential amino acids, 100 units/ml penicillin, and 100 µg/ml streptomycin.

TUBO (24) was cloned from a spontaneous mammary tumor in a BALB NeuT (NeuT) (25) mouse. TUBO grew progressively in wild type BALB/c mice and gave rise to tumors which were histologically similar to autochthonous tumors in BALB NeuT females. Bam1a cell was established in soft agar from another BALB NeuT spontaneous mammary tumor, then maintained as a cell line in monolayer culture. Bam IR-5 variant was derived from Bam1a by culturing in increasing concentrations of gefitinib until stable growth was achieved in the presence of 5 µM gefitinib (26). Gefitinib (Iressa, ZD1839, 4-(3-chloro-4-fluoroanilino)-7-methoxy-6-(3-morpholinopropoxy) quinazoline, Zeneca Pharmaceuticals, Macclesfield, Cheshire) is a receptor tyrosine kinase inhibitor.

Antigen presenting cells (APC) 3T3/KB and 3T3/NKB were generated as previously described (27). Briefly, BALB/c NIH 3T3 fibroblasts were transfected with K^d and B7.1 (KB), or with K^d, B7.1, and neu (NKB). Stable clones were selected and maintained in medium supplemented with 0.8 mg/ml G418 and 7.5 µg/ml of puromycin (3T3/KB) or 0.8 mg/ml G418 and 0.8 mg/ml of zeocin (3T3/NKB).

D2F2 was derived from a mouse mammary tumor that arose in a BALB/c hyperplastic alveolar nodule line, D2 (28). D2F2 cells were co-transfected with pRSV/neo and pCMV/neu, which encodes wild type rat *neu* to establish D2F2/neu (29). Transfected cells were maintained in medium supplemented with 0.8 mg/ml of G418 (Geneticin, Invitrogen).

DNA Immunization

pcDNA/neuTM encoding the extracellular and transmembrane domains of rat neu was previously described (24).

pCMV/cytneu (pcytneu) was constructed by deleting the ER signal sequence from pCMV/neu with a polymerase chain reaction (PCR) strategy (30). The first 684 bp of the protein coding region excluding the ER signal sequence was amplified using the high fidelity DNA polymerase Pfu (Stratagene, La Jolla, CA). The upper primer β, 5'-GCGGGGGAGCTCCGCCACCATGGGCACCCCAAGTGTGTAC-3', is homologous to the Kozak consensus ribosome binding site (Kozak, 1986), the initiation codon ATG and 15 bp immediately downstream from the ER signal sequence, but excludes the 72 bp signal sequence itself. The lower primer β, 5'-GTGGAGGCAGGCCAGGCAGTCAGAATGC-3', contains a naturally occurring BsmI site. This PCR product was digested with SacI and BsmI and used to replace the corresponding region in pCMV/neu to generate the plasmid pCMV/cytneu (pcytneu). The recombinant cytneu is designed to direct the synthesis of a cytoplasmic protein.

pEFBos/GM-CSF (pGM-CSF) encoding murine GM-CSF was provided by Dr. N. Nishisaka at Osaka University, Osaka, Japan. pCMV is the control empty vector. Mice were injected in the quadriceps muscle with plasmid DNA as previously described (30). Intramuscular DNA injection was followed immediately by square wave electroporation over the injection site using a BTX830 (BTX Harvard Apparatus, Holliston, MA) as we previously described (29). A tweezer electrode was used to deliver 8 pulses at 100V for 25 msec per pulse.

T cell depletion

To deplete CD4 or CD8 T cells, mice received i.p. GK1.5 or 2.43 mAb (ATCC), respectively, in the form of ascites fluid. Mice were treated once or twice before tumor challenge and then 1-2 times per week until completion of the experiment. T cell depletion was verified by FACS analysis using PBL.

Tumor challenge

Mice were challenged s.c. with 2.5×10^5 (TUBO or D2F2/neu) or 5.0×10^5 (Bam1a or Bam IR-5) cells in the flank. Tumor growth was monitored by weekly palpation and mice were sacrificed when any one dimension of the tumor reached 20 mm. Differences in tumor incidence were analyzed by the Log rank test.

Measurement of anti-neu antibody by flow cytometry

To measure anti-neu antibody, 3T3/NKB cells were incubated with serially diluted immune mouse sera. Briefly, phycoerythrin (PE) conjugated goat-anti-mouse antibody directed to the γ -chain of mouse IgG (Jackson ImmunoResearch, West Grove, PA) was used to detect bound antibody by flow cytometry. Normal mouse serum or isotype matched mAb was the negative control. A standard curve for neu binding was generated using c-erbB2/c-neu mAb (Ab4, clone 7.16.4; Calbiochem, San Diego, CA). Antibody concentrations in the test sera were calculated by linear regression analysis based on the standard curve as we previously reported (31). Flow cytometric analysis was performed with a FACSCalibur (Becton Dickinson, San Jose, CA). Differences in antibody concentration were analyzed by the Student's t test.

Measurement of IFN- γ secreting T cells by ELISPOT Assay

Neu reactive T cells were measured by ELISPOT assay (29). PBL or spleen cells were suspended in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 100 units/ml penicillin and 100 μ g/ml streptomycin. A total of 4×10^5 spleen cells were added to each well of a 96-well HTS IP plate (Millipore, Bedford, MA) which was pre-coated with 2.5 μ g/ml rat anti-mouse IFN- γ (clone R4-6A2, BD Pharmingen, San Diego, CA). 3T3/NKB cells were added as antigen presenting cells (APC) over the spleen cells. 3T3/KB cells were used as control. The ratio of spleen cells to APC was 10:1. Following 48 hr incubation, cells were removed and 2 μ g/ml biotinylated rat anti-mouse-IFN- γ (clone XMG 1.2, Pharmingen) was added. Plates were incubated for another 12 hours at 4°C, then washed to remove unbound antibody. Bound antibody was detected by incubating the plates with 0.9 μ g/ml avidin-horseradish peroxidase (Pharmingen) for 2 hours at room temperature. The substrate 3-amino-9-ethylcarbazole (AEC; Pharmingen) in 0.1 M acetic acid and 0.003% hydrogen peroxide was added and the plate was incubated for 3-5 minutes. AEC solution was discarded and the plates were washed six times with water. The visualized cytokine spots were enumerated with the ImmunoSpot analyzer (CTL, Cleveland, OH) and the results expressed as the number of cytokine producing cells per 10^6 cells. Data were analyzed using the Student's t test.

Proliferation assay

Cell proliferation was measured indirectly by mitochondria metabolic activity using a modified MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide) assay (32). Briefly, quadruplicate wells of cells were plated in flat bottom 96-well plates at 10,000 cells per well. Approximately 16 hours after plating, when cells reached 40-50% of confluence, dilutions of anti-neu mAb 7.16.4 or gefitinib were added. Replicate plates were terminated at 24, 48, 72, 96 and 120 hours post treatment. At each time point, 10-20 μ l of 5 mg/ml MTT in PBS was added and incubated for 4 h at 37°C before the stop reagent (Isopropanol with 0.04N HCl) was added and the absorbance measured at 600-650 nm.

Measurement of cell surface neu and MHC class 1 K^d expression by flow cytometry

Cells were stained with anti-neu mAb 7.16.4 or anti-K^d mAb SF1.1, followed by PE-conjugated affinity pure F(ab')₂ fragment goat anti-mouse IgG Fc (Jackson ImmunoResearch, West Grove, PA) at 1:200 on ice for 25 min. After staining, samples were washed twice and analyzed by flow cytometry using a FACSCalibur (Becton Dickinson, Mountain View, CA) flow

cytometer. Between 10,000 and 20,000 events were collected for each sample and the data were analyzed using flowjo software package.

Western blot analysis

Whole cell lysates were prepared as previously described after cells were incubated with indicated concentrations of gefitinib or anti-neu mAb or immune sera (26). Equal amounts of protein were loaded onto a 10% SDS gel and transferred to Immobilon-P membranes. After blocking, primary antibodies to phospho-Akt (Cell Signaling, Danvers, MA) were added to detect the target proteins. The membrane was washed and labeled for 90 minutes at room temperature with Peroxidase-conjugated AffiniPure Goat anti-mouse or goat anti-rabbit secondary Abs (Jackson ImmunoResearch Laboratories). The membranes were developed with enhanced SuperSignal® West Pico Chemiluminescent Substrate (Pierce Biotechnology, Inc., Rockford, IL) and imaged with Kodak-MR film. For normalization, blots were stripped and reprobed for β -actin.

Spectral Karyotyping (SKY) analysis

Cells were treated with colcemid for 2 hours and chromosomal slides were prepared using the standard protocols including hypotonic treatment, fixation, and air-drying as we described (33). After pepsin treatment and fixation with formaldehyde followed by dehydration, the chromosomal slides were denatured and hybridized with denatured painting probes (SkyPaint) for 48 hrs at 37°C. After slide washing, the chromosomes were counter-stained with DAPI and mounted with anti-fade (34). 30-50 mitotic figures from each cell line were randomly selected for SKY images. The selection criterion were high quality hybridization signals and minimal chromosomal overlapping. The presence of chromosomal abnormalities was not a selection criterion. This procedure was used for both the spectral and DAPI images.

Chromosomes were karyotyped by the color and the size of each chromosome using software developed by Applied Spectral Imaging Inc. (Carlsbad, CA). A unique single color was assigned to individual chromosomes. The presence of mixed colors along a chromosomal arm indicated a translocation (indicated by arrows). Clonal chromosomal aberrations (CCAs) referred to identical or closely related chromosomal aberrations occurring at least twice in 20-50 mitotic figures (35). When CCAs dominated a cell population the tumor was considered more stable and homogenous. The levels of non-clonal chromosomal aberrations (NCCA) were defined by occurrence at a frequency of less than 4% among 50-100 mitotic figures and correlated with overall genetic instability, indicating a non-linear pattern of cancer initiation and progression (35).

Results

Characterization of neu⁺ tumor cell lines

The efficacy of neu targeted therapy was tested with neu expressing BALB/c mammary tumor lines TUBO, Bam1a, Bam IR-5 and D2F2/neu. TUBO (24) was established from a NeuT spontaneous mammary tumor (25). Bam1a was derived independently from another NeuT spontaneous mammary tumor by soft agar culture (26). Bam IR-5 was derived from Bam1a by continuous culture in increasing concentrations of gefitinib to become resistant to 5 μ M of gefitinib. D2F2/neu was generated by co-transfecting BALB/c mammary tumor cell D2F2 (28) with pCMV/neu and pRSV/neo (29). All test cells, except D2F2, expressed neu as measured by flow cytometry using anti-neu mAb, 7.16.4 (Figure 1A, top row, open histogram). In addition, all test cells expressed MHC class I, K^d, (Figure 1A, bottom row, open histogram). These cells may represent Her-2/neu positive breast cancer cells eligible for Her-2/neu targeted therapy.

Inhibition of tumor cell proliferation by RTKI and anti-neu mAb

The effect of RTKI and anti-neu mAb on cell proliferation was measured by MTT assay after 24 to 120 hours of incubation with the test agent (Figure 1B). Reduction of TUBO cell proliferation was observed at 1 μ M or higher concentrations of gefitinib. Reduction in Bam1a cell proliferation was observed at 0.25 μ M gefitinib (Figure 1B). Although both cell lines were derived from spontaneous NeuT mammary tumors, Bam1a cells were more sensitive to RTKI. Bam IR-5 and D2F2/neu cells continued to proliferate even in the presence of 4 μ M gefitinib, showing their resistance to RTKI, whether acquired or intrinsic.

To test the direct effect of anti-neu mAb, tumor cells were incubated with 0.025 to 2.5 μ g/ml of anti-neu mAb. TUBO and Bam1a cell proliferation was significantly inhibited by 0.25 μ g/ml or higher concentrations of mAb ($p < 0.05$) with greater inhibition observed in Bam1a cells. Inhibitory activity was substantially reduced in Bam IR-5 since 2.5 μ g/ml mAb was required to induce growth inhibition. D2F2/neu cells were not affected, even at 2.5 μ g/ml. Therefore, cells that are resistant to RTKI are resistant or less sensitive to anti-neu mAb.

Effect of targeted therapy on neu expression and neu mediated signaling

Inhibition of tumor cell proliferation by RTKI or anti-neu Ab may be mediated via a common or different pathway and may indicate how tumor cells would respond to Her-2/neu vaccination. The effects on neu expression and Akt phosphorylation were analyzed. Cells were incubated with 4 μ M of gefitinib for 2 to 24 hrs before expression of neu on the cell surface was measured by flow cytometry. Figure 2A shows representative results from 8 hr culture. Treatment with gefitinib increased neu expression on TUBO and Bam1a cells, with a very modest effect on Bam IR-5 and no effect on D2F2/neu cells. This was consistent with our earlier observation by Western blotting showing sustained total Her-2/neu level in gefitinib treated Bam1a cells despite a marked reduction in phosphorylated Her-2/neu (26). Therefore, inhibition of TUBO and Bam1a cell proliferation by RTKI was not due to loss of neu expression.

Incubation with 2.5 μ g/ml anti-neu mAb, on the other hand, down-modulated neu expression on TUBO, Bam1a, and Bam IR-5 cells and this may contribute, in part, to reduced tumor growth (Figure 1B). Consistent with this finding is our observation by Western blot analysis that antibody binding induces degradation of activated Her-2/neu in Bam1a and BamIR-5 cells (not shown).

Since Akt phosphorylation was critical in neu-mediated signaling it was measured by Western blotting. Figure 2B shows representative results from 2hr incubation with gefitinib or anti-neu mAb. In TUBO and Bam1a cells, Akt phosphorylation was reduced by gefitinib treatment, with modest effect on Bam IR-5 and no effect on D2F2/neu cells (26). Therefore, gefitinib dampened neu mediated signaling in TUBO and Bam1a cells, correlating with the growth inhibitory effect of the drug. Reduction of phospho-Akt was also induced by anti-neu mAb in TUBO and Bam1a cells, consistent with the growth inhibitory activity (Figure 1B). A modest effect was induced in Bam IR-5, consistent with the modest growth inhibition by mAb (Figure 1B). D2F2/neu cells that were resistant to RTKI and mAb showed little change in Akt phosphorylation after treatment by either agent, indicating the use of independent signaling pathways for their growth and survival.

Spectral karyotyping analysis of neu expressing cell lines

The differential sensitivity of TUBO, Bam1a, Bam IR5, and D2F2/neu cells to gefitinib and antibody may represent intrinsic or induced changes of Her-2/neu⁺ tumor cells during therapy. Changes beyond neu mediated signaling may render the tumors refractory to other therapies. Since genomic instability has been correlated with tumor progression and disposition to drug

resistance, neu expressing tumor cells were subjected to spectral karyotyping (SKY) analysis (Figure 3). As chromosomal instability escalates, the cell population forms various combinations of NCCAs/CCAs (36). Table I shows chromosomal aberrations in neu expressing tumor lines, including CCAs, NCCAs, and chromosome fragments.

Although both TUBO and Bam1a were derived from NeuT spontaneous mammary tumors, significant disparities exist in their karyotypes. CCAs with chromosomal translocations t(15; 17), t(4; 10), and t(4, 13) were found only in TUBO cells. Similarly, NCCAs and chromosome fragments were found in TUBO, not Bam1a cells, showing more severe chromosomal aberrations in TUBO cells, correlating with their reduced drug sensitivity (Figure 1).

Bam IR-5 cells which are resistant to gefitinib and anti-neu mAb showed one CCA, 15% NCCAs, and many abnormal centromeres (not shown). Bam IR-5 cells also exhibited more trisomies of chromosome 6, 13 and 16, and monosomies of chromosome 18 and 19 relative to the parental Bam1a (Figure 3). It is not clear if these aberrations occurred after gefitinib treatment or if cells with pre-existing aberrations survived the drug selection. Regardless, continuous treatment with RTKI resulted in a cell population with drug resistance and more profound chromosomal aberrations.

When comparing D2F2/neu with D2F2 cells, they shared one CCA at t(11;16) and chromosome fragments X, 13, 3, and 1 (Table 1), verifying the common origin of these cells. But the transfected D2F2/neu cells exhibited a more stable karyotype. Therefore, the process of generating the transfected cell line selected for a less aberrant clone which expressed neu at a high level. Despite a more stable karyotype, D2F2/neu remained refractory to Her-2 targeted drugs and mAb.

These four cell lines, all expressing neu, showed divergent sensitivity to RTKI and anti-neu antibody as a result of genetic aberrations and different usage of signaling pathways. In human cancers, Her-2 positive cells with heterogeneous karyotypes may co-exist. Upon treatment with Her-2/neu targeting agents, the selection pressure would eliminate sensitive cells while those with greater chromosomal aberrations and drug resistance may expand.

Inhibition of tumor growth by neu DNA vaccination

To test if Her-2/neu vaccination can inhibit tumor growth regardless of their chromosomal aberrations and resistance to neu targeted therapy, BALB/c mice were electrovaccinated twice with pGM-CSF and pneuTM encoding the extracellular and transmembrane domains of neu (Figure 4). At 2 wks after the second immunization, an average of 19.2 ± 9.5 μ g/ml anti-neu antibody was induced (Figure 4A). T cell response was measured using IFN- γ ELISPOT assay using peripheral blood leukocytes (PBL). After incubation with 3T3/NKB cells that were engineered to express neu, K^d, and B7.1 (CD80), $150 \pm 50/10^6$ PBL IFN- γ secreting cells were detected in immunized mice, significantly higher than that in control mice receiving pCMV vector (Figure 4B).

To determine if anti-neu immunity from DNA vaccination protected mice from tumor growth, immunized mice were inoculated s.c. with TUBO, Bam1a, Bam IR-5, or D2F2/neu. In mice that received control vectors, 100% of the inoculated mice developed progressively growing tumors and mice were sacrificed when tumors reached 20 $\times$ 20 mm (Figure 4C). In pneuTM vaccinated mice, complete protection was achieved against every challenging tumor: TUBO, Bam1a, Bam IR-5, or D2F2/neu. Therefore, neu DNA vaccination protected mice from neu expressing tumors, regardless of their chromosomal aberration or resistance to neu targeted therapy.

To distinguish the effect of anti-neu Ab on different tumor cells, CD4⁺ and CD8⁺ T cells in some immunized mice were depleted with specific mAb prior to tumor cell inoculation and then at weekly intervals throughout the experiment. Complete protection against TUBO and Bam1a tumors was achieved even after T cell depletion, showing the activity of vaccine induced anti-neu antibody against TUBO and Bam1a cells. Protection against Bam IR5 and D2F2/neu, however, was compromised when T cells were depleted. In 3/7 Bam IR-5 and 4/6 D2F2/neu inoculated mice, tumors grew progressively. Therefore, T cell immunity may not be required in controlling drug-sensitive tumors, but are indispensable for controlling drug-resistant tumors, although anti-neu Ab may contribute to tumor rejection also by direct suppression of Her-2 signaling or facilitation of ADCC.

Bam IR-5 and D2F2/neu tumors that grew in vaccinated, T cell depleted mice were excised (Figure 4D), plated in monolayer culture and their expression of neu was measured. Bam IR-5 tumor outgrowth expressed comparable levels of neu as cells maintained in culture. But D2F2/neu cells completely lost neu expression after growing in mice. These results indicate that neu mediated signaling is still critical to the survival of BAM IR-5 cells, thus the sustained expression, yet dispensable in D2F2/neu cells which may use alternate signaling pathways for survival.

Effect of pcytneu DNA vaccine on tumor growth

To test if CTL alone could control neu⁺ tumors, pcytneu vaccine was generated to encode cytoplasmic neu with a truncated endoplasmic reticulum (ER) signal peptide. Similar to pcytE2 that we previously reported (37), pcytneu was released into the cytoplasm upon synthesis and rapidly degraded by the proteasome (not shown). Processing of proteins through this pathway would result in the complete repertoire of neu peptides for MHC class I presentation, but mature protein was not expressed on the cell surface for antibody recognition.

BALB/c mice were vaccinated twice with pcytneu and pGM-CSF (Figure 5). Neu Abs were not detected in any of the mice as expected (not shown). Neu reactive T cells from six immunized mice were measured individually and an average of $154 \pm 60/10^6$ splenocytes of IFN- γ producing cells were detected (Figure 5A). Therefore, vaccination with pcytneu activated T cells without inducing an antibody response. To test if T cells alone can protect mice from neu⁺ tumor, immunized mice were challenged s.c. with TUBO, Bam1a, Bam IR5 or D2F2/neu cells and tumor growth was measured weekly (Figure 5B). All four tumors were rejected by pcytneu vaccinated mice, showing control of neu expressing tumors by CTL, regardless of their drug sensitivity, and that vaccine-induced anti-neu antibody was not required for complete tumor rejection.

Discussion

A panel of neu expressing BALB/c mammary tumor cell lines, TUBO, Bam1a, Bam IR-5, and D2F2/neu, have been established to represent three types of Her-2⁺ human breast cancers. In a portion of Her-2⁺ breast cancers Trastuzumab monotherapy inhibits tumor growth (11). TUBO and Bam1a cells which are highly sensitive to anti-neu antibody and RTKI would represent these responsive cancer cells. In Bam IR-5 cells, neu L726I mutation induced by gefitinib corresponds to Her-2 L726F mutation in human breast cancer cells that are resistant to another RTKI lapatinib (38). Therefore, Bam IR-5 represents treatment induced drug-resistant tumor cells. The majority of Her-2 expressing human tumors have intrinsic resistance to Trastuzumab because only 11-23% of metastatic breast cancer patients respond to antibody monotherapy (11;39). D2F2/neu which are refractory to Her-2 targeted therapeutics without prior selection is a representative of these resistant cells. When developing Her-2 based immunotherapy or vaccines, all three types of Her-2/neu⁺ tumors should be tested to fully assess treatment efficacy. The propensity to develop cerebral metastases after Trastuzumab

treatment suggests therapy-induced genetic alterations or the selection of aggressive tumor cells (15;16), warranting vaccines that can initiate or amplify Her-2 immunity to control drug resistant tumor cells.

Although TUBO and Bam1a are both derived from spontaneous NeuT mammary tumors and they express comparable levels of neu, more chromosomal aberrations are found in TUBO cells, including the translocation of chromosome 4 which has been reported in other neu induced tumors (40). It is not clear whether the disparities between TUBO and Bam1a cells indicate heterogeneity of neu induced tumors or changes induced during tissue culture selection. However, increased chromosomal aberrations in TUBO cells correlate with their reduced sensitivity to gefitinib when compared with Bam1a cells, suggesting the use of alternative survival and growth signaling pathways in TUBO cells.

Bam IR-5 cells established by prolonged culture in gefitinib have developed CCAs, NCCAs, trisomies and monosomies which were not detected in the parent Bam1a cells. The point mutation of leucine 726 to isoleucine 726 in the ATP binding pocket of the neu tyrosine kinase domain may change the binding of gefitinib to Her-2 and mediate their resistance to drug and antibody treatment, although other genetic changes such as those revealed by SKY analysis may also contribute to the resistance.

Neu transfected D2F2/neu cells are resistant to drug and antibody treatment both *in vitro* and *in vivo*, showing their independence of neu in survival and proliferation. The common origin of D2F2 and D2F2/neu cells is evidenced by the shared chromosome 11 and 16 translocations which occur in some hormone-induced tumors (41). It is of interest that expression of neu resulted in a less aberrant SKY profile, suggesting the transfection and expansion of a cell with less aberration.

Whether gefitinib resistance is a result of drug selection as in Bam IR-5 or intrinsic resistance as in D2F2/neu, these cells are also resistant to antibody treatment, indicating shared escape mechanism. But, all test cells are rejected by pcytneu. vaccination which induces CTL without Ab response. We reported previously using pcytE2 vaccinated mice that depletion of CD8, but not CD4 T cells significantly reduced anti-tumor immunity, showing the induction of CTL which mediated tumor rejection (37). Therefore, a strong CTL response to the complete repertoire of neu peptides abolishes tumor growth regardless of their sensitivity to neu targeted therapy. When a strong humoral response is induced without effector T cells, as in T cell depleted, pneuTM immunized mice, only drug sensitive cells were completely rejected, with partial protection against drug resistant cells. In patients whose tumors are refractory to drug and antibody therapy, induction of comprehensive immunity by active vaccination will be critical to their long term protection.

Treatment with gefitinib increased neu expression. Since neu activation is required to induce receptor endocytosis and degradation, inactivation of the kinase domain by gefitinib binding may trigger a compensatory increase in Her-2/neu surface expression. Binding of Her-2⁺ breast cancer cells with Trastuzumab has been associated with decreased Her-2 surface expression (42) with increased internalization and degradation of Her-2 (43). Priming of endogenous Her-2 immunity and increased tumor cell lysis by CTL has also been associated with Trastuzumab treatment of breast cancer (44) (45). In Bam1a and TUBO cells, treatment with anti-neu mAb reduced neu expression, Akt phosphorylation, and cell proliferation. Anti-neu immunity may also be enhanced by anti-neu antibody binding to tumor cells.

It is of note that even D2F2/neu cells which are resistant to antibody *in vitro* are partially inhibited by vaccine induced antibodies *in vivo*, suggesting effector mechanisms such as ADCC in tumor growth control. D2F2/neu cells that survive immune attack *in vivo* completely lost neu expression, indicating the selection of antigen negative tumor cells in vaccinated hosts,

whereas Bam IR-5 cells maintained neu expression in immunized hosts, showing their continued dependence on neu for tumor outgrowth.

Regarding the specificity and safety of Her-2/neu vaccination, mice immunized with Her-2/neu DNA could not reject D2F2 cells which did not express Her-2/neu (not shown), showing specific recognition of Her-2/neu by DNA vaccination (Figure 4). Little to no binding of human epidermal growth factor receptor (EGFR, Her-1) transiently expressed on 3T3 cells was detected with Her-2 immune sera (not shown). Immunized mice do not show any apparent abnormality for at least one year except for their anti-Her-2/neu immunity (not shown) further showing the specificity of Her-2/neu DNA vaccination. The complete blood count of untreated and DNA vaccinated Her-2 Tg mice did not show obvious differences (not shown). Therefore, the induction of Her-2/neu specific immunity without noticeable toxicity supports the efficacy of Her-2/neu DNA vaccine.

Taken together, we have demonstrated the benefit of DNA vaccination in the management of diverse tumors that exhibit differential sensitivity or resistance to Her-2 targeted therapies. Anti-neu antibody can suppress tumor growth either directly or through mechanisms such as ADCC and may amplify T cell immunity. A strong Her-2/neu-specific T cell response continues to be the goal of Her-2 vaccination as drug-sensitive and -resistant tumors are both controlled by T cells.

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Figure 1. Characterization of neu⁺ tumor cell lines. (A) Expression of neu and MHC class I K^d. TUBO, Bam1a, Bam IR-5, D2F2/neu, and D2F2 cells were stained with anti-neu mAb, 7.16.4 (top row, open histogram) or mAb SF1.1 for K^d (bottom row, open histogram). Isotype controls were represented as shaded histograms. Secondary antibody was PE-conjugated goat anti-mouse IgG Fc. (B) Inhibition of tumor cell proliferation by RTKI and anti-neu antibody. Cells were plated in monolayer and incubated with gefitinib (0.25–4 μ M, top row) or anti-neu mAb (0.025–2.5 μ g/ml, bottom row). Control groups received DMSO or isotype control Ab. Relative cell number, reflected by metabolic activity, was evaluated at indicated time with MTT assay and graphed as percent of control. Each symbol represents the mean $\pm$ SD of 4 replicates.

Figure 2.

Effect of RTKI or anti-neu mAb on neu⁺ tumor cell lines. (A) Effect of gefitinib or anti-neu mAb on neu expression. TUBO, Bam1a, Bam IR-5, and D2F2/neu cells in monolayer culture were incubated with 2.5 μ g/ml of mAb 7.16.4 or 4 μ M of gefitinib for 8 hours (open histogram, grey outline). Control groups were treated with DMSO or normal mouse serum (open histogram, black outline). After the incubation, cells were stained with Ab4 and PE-conjugated goat anti-mouse IgG Fc. Isotype control was shown as shaded histogram. (B) Effect of gefitinib or anti-neu mAb on Akt phosphorylation. Cells were incubated for 2 hours in the presence of gefitinib (μ M) or anti-neu mAb 7.16.4 (μ g/ml). Whole cell lysates were resolved in SDS-PAGE. Blots were probed with phospho-Akt or control actin Abs. The concentrations of gefitinib from lanes 1 to 4 were 0, 0.25, 1, and 4 μ M, respectively. The concentrations of mAb 7.16.4 from lanes 2-4 were 0.025, 0.25, and 2.5 μ g/ml, respectively. Lane 1 was IgG_{2a} isotype control at 2.5 μ g/ml

Figure 3.

Representative karyotypes of neu-expressing cell lines. Spectral karyotyping (SKY) analysis was performed on metaphase preparations of each cell line. Full karyotypes of each tumor cell line were shown. Arrows indicate translocations summarized in Table 1.

Figure 4.

Rejection of drug-sensitive and -resistant tumors by pneuTM DNA vaccination. BALB/c mice were electrovaccinated twice, i.m., 2 weeks apart, with either pneuTM and pGM-CSF or control vector. Sera were collected 2 weeks after the final immunization. Also at 2 weeks after the final immunization, some mice were treated with mAb GK1.5 and 2.43 twice in one week to deplete CD4 and CD8 T cells. mAb treatment continued weekly throughout the remainder of the experiment. (A) Anti-neu antibody was measured by flow cytometry and antibody concentration was calculated by regression analysis as described in materials and methods. (B) PBL were collected and cells from mice within each group were pooled and stimulated with 3T3/KB or 3T3/NKB. The ratio of PBL to APC was 10:1. IFN- γ producing cells were

enumerated by ELISPOT assay. Results were expressed as number of spots per 10^6 PBL and analyzed by Student's t test; * indicates $p < 0.05$ when compared with the pCMV vaccinated control group. (C) Mice were challenged with TUBO, Bam1a, Bam IR-5, or D2F2/neu, 3 weeks after the last vaccination. Tumor growth was measured weekly by palpation and the results expressed as % tumor free mice. There were 6-10 mice in each group. Results were analyzed by Log rank test. * indicates $p < 0.05$, ** indicates $p < 0.005$ and *** indicates $p < 0.0005$ when compared with the pCMV vaccinated control group. (D) Expression of neu by Bam IR-5 and D2F2/neu tumor outgrowths. Tumors were removed from mice, single cell suspensions were prepared by enzymatic dissociation, and expression of neu on the cell surface was measured using flow cytometry (lower panel). Cells maintained in monolayer culture were used as controls (upper panel). Open histograms represent cells stained with mAb 7.16.4 and filled histograms are isotype controls.

Figure 5.

Effect of pcytneu DNA vaccination on neu tumor growth. BALB/c mice were electrovaccinated twice, i.m., 2 weeks apart with pcytneu and pGM-CSF or control vector and pGM-CSF. (A) IFN- γ secreting T cell response in immunized mice was measured by ELISPOT assay. Splenocytes were collected 2 weeks after last vaccination. Splenocytes from individual mice were incubated with 3T3/KB or 3T3/NKB cells. Each symbol represents the mean $\pm$ SD for individual mouse in 4 replicate wells. Results were expressed as number of spots per 10⁶ splenocytes and analyzed by Student's t test. (B) At 3 weeks after final immunization, the remaining mice were inoculated with TUBO, Bam1a, Bam IR-5, or D2F2/neu cells. Tumor growth was measured weekly by palpation and the results expressed as % tumor free mice. There were 6-7 mice in each group. Results were analyzed by Log rank test. * indicates

$p < 0.05$ when compared with the control group. ** indicates $p < 0.005$ when compared with the control group.

Table 1

Spectral karyotyping analysis of neu-expressing cell lines TUBO, Bam1a, Bam IR-5, D2F2/neu, and D2F2. Clonal chromosome aberrations (CCAs), non-clonal chromosome aberrations (NCCAs), and chromosome fragments were summarized

Cell Lines	Specific clonal chromosome aberrations (CCAs)		Non-clonal chromosome aberrations (NCCAs)	Chromosome Fragments
TUBO	t(15;17) t(4;10) t(4;13)	(94%) (94%) (89%)	22%	17,11,10
Bam1a	Non-detected		Structural aberrations	
Bam IR-5	i(16;16)	(35%)	15%, many abnormal centromeres	
D2F2/neu	i(X;X) t(11;16)	(89%) (17%)	14%	X,13,6,3,1
D2F2	t(11;1;12) i(13;13) t(X;14) t(1;11) t(11;12;1) t(11;16) t(5;3;2)	(89%) (84%) (70%) (43%) (41%) (11%) (8%)	35%	X,17,13,11,3,1

t = translocation

i = isochromosome