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Vaccination with the extracellular domain of p185^{neu} prevents mammary tumor development in *neu* transgenic mice

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Abstract The HER2/*neu* oncogene product, p185$^{HER2/neu}$, is overexpressed on the surface of many human breast cancers. Strains of transgenic mice have been developed that express the rat *neu* oncogene in mammary epithelial cells and develop spontaneous mammary tumors that overexpress p185^{neu}. This model provides an ideal system for testing interventions to prevent tumor development. In this study, we immunized *neu*-transgenic mice with a vaccine consisting of the extracellular domain of p185^{neu} (NeuECD). Immunized mice developed Neu-specific humoral immune responses, as measured by circulating anti-Neu antibodies in their sera, and cellular immune responses, as measured by lymphocyte proliferation to NeuECD in vitro. In addition, the subsequent development of mammary tumors was significantly lower in immunized mice than in controls and vaccine treatment was associated with a significant increase in median survival.

Key words Breast cancer vaccine · HER2/*neu* · Tumor immunotherapy · Transgenic mice

Introduction

The HER2/*neu* protooncogene encodes a growth factor receptor, p185$^{HER2/neu}$, with protein tyrosine kinase activity. Amplification and/or overexpression of HER2/*neu* appears to play a crucial role in the pathogenesis of many human cancers. Among the over 185 000 new cases of breast cancer diagnosed each year, overexpression of HER2/*neu* occurs in approximately 30% of invasive breast carcinomas and in 50% of ductal carcinoma in situ and contributes to a poor clinical outcome [29, 47–49, 51]. In addition to breast cancer, HER2-overexpression also occurs frequently in other malignant diseases, including ovarian cancer [3, 48], endometrial cancer [4], non-small-cell lung cancer [26], gastric cancer [40, 55, 56], bladder cancer [59], and prostate cancer [60].

The p185$^{HER2/neu}$ protein is an attractive target for immunotherapeutic interventions. It is expressed only at low levels in certain normal adult tissues [44] but at high levels in tumors. In addition, HER2/*neu* overexpression occurs relatively homogeneously within primary tumors, and is maintained at synchronous or metachronous metastatic sites, suggesting a continuous requirement for HER2 overexpression throughout the malignant process [35]. This is in contrast to most other tumor-associated antigens, which are often heterogeneously expressed and/or can undergo down-modulation of expression without significantly affecting tumor growth. Passive immunotherapy with anti-HER2/*neu* monoclonal antibodies has demonstrated efficacy in animal models as well as in clinical trials [2, 46].

Several transgenic mouse models of breast cancer have been developed [1, 7, 21, 33, 53]. The N202 transgenic mouse line expresses a wild-type rat *neu* gene under the control of the murine mammary tumor virus (MMTV) 3′ long terminal repeat. Female N202 mice develop mammary lesions strikingly similar, on a gross and histological level, to human breast cancer. Here we show that immunization of N202 *neu* transgenic mice with the extracellular domain of p185$^{HER2/neu}$ (NeuECD) induces anti-Neu humoral and cellular immunity and prevents mammary tumor development in a significant proportion of immunized animals. These results strongly suggest that transgenic animal studies can provide a design environment in which tumor prevention strategies can be evaluated.

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Materials and methods

Cloning and expression of the rat Neu extracellular domain

Specific oligonucleotides were synthesized on the basis of the published rat *neu* DNA sequence. Total cellular RNA was extracted from DHFR/G8 cells (NIH3T3 cells transfected with rat *neu* [22]) and used as a template in reverse transcription/polymerase chain reaction (RT-PCR) to generate the sequence encoding the rat *neu* extracellular domain (ECD). The NeuECD protein was produced by constructing a fusion between amino acids 1–53 of the herpes simplex virus type 1 glycoprotein D (gD) [39] to amino acids 57–687 of the Neu protein. This was cloned into the pRK5 expression vector under the control of a cytomegalovirus promoter. This construct was transiently transfected into 293 cells using a calcium phosphate precipitation protocol.

Purification of NeuECD protein

Purified rat NeuECD protein was obtained from approximately 15 l supernatant from 293 transfectants. This was concentrated to 1.0 l by ultrafiltration through a Filtron Ultrasette unit (30 kDa molecular mass cut-off). This material was passed through a 40-ml anti-gD immunoaffinity column, washed with phosphate-buffered saline (PBS) then PBS containing 1 M NaCl, and eluted with 0.1 M acetic acid/0.5 M NaCl, pH 2.9. Eluted fractions were collected and neutralized with 1 M TRIS, pH 8.0, then dialyzed against three changes of PBS. The final product was shown to have a molecular mass of approximately 82 kDa and a purity of about 94% by polyacrylamide gel electrophoresis. To identify potentially contaminating murine IgG that might have leached off the affinity column, a sample of the NeuECD protein was electrophoresed, blotted onto polyvinylidene difluoride membrane, and probed with an horseradish peroxidase conjugated anti-(mouse IgG). No murine IgG was detected.

Genotyping of transgenic mice

Breeding pairs of MMTV/*neu*-transgenic mice (N202 strain) were generously provided by Dr. William Muller, McMaster University. Since these animals were heterozygous for the *neu* transgene, pups were genotyped by PCR at weaning. DNA was prepared from tail- or ear-punch tissues and subjected to a standard PCR reaction [1 × PCR buffer (Boehringer Mannheim), 2.5 mM $MgCl_2$, 200 μM dNTP mix, 0.6 U Taq polymerase (Boehringer Mannheim), 2.1 pM each forward and reverse primers]. The primers used for genotyping amplify the region corresponding to nucleotides 1492–2117 of the rat *neu* cDNA.

Vaccines

Groups of 10 to 12-week-old transgenic and non-transgenic female mice were injected into the footpad with 6.7 μg NeuECD protein or a control antigen (chicken serum albumin, CSA) emulsified in complete Freund's adjuvant (CFA). A boost of 6.7 μg NeuECD or CSA, emulsified in incomplete Freund's adjuvant (IFA) was administered subcutaneously 14 weeks after the initial immunization. Palpable mammary tumors were measured on a weekly basis and tumor volume was calculated as (length × width^2)/2. The total tumor burden is calculated as the sum total of tumor volumes for all tumors present on a given mouse. Tumor incidence curves were analyzed by Wilcoxon's two-sample test.

ELISA assays

Sera from immunized mice were obtained at 2, 4, 8, 16, 22, and 31 weeks after the primary immunization and analyzed for anti-Neu reactivity in enzyme-linked immunosarbent assays (ELISA). Ninety-six-well microtiter plates were coated with NeuECD (0.75 μg/ml) or CSA (1.0 μg/ml) for 2 h at room temperature or overnight at 4 °C. All subsequent incubations were performed at room temperature. Non-specific binding sites were blocked with PBS containing 5% non-fat dry milk for 1 h, the plates were washed in PBS + 0.05% Tween 20 (PBS/Tween), and then incubated for 1 h with sera diluted over 12 wells, in triplicate. Plates were then washed in PBS/Tween and incubated with sheep anti-(mouse IgG) conjugated to horseradish peroxidase for 1 h. After another wash in PBS/Tween, substrate was added to each well and reactions were developed for 15–30 min. Reactions were stopped with the addition of 50 μl/well 1 M phosphoric acid. Absorbance was read at 450 nm on a microtiter plate reader (Vmax, Molecular Dynamics). Averages of triplicate determinations were plotted for each point in the titration series and a sigmoid titration curve was obtained. Curve-fitting equations were used to determine the titer for each serum, which is defined as the dilution at which half-maximal binding occurred. Antisera were also tested in ELISA against an irrelevant gD fusion protein and little if any anti-gD reactivity was observed.

Cellular proliferation assays

Draining lymph nodes were harvested from transgenic and non-transgenic mice immunized with NeuECD in CFA. Single-cell suspensions were prepared and plated at 5×10^5 cells/well in a 96-well microtiter plate. Cells were incubated with various concentrations of NeuECD protein, in triplicate, for 72 h. The cultures were then pulsed with 1 μCi/well [^{3}H] thymidine for 18 h and harvested, and [^{3}H] thymidine incorporation was measured in a scintillation counter. Results are presented as mean thymidine uptake ± SEM.

Results

To determine whether an anti-HER2/*neu* vaccine could induce an immune response in *neu*-transgenic mice, groups of mice were immunized with a recombinant NeuECD protein, emulsified in CFA, and administered either via the foot pad or subcutaneously. Immunized mice developed antibodies to NeuECD in a dose-dependent manner (data not shown). In a separate experiment, draining lymph node lymphocytes were harvested from transgenic mice and non-transgenic littermates immunized with NeuECD in CFA. When lymphocytes were subsequently exposed to NeuECD in vitro, strong proliferative responses to the immunogen were obtained in non-transgenic as well as transgenic mice (Fig. 1). Lymphocytes from non-immunized mice demonstrated little if any proliferative response to NeuECD in vitro (data not shown). These results are consistent with those of other groups demonstrating immunity to HER2/*neu* [11, 12, 15, 16, 34].

To study the effect of a NeuECD-based vaccine on mammary tumorigenesis, a group of 16 female N202 transgenic mice were immunized at 10–12 weeks of age (prior to the development of tumors) with recombinant NeuECD protein emulsified in CFA, and subsequently boosted with NeuECD in IFA. A control group of 10 female N202 transgenic mice was immunized and boosted with chicken serum albumin (CSA), using the same adjuvant and schedule. By 4 weeks after immunization, high titers of anti-NeuECD antibodies (or anti-CSA antibodies in the control immunized mice) were

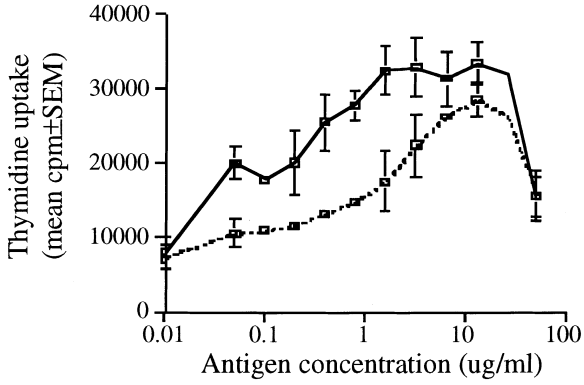

Fig. 1 Cellular response to NeuECD vaccine. In vitro proliferation of lymphocytes isolated from mice immunized with Neu extracellular domain (NeuECD) + complete Freund's adjuvant (CFA). — Transgenic mice, - - - non-transgenic littermates. Results are presented as the mean thymidine uptake ± SEM for triplicate samples in the assay.

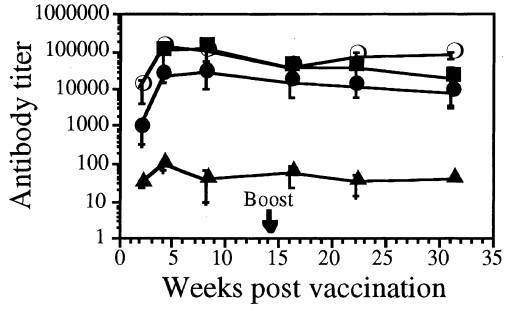

Fig. 2 Humoral response to NeuEDC vaccine. Sera from immunized or non-immunized mice were collected at various times and tested in enzyme-linked immunosorbent assays for reactivity against NeuECD (○, ●, ▲) or against chicken serum albumin (CSA; ■). ●, ▲, ■ Transgenic mice; ○ nontransgenic mice. ▲ Unimmunized; ●, ○ immmunized with NeuECD; ■ immunized with CSA. Each point represents the average titers of 8–16 mice, depending on the group

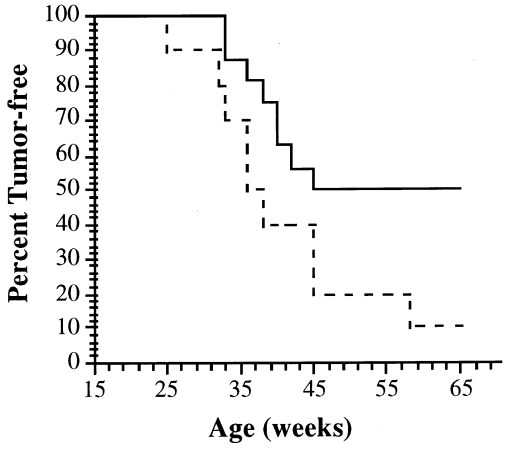

Fig. 3 Tumor incidence in immunized mice. Transgenic mice were immunized with CSA + CFA (10 mice; - - -) or NeuECD + CFA (16 mice; —) and were monitored for tumor growth. NeuECD immunization significantly prolonged tumor-free survival as compared to CSA immunization ($P = 0.001$; Wilcoxon's two-sample test)

observed in the serum and were sustained over 30 weeks after immunization (Fig. 2).

Tumor growth in these mice was inhibited or prevented by the NeuECD vaccine. As shown in Fig. 3, NeuECD immunization prevented tumor growth in 50% (8/16) of the animals immunized. In contrast, 90% (9/10) of the control mice developed mammary tumors. Figure 4 displays the growth of tumors that developed in these NeuECD- and CSA-immunized mice. Although immunization with NeuECD delayed the onset of tumor growth (Fig. 4B), once tumors began to grow they appeared to do so at the same rate as those in the control immunized mice (Fig. 4A). Tumors that developed in NeuECD-immunized mice were still Neu-positive by immunohistochemistry, indicating that escape from immunotherapy was not due to loss of antigen expression.

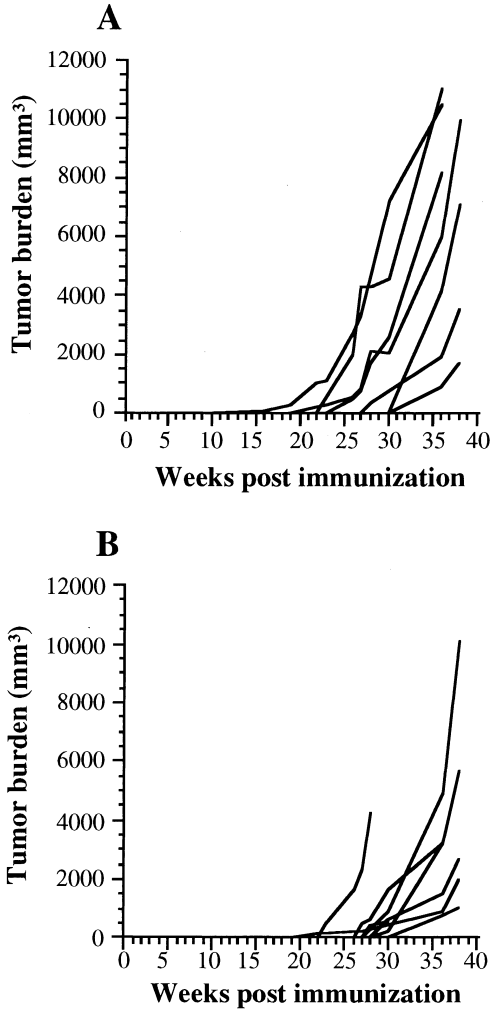

Fig. 4A, B Tumor growth in immunized mice. *neu*-transgenic mice were immunized with CSA + CFA (**A**) or NeuECD + CFA (**B**) and tumor growth was monitored over time. Each curve represents the tumor burden of an individual mouse. The volume of individual tumors was calculated as (length × width^2)/2. Tumor burden represents the sum total of the volumes of all tumors on an individual animal

Discussion

We have demonstrated that immunization of *neu*-transgenic mice with a recombinant protein containing the extracellular domain of $p185^{neu}$ generates specific humoral and cellular immune responses and prevents the formation of mammary tumors in a significant proportion of immunized mice. This may at first seem surprising since $p185^{neu}$ is a self protein in these transgenic mice and immunotolerance should prevent or limit reactivity towards self proteins. Indeed, early attempts to immunize rats using a recombinant vaccinia virus vector expressing rat NeuECD [5] or using whole rat Neu protein [12] failed. Transgenic mouse studies have shown that self-reactive T cells can be found in the peripheral T cell pool [6, 20, 32, 36–38, 42, 43, 52, 54, 58]. It may be that the presence of self-reactive anti-Neu cells in our *neu*-transgenic mice is simply due to the absence of thymic expression of the *neu* transgene, as has been shown in other transgenic models [25, 27, 30, 50]. Alternatively, autoreactive T cells may escape deletion by virtue of their low avidity for the self antigen [9, 36, 42, 43, 52, 58].

Existent humoral and cellular immune responses to HER2/*neu* have been demonstrated in some patients with HER2/*neu*-overexpressing tumors, and HER2/*neu*-peptide-specific T cells can be generated in vitro from patients as well as normal individuals, indicating the presence of HER2/*neu*-self-reactive cells in the periphery [8, 10, 17, 18, 24, 41, 45, 57]. Thus appropriately constructed HER2/*neu* vaccines may be capable of mobilizing these autoreactive cells. Disis et al. [12] have shown that a *neu*-peptide-based vaccine, but not a whole-protein vaccine, can elicit humoral and cellular responses in rats, although anticancer activity was not studied. These results, along with the results we report here demonstrating antitumor activity of a NeuECD vaccine, suggest that HER2/*neu* vaccines may prove efficacious in inducing or boosting immunity to HER2/*neu* in patients with HER2/*neu*-overexpressing tumors.

Although we observed prevention of mammary tumor development in 50% of NeuECD-immunized mice, the question arises of why tumors grew in the other 50%. There was no difference in the humoral immune responses between mice that developed tumors and those that did not; however, we cannot rule out the possibility of differences in cellular immune respones at this time. Antigen-loss variants do not appear to account for the tumors that grew in NeuECD-immunized mice since they all stained positively for $p185^{neu}$. Green and colleagues have shown that tumors expressing both activated *neu* and activated *ras* oncogenes are resistant to the growth-inhibitory effects of anti-Neu mAb [13]. Thus, if anti-Neu antibodies elicited by the NeuECD vaccine are responsible for preventing tumor growth in our studies, activation of other oncogenes, such as *ras*, may account for those tumors that escaped therapy.

Another possible mode of immunological escape is resistance to killing. Many HER2/*neu*-positive tumors are resistant to natural killer (NK) cells and to tumor-necrosis-factor(TNF)-mediated cytotoxicity [28], although the mechanism(s) of this resistance is not well understood. Sensitivity to NK or TNF-mediated cytotoxicity of mammary tumors arising in the N202 *neu*-transgenic line has not been examined and whether the tumors that escaped in our studies are NK- or TNF-resistant also remains to be determined. To address the question of escape fully will require further experiments, especially with respect to the mechanism of the antitumor response. Ongoing experiments in our laboratory are aimed at more finely dissecting the humoral and cellular responses generated by this vaccine, including assays for cytotoxic T cell activity, antibody-dependent cellular cytotoxicity assays, and direct anti-proliferative effects of anti-NeuECD sera.

An alternative explanation for why tumors grew in 50% of the NeuECD-immunized mice may be found in the "danger" model proposed by Matzinger and Fuchs [19, 31]. In this model, the immune system does not distinguish self from nonself, but rather responds to "danger", that is, anything causing tissue distress or lytic cell death. According to the danger model, the immune system fails to eradicate tumors because they are not seen as dangerous, they cause no immediate cell death or stress to alert local antigen-presenting cells. In addition, most tumor cells can only supply signal 1 to T cells specific for tumor antigens, but not a costimulatory signal 2, thus resulting in deletion of tumor-specific T cells. The danger model implies that it is important to keep the immune system on continuous alert against the tumor antigens, for example by repeated immunizations, until all tumor cells have been eradicated. As the danger signals disappear the antitumor response will die down. In addition, the tumor cells will deliver signal 1 but not signal 2 to tumor-specific memory T cells as they rest down, resulting in their deletion.

Since tumors do not arise at the same time in the *neu*-transgenic mice and since we only immunized twice and not continuously, it is possible that mice in which tumors began to develop early, when there were still danger signals present from the immunization, had their tumors eradicated. On the other hand, in mice in which tumors began to develop later, the danger signals induced by the immunizations may have already waned. These tumors were not seen as "dangerous" and hence continued to develop. We are currently testing this hypothesis by administering the vaccine repeatedly (every 4–6 weeks) for 50–60 weeks, at which point 80%–100% of the control mice will have developed tumors.

There are a number of concerns that arise with respect to immunization with HER2/*neu* vaccines. One concern is that the polyclonal humoral response generated may contain immunoglobulins that can activate the HER2/*neu* receptor, as has been found with some mAb, and lead to increased cell growth rather than inhibition. However, at least with mAb, in a mixture of antibodies containing some that are activating and some that are inhibitory, the inhibitory effect dominates [23].

A second concern is that of generating autoimmune toxicity against normal tissues that express the HER2/*neu* gene. $p185^{neu}$ is expressed at low levels in some normal adult tissues including skin, digestive tract epithelium, breast, ovary, hepatocytes, and alveoli [44]. However, there are several lines of evidence suggesting that HER2/*neu* immunity can be generated without destruction of normal tissue. First, cancer patients with existing immune responses to HER2/*neu* do not show signs of autoimmune toxicity. Second, treatment of rats with anti-(rat Neu) mAb does not cause any toxicities, yet still produced antitumor activity [14]. Finally, in rats immunized with *neu* peptide vaccines, immune responses to *neu* were induced, but normal tissues showed no evidence of lymphocyte infiltration or tissue destruction [12]. However, this vaccination regimen was not tested for anticancer activity. It is possible that increasing the anti-*neu* immunity to a level necessary to destroy cancer tissue in vivo may also increase levels of autoimmune reactivity against normal tissues to the point of inducing toxicity. Autoimmune toxicity was not evident in our studies, suggesting that successful antitumor immunity can be elicited without destruction of normal tissue.

New and innovative treatment strategies are clearly needed to improve outcomes in breast cancer, which too frequently recurs or progresses despite aggressive multimodality therapy. Ductal carcinoma in situ, the earliest manifestation of malignant breast cancer, is rapidly rising in incidence and represents both a problem, in that optimal management remains extremely controversial, and an opportunity, in that novel treatment strategies may be especially appropriate for this early stage of the disease. Indeed, ductal carcinoma in situ represents the best chance for truly preventative interventions, such as the HER2/*neu* vaccine strategy described in this report, since the burden of disease is low and the patient's immunocompetence is high.

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