Immunological Prevention of a Multigene Cancer Syndrome

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ABSTRACT

Vaccines effectively prevent the onset of tumors in transgenic mice carrying activated oncoproteins; however, human tumors are caused by combined alterations in oncoprobes and oncosuppressor genes. We evaluated the impact of prophylactic vaccines in HER-2/neu transgenic, p53 wild-type/null mice that succumb to an aggressive cancer syndrome comprising mammary and salivary gland carcinomas and rhabdomyosarcoma. A vaccine made of allogeneic mammary carcinoma cells expressing HER-2/neu and interleukin 12 afforded long-term protection from tumor onset. Tumor prevention was mediated by T cell–derived cytokines, in particular γ-interferon, and by anti–HER-2/neu antibodies. HER-2/neu expression was inhibited in target tissues of vaccinated mice, and somatic loss of the wild-type p53 allele did not occur. A highly effective vaccine against a single oncoprotein induced a powerful immune response that arrested multistep carcinogenesis in distinct target tissues.

INTRODUCTION

The immune system effectively delays the onset and reduces the incidence of tumors, as illustrated by the enormous increase in neoplastic growth, spontaneous and carcinogen induced, observed in severely immunodepressed mice (1, 2). Unfortunately, the existence of tumors in mice and humans clearly shows that spontaneous immune responses are not sufficient for a complete prevention of carcinogenesis. Conversely, it has been shown that a prophylactic activation of the immune system with vaccines and cytokines could result in a significant reduction in tumorigenesis (3).

The effectiveness of tumor immunoprevention has been clearly shown for carcinogen-induced tumors (4) and for spontaneous tumors originating in transgenic mouse models (5). A wide array of different immunologic strategies was used to prevent carcinomas in HER-2/neu transgenic mice, including passive administration of anti–HER-2/neu antibodies (6), modulators of the immune response like interleukin 12 (IL-12) (7, 8), α-galactosylceramide (9), or bacterial CpG sequences (10), and various HER-2/neu–specific vaccines based on whole cells (11), DNA (12–16), peptides (17), protein (18), or heat shock proteins (19). An almost complete immunoprevention of mammary carcinogenesis was obtained with a cell vaccine combining different immunologic stimuli (20).

Immunoprevention studies were performed in mice transgenic for oncoprobes; however, a hallmark of human neoplastic development is the cooperation of oncogene activation with oncosuppressor gene inactivation (21). Most events leading to the inactivation of oncosuppressor genes entail the absence of the corresponding protein, thus leaving no target antigen for the immune system. A second issue relevant to immunoprevention is the occurrence of different tumor histotypes in individuals with identical inherited genetic defects. At present, it is not known whether cell vaccines made of a given cell type may afford protection from tumors of a different histologic origin.

To address these issues, we investigated cancer immunoprevention in a multigenic cancer syndrome. Bigenic mice carrying an activated HER-2/neu transgene and one p53 null allele develop multiple tumor types that include mammary and salivary carcinomas (22) and rhabdomyosarcomas (23). This model system resembles human Li-Fraumeni syndrome and other cancer syndromes in which multiple mesenchymal and epithelial tumors occur in different districts in the same individual. We tested the prophylactic activity of a cell vaccine named “triplex” because it contains three immunogenic signals, namely, p185 (i.e., the gene product of HER-2/neu), combined with allogeneic major histocompatibility complex class I glycoproteins and IL-12 (20). We show here that the triplex vaccine is highly effective in preventing the onset of multiple tumor histotypes generated by the combination of p53 knockout and HER-2/neu activation through the inhibition of specific molecular events.

MATERIALS AND METHODS

Mice. Mice transgenic for a mutant rat neu oncogene driven by an MMTV-LTR and knockout for the p53 oncosuppressor gene (referred to as BALB/p53neu) were obtained by crossing BALB/c HER-2/neu (BALB/neuT) male mice with BALB/c p53+/− female mice; offspring bearing the p53+/−/neu+/− genotype then were selected by PCR analysis (23). Parental BALB/c p53+/+ mice (BALB/c-<Trp53<sub>H11001</sub>><sub>H11002</sub>><sub>H11001</sub>)) were purchased from The Jackson Laboratory (Bar Harbor, ME). BALB/c HER-2/neu transgenic mice were bred in our animal facilities. To study the role of antibodies in tumor prevention, BALB-p53−/− male mice and BALB-NeuT male mice were independently crossed with female BALB/c μMT mice, knockout for the immunoglobulin μ chain gene (24), a gift from Dr. Thomas Blankenstein (Max-Devber B Center for Molecular Medicine, Berlin, Germany). Offspring were interbred, and mice transgenic for the HER-2/neu oncogene (neu<sup>+/−</sup>), knockout for one p53 allele (p53<sup>−/−</sup>), and lacking B220-positive B cells (μMT/μMT) were selected. The level of B220-positive B cells was monitored by flow cytometry using monoclonal antibody RA3–6B2 (PharMingen, San Diego, CA). All of the experiments with animals were in accord with institution guidelines and were approved by the institution committee for animal use and care.

Cell Lines. TT12 cells (here referred to as Neu/H2-2<sup>+</sup>) cells) were derived (25) from a mammary carcinoma developed in FVB-NeuN<sup>−</sup> male mice. Neu/H2-2<sup>+</sup> cells express high levels of p185<sup>neu</sup> and are allogeneic to BALB/c mice. TT12 cells were transfected with a polycistronic plasmid pIL12-<sub>IR5</sub> containing murine IL-12 and IL-18, and stable transfectants (here referred to as Neu/H2-2<sup>+</sup>/IL-12 cells) were obtained and cultured as described previously (27).

Vaccination Protocol. Starting at the 6th week of age, BALB-p53neu female and male mice received successive 4-week courses of twice-weekly intraperitoneal vaccinations with 2 × 10<sup>5</sup> mitomycin-blocked Neu/H2-2<sup>+</sup>/IL-12 cells in 0.4 mL of PBS for 2 weeks, followed by 2 weeks of rest. The vaccination protocol of mice treated with Neu/H2-2<sup>+</sup> cells combined with systemic IL-12 was reported previously (20). Briefly, mice received 4-week courses of twice-weekly intraperitoneal vaccinations with 2 × 10<sup>5</sup> cells for 2 weeks, followed by 1 week of five daily intraperitoneal administrations of recombinant mouse IL-12 (50 ng in the first course, 100 ng thereafter) provided by S. Wolf (Genetics Institute, Andover, MA) in 0.2 mL of PBS.
supplemented with 0.01% mouse serum albumin (Sigma Aldrich, St. Louis, MO), followed by 1 week of rest. Control BALB-p53neu mice included untreated mice, mice treated with systemic IL-12, and mice treated with Neu/H-2<sup>q</sup> cells. The vaccination courses were repeated until mice were killed or were 1 year old. Tumor development was monitored weekly.

**Morphologic and Immunohistochemical Analysis.** Tissue samples obtained from BALB-p53neu mice at the indicated times were processed as described previously (28). For histologic evaluation, formalin-fixed tissue sections were stained with H&E. For immunohistochemical analyses, the following antibodies were used: anti-p185<sub>neu</sub> (C-18; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-proliferating cell nuclear antigen (anti-PCNA; Ylem, Rome, Italy), anti-CD11b/CD18 (Mac-1; clone M1/70.5), anti-CD8 (LyT2; clone YTS 169.4), and anti-CD4 (L3T4; clone YTS.191.1.2; all from Sera-lab, Crawley Down, Sussex, United Kingdom), anti-NK (asialo GM1; Wako Chemicals GmbH, Neuss, Germany), and anti-GR1 (granulocytes; clone RB6-8C5; American Type Culture Collection, Manassas, VA).

**PCR.** DNA and RNA were extracted from salivary gland tissue samples of BALB-p53neu mice at the indicated times by snap freezing and pulverization, followed by phenol-chloroform method. The p53 status was evaluated by a multiplex PCR on genomic DNA (0.1 μg each sample), amplifying simultaneously the p53 wild-type allele and p53 knockout allele. The amplification conditions and primer sequences were derived from The Jackson Laboratory. To study gene expression, RNA (1 μg each sample) was retrotranscribed, and semiquantitative reverse transcription-PCR was performed as described previously (29) with specific primer pairs for glyceraldehyde-3-phosphate dehydrogenase, γ-interferon (IFN-γ), and IL-4 (all from Clontech, Palo Alto, CA), rat HER-2<sub>neu</sub> (5'-AGG GCA ACT TGG AGC TTA CCT ACG-3', 5'-GGG TTC TGC CTG GGG TGG A-3'), and inducible protein 10 (20).

**Cell-mediated Response.** Spleen cells were collected from treated and control BALB-p53neu mice at the indicated times. Unseparated splenocytes or fractionated subsets, positively selected by paramagnetic beads conjugated with specific monoclonal antibodies (anti-CD4, anti-CD8, anti–CD45RB-220, and anti–NK-DX5; all from Miltenyi Biotec, Bergisch Gladbach, Germany) were cultured for 6 days at 37°C in Roswell Park Memorial Institute 1640 supplemented with 10% fetal bovine serum, containing 10 units/mL recombinant mouse IL-2 (Peprotech, Rocky Hill, NJ), alone or in the presence of mitomycin C-treated Neu/H-2<sup>q</sup> cells (10:1 splenocyte/target cell ratio). Supernatants were assayed for IFN-γ and IL-4 content by ELISA assay from Endogen (Woburn, MA).

**Antibody Response.** Antibody production by treated and control mice against Neu/H-2<sup>q</sup> cells was determined by cytofluorometric analysis of tumor-free mice sera as reported previously (20).

**RESULTS**

**The Cancer Syndrome of BALB-p53neu Mice.** BALB-p53neu mice derive from a cross between mice carrying one mutant HER-2<sub>neu</sub> transgene under transcriptional control of the mouse mammary tumor virus promoter and heterozygous p53 wild-type/null mice. All of the BALB-p53neu mice develop salivary gland carcinomas in the parotids at 13 to 15 weeks of age (22, 23). Female mice also are prone to mammary carcinoma with a longer latency time (~19 to 20 weeks); thus, growth of salivary tumors precedes the complete development of mammary carcinogenesis. At sacrifice, ~30% of female mice are affected by both types of tumor. In male BALB-p53neu mice, pelvic rhabdomyosarcomas (23) show latency time and growth rate similar to those of salivary gland carcinomas; thus, lethality can result from either type of neoplasm.

**Vaccination of BALB-p53neu Mice Prevents Tumor Development.** We previously reported an almost complete prevention of mammary carcinogenesis in HER-2<sub>neu</sub> transgenic mice using the triplex vaccine made of allogeneic HER-2<sub>neu</sub> transgenic mammary carcinoma cells (named Neu/H-2<sup>q</sup>/IL-12) (20). To test the efficacy of this prophylactic approach against the complex carcinogenesis of BALB-p53neu mice, we started vaccinations at 6 weeks of age, when mammary and salivary glands are affected by atypical hyperplasia and no sign of rhabdomyosarcomagenesis is detectable in the pelvis. We used two types of vaccine that differed in the source of IL-12: one made of IL-12–transduced allogeneic mammary carcinoma cells, designated as Neu/H-2<sup>q</sup>/IL-12, and the other with cells plus exogenous recombinant IL-12, designated as Neu/H-2<sup>q</sup> + IL-12.

Vaccination of female BALB-p53neu mice resulted in an impressive long-term inhibition of carcinogenesis in the salivary and mammary glands (Fig. 1A). More than 60% of vaccinated females were completely tumor free at 1 year of age, whereas all of the untreated mice developed tumors by 20 weeks of age. Vaccination of male mice resulted in a lower proportion (25% to 40%) of tumor-free individuals at 1 year of age (Fig. 1B).

Although vaccination with IL-12–transduced cells resulted in a higher proportion of tumor-free mice than the administration of cells + IL-12 in both sexes, the difference did not reach statistical significance. All of the mice receiving either IL-12 or Neu/H-2<sup>q</sup> cells succumbed to tumors with a latency time similar to that of untreated mice (Fig. 1).

To better understand the lower preventive efficacy of immunoprevention in male mice, the incidence of rhabdomyosarcomas and salivary gland carcinomas was studied independently. Fig. 2 shows that the proportion of vaccinated male mice free from salivary gland tumors was similar to that of vaccinated females (compare with Fig. 1A). A higher proportion of male mice succumbed to rhabdomyosarcomas.
coma, suggesting that the latter tumor was the main determinant of the preventive efficacy of the vaccine in males.

Morphologic Aspects of Tumor Immunoprevention. Histologic examination of all of the sites of tumor development showed that vaccinated, long-term survivors up to 1 year of age were completely free from preneoplastic or neoplastic lesions, thus confirming at the microscopic level the tumor preventive efficacy of the vaccine.

We performed an in-depth analysis of tumor progression in the salivary glands because salivary gland carcinoma is the most aggressive and prevalent tumor originating in BALB-p53neu mice of both sexes. Whereas submandibular and sublingual glands appeared free from evident neoplastic lesions, parotid glands from 8-week-old mice showed, bilaterally, multiple foci of atypical hyperplasia expressing p185neu antigen and PCNA and deriving from intercalated ducts and acinic cells (Fig. 3A). In some cases, in situ carcinoma already was detectable. Poorly differentiated acinic cell carcinoma or undifferentiated carcinoma, including areas of malignant mixed tumor, developed in parotid glands of all of the mice within 20 weeks of age. The carcinogenic process was accompanied by a progressive increase in proliferating (PCNA-positive) cells expressing surface p185neu (Fig. 3A). After three courses of triplex vaccination, at 17 weeks of age, both parotid glands were almost free from neoplastic and preneoplastic lesions; membrane p185neu expression was not detectable; and the expression of PCNA was comparable with that of normal glands (Fig. 3A). At this time, focal infiltrate mainly constituted of GR1+ granulocytes and asialo GM-1+ natural killer cells could be observed close...
By allele-specific PCR, we then analyzed the status of p53 in successive stages of salivary gland carcinogenesis. In tissues not affected by carcinogenesis (e.g., tail), the wild-type to knockout allele ratio was ∼1:1 (Fig. 4B), whereas in salivary carcinomas of untreated mice, we observed an almost complete disappearance of the wild-type allele (Fig. 4B). In contrast, almost all of the parotid glands of vaccinated, long-term tumor-free mice displayed a 1:1 ratio with no loss of the wild-type p53 allele (Fig. 4B). Persistence of the wild-type p53 allele indicates that vaccination prevented an additional molecular hit required for carcinogenesis.

Cell-mediated Mechanisms in Tumor Prevention. We previously found that the inhibition of mammary carcinoma onset in HER-2/neu transgenic mice rests on T cell–derived cytokines like IFN-γ and specific antibodies, whereas T cell–mediated cytotoxicity does not play a major role (20). The study of cytotoxic T-cell activity in vaccinated BALB-p53neu mice showed only a marginal induction of lytic activity against HER-2/neu–positive carcinoma cells (data not shown).

In the salivary gland of vaccinated mice, we found a strong induction of IFN-γ, the main downstream mediator of IL-12 and of tertiary chemokines with antiangiogenic activity, such as monokine induced by IFN-γ and inducible protein 10 (without in vitro restimulation (Fig. 5A). Spleen cells from vaccinated mice also displayed strong proliferative activity (data not shown) and cytokine secretion even without in vitro restimulation (Fig. 5B). Abundant IFN-γ release by splenic CD4, CD8, and natural killer cells was observed after in vitro restimulation with Neu/H-2K cells (Fig. 5B). It is interesting to note that the cytokines produced by CD4 cells included IFN-γ and IL-4, thus suggesting that the vaccine elicited a broad, nonpolarized immune response.

Antibody Dependency of Tumor Immunoprevention. IFN-γ is thought to play an immunoregulatory role in immunoprevention, and the most important final effectors are anti-p185neu antibodies (30). The study of the antibody response of BALB-p53neu receiving the complete vaccine or individual components showed that only the complete vaccine was able to induce a strong antibody response (Fig. 6). Analysis of early antibody responses after the first cycle of vaccination revealed a suboptimal antibody response in a minority of vaccinated mice (Fig. 6A). Mice with high antibody levels after the first cycle of vaccination developed even higher antibody levels in the course of subsequent vaccinations (Fig. 6B). We could not extensively investigate the kinetics of antibody responses in mice with low initial antibody titers because of early tumor development. Stratification of the survival of vaccinated mice shown in Fig. 1 by the intensity of the early antibody response showed that early responders were long survivors, whereas late responders rapidly succumbed to tumors (Fig. 6C).

This result indicates that antibody levels elicited by the first vaccination cycle, at 9 weeks of age, are predictive of long-term (1-year) survival and of freedom from tumor onset.

To formally show that antibodies are a major determinant of immunoprevention in vaccinated mice, we crossed BALB-p53neu mice with B cell–deficient μMT mice. Vaccination of antibody-deficient BALB-p53neu-μMT mice was completely ineffective (Fig. 6D). We conclude that the major protective mechanism elicited by the vaccine was a strong specific, long-term antibody response.

DISCUSSION

We have shown here that a vaccine prevents a complex cancer syndrome caused by combined alterations in oncogenes and oncosuppressor genes. Until now, immunoprevention has been investigated exclusively in oncogene-transgenic mice and not in oncosuppressor knockout mice. A combination of oncogene activation and oncosup-
Antivaccine antibodies after one vaccination course. Each symbol represents the binding to Neu/H-2^q cells of diluted (1:65) sera obtained after the first course of the indicated treatments (9 weeks of age) as determined by cytofluorometric analysis. The mean fluorescence intensity (MFI) of each serum was normalized by glyceraldehyde-3-phosphate dehydrogenase intensity. P values were determined by the Student’s t test.

Protection from carcinogenesis afforded by the vaccine rested mainly on the inhibition of HER-2/neu expression in target tissues. In HER-2/neu transgenic mice, expression of p185^{neu} is an absolute requirement for the early steps of tumor progression, as shown by the regression of established tumors after HER-2/neu deinduction in mice bearing an inducible transgene (31). We found that spontaneous loss of p185^{neu} expression in vitro abolished the tumorigenicity of HER-2/neu transgenic mammary carcinoma cells (25).

The block of HER-2/neu expression in vaccinated mice was sufficient to prevent the occurrence of subsequent events in tumor progression, in particular, the loss of the remaining p53 wild-type allele. Because the assessment of p53 status was performed on whole salivary glands, we could not exclude the existence of rare p53-null cells. If such cells exist, it remains to be determined whether they are tumorigenic; in a single vaccinated mouse, we found evidence of p53 allele loss in the absence of overt neoplastic growth (see Fig. 4). To address this point, it will be interesting to analyze p53 status at the clonal level during neoplastic progression and prevention.

In theory, loss of the wild-type p53 allele should be independent of the immune response elicited by the vaccine. However, cell proliferation driven by HER-2/neu enhances the probability of additional genetic damage affecting p53; thus, the immunologic inhibition of HER-2/neu expression also entails a reduced probability of p53 loss. In a hierarchy of events leading to tumor development, the abrogation of events featuring antigenic targets can bring about a complete arrest of the carcinogenic process. In the model system used here, expression of the antigenic oncogene precedes loss of p53, but in principle immunoprevention also should work if loss of a caretaker oncosuppressor gene (21) is the first event in the process, as long as suitable downstream targets of immune response (e.g., receptor tyrosine kinases) are expressed as later events (30).

Vaccination with mammary carcinoma cells was highly effective in
preventing mammary carcinoma of female BALB-p53neu mice and salivary gland carcinoma of male and female mice but was less effective against rhabdomyosarcoma of male mice. There are several possible explanations of this difference, including the histologic origin of tumors (i.e., epithelial versus mesenchymal) and the anatomic site of onset. The most plausible explanation is a reduced immune recognition of rhabdomyosarcoma cells caused by the low expression of p185$^{\text{neu}}$. We have shown previously that in rhabdomyosarcoma cells of BALB-p53neu the surface level of p185$^{\text{neu}}$ is one to two logs lower than in carcinomas (23). By conventional immunohistochemistry, p185$^{\text{neu}}$ was barely detectable in rhabdomyosarcoma (comparable with a 0+ or 1+ score), whereas it was strongly expressed in carcinomas (a 3+ score). In human breast carcinoma, it has been clearly shown that HER-2–positive 3+ cases are responsive to therapeutic treatments with anti–HER-2 monoclonal antibodies, whereas cases as a whole point to a major role of vaccine-induced anti–HER-2 immune responses in which CTL activity predominates, whereas humoral responses with a low systemic toxicity, whereas immunotherapy is better served by acute, transient CTL responses. A difference between cancer immunoprevention and cancer immunotherapy is that immunoprevention must be based on long-lasting, whereas prevention from subsequent reinfestations is mediated by neutralizing antibodies (36). A clear demonstration of the key role of antibodies in our system was provided by the loss of tumor prevention in vaccinated BALB-p53neu mice lacking antibody production. The various arms of the immune system are tightly interconnected, and it is virtually impossible to abrogate just one type of response; thus, B cell–deficient mice may harbor secondary defects in their T-cell responses. However, our results as a whole point to a major role of vaccine-induced anti–HER-2/neu antibodies in the prevention of mammary carcinoma. We previously have shown that vaccine-elicited antibodies against p185$^{\text{neu}}$ have multiple antitumor activities, as does Herceptin administered in human neoplasia (15, 20). Immune functions like complement-mediated cytotoxicity and antibody-dependent cell-mediated cytotoxicity are complemented by direct activities stemming from antibody-p185$^{\text{neu}}$ molecular interactions at the cell surface that determine inhibition of receptor homodimerization and heterodimerization, inhibition of receptor recycling, and inhibition of mitogenic signal transduction (30, 37). An additional reason for the importance of humoral responses against tumor progression is that antibodies efficiently bind tumor cells regardless of major histocompatibility complex expression, whereas the T-cell receptor binds a peptide–major histocompatibility complex that is frequently underexpressed by tumor cells to escape immune recognition (38–40).

The demonstration provided here for the first time that immune strategies can effectively prevent a multigene cancer syndrome, along with recent improvements that further increase the efficacy of prophylactic DNA and cell vaccines (20, 41–43), indicates that cancer immunoprevention has reached a convincing preclinical stage.

However, future developments will have to consider specific features of the transgenic models. The target antigen in this system is encoded by a xenogeneic transgene; thus, one may question whether it is protected by immune tolerance as human tumor antigens are. Available experimental evidence suggests that HER-2/neu transgenic mice are tolerant to rat p185 (44), thus implying that induction of immune responses by vaccines entails a break of tolerance.

Tumors that originate as a consequence of activated HER-2/neu expression are exquisitely dependent on HER-2/neu–driven signal transduction and are uniformly inhibited by anti-p185 antibodies. In contrast, only subsets of human carcinomas overexpressing HER-2 respond to anti-p185 trastuzumab. Recent results elucidating the molecular mechanisms of trastuzumab resistance provide additional targets for the development of combination approaches (45).

**REFERENCES**


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