Host Perforin Reduces Tumor Number but Does Not Increase Survival in Oncogene-Driven Mammary Adenocarcinoma

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Abstract

The concept of tumor immune surveillance has been supported by several recent studies in mice which show that immune effector mechanisms suppress hematologic malignancies. However, because the most common forms of human cancer are epithelial in origin, and comparatively very little data supports the immune surveillance of epithelial malignancies, we have chosen to evaluate the role of perforin-mediated cytotoxicity in the prevention of BALB/c Her2/neu-induced mammary cancer. Interestingly, perforin significantly delayed the onset of mammary tumorigenesis and reduced the number of mammary tumors without improving survival. Natural killer cell, but not CD8+ T cell, depletion resulted in a similar phenotype to perforin deficiency in this regard. Histologic analysis further indicated that the effect of perforin was most evident during the earliest stages of carcinogenesis rather than prior to or during the hyperplastic phase. This data suggests that perforin may mediate some suppression of epithelial carcinogenesis by intervening early in the tumor development process. [Cancer Res 2007;67(11):5454–60]

Introduction

Until recently, immune surveillance was considered irrelevant for tumor development and only cancers with a viral etiology seemed to be suitable targets for immune-based cancer therapies. With the emergence of suitable tumor antigens on epithelial cancers, gene-targeting technology in mice, and neutralizing monoclonal antibodies (mAb) specific for immune effector molecules and lymphocyte subsets, our understanding of the immune system has blossomed and the involvement of the immune system in the process of carcinogenesis is once again being explored (1–3).

Although there have been many studies showing the ability of the immune system to recognize and inhibit experimental tumor cell lines (4–9), there have been relatively few reports investigating immune recognition and elimination of spontaneous tumors (10–12). In particular, the importance of immune molecules such as perforin (pfp) and IFN-γ in controlling hematologic malignancies have been established (10, 12–15), and several reports showing the importance of IFN-γ in the immune surveillance of spontaneous carcinomas have been published (11, 12, 14, 16). Despite the wider acceptance that immune effector molecules may extrinsically suppress tumor development, there have been no studies to determine at what stage this control may be exerted.

Transgenic mouse models of carcinogenesis provide valuable insights into disease progression because many of these have distinct stages comparable to those observed in human carcinomas. BALB/c Her2/neu mice express activated rat Her2/neu under the mouse mammary tumor virus promoter, and female mice undergo staged development of spontaneous mammary tumors within 4 months of age (17–19). These mice undergo distinct, progressive, well-defined stages of carcinogenesis and therefore the effects of the immune system on specific stages of carcinogenesis can be determined.

Natural immune surveillance has not been extensively studied in Her2/neu transgenic mice, and in particular, the role of lymphocyte-mediated cytotoxicity in inhibiting spontaneous epithelial tumor development is poorly understood. The relative short latency period and high penetrance of mammary carcinoma in Her2/neu mice diminishes the problems associated with spontaneous tumor development in a perforin-deficient background because perforin-deficient mice develop spontaneous lymphomas from 14 months of life. Therefore, BALB/c Her2/neu transgenic mice were either generated on a perforin-deficient background or depleted of natural killer (NK) cells or CD8+ T cells and monitored for tumor development. NK cells and perforin were shown to have a subtle, but significant early, effect in delaying this form of mammary carcinoma.

Materials and Methods

Mice. Inbred BALB/c Her2/neu transgenic mice (BALB/c Her2/neu; backcrossed to BALB/c for >20 generations and kindly provided by Dr. G. Forni, University of Torino, Italy) were bred and maintained at the Peter MacCallum Cancer Centre, East Melbourne, Australia. BALB/c Her2/neu transgenic mice homozygous (pfp+/+), heterozygous (pfp+/−), or null (pfp−/−) for perforin were generated by several intercrosses of BALB/c Her2/neu mice with BALB/c pfp+/+ mice and the subsequent offspring screened for the Her2/neu transgene as previously described (17). All mice were routinely screened for viruses, parasites, and other microbes and tested negative over the entire course of the experiment.

BALB/c Her2/neu pfp+/+, BALB/c Her2/neu pfp+/−, and BALB/c Her2/neu pfp−/− mice were monitored twice or thrice a week and when the total area of mammary tumors exceeded 1,000 mm2, or when one tumor exceeded 150 mm2, mice were sacrificed (for ethical reasons) and autopsies were done on some of the mice. Mean life span ± SE were calculated and probability of significance determined using a Mann-Whitney rank sum U test. We did not observe abnormal peripheral lymphoid organs or immunoproliferative syndromes in any mice at the time of sacrifice. All experiments were done in accordance with guidelines set out by the Peter MacCallum Cancer Centre Animal Experimental Ethics Committee.
Some groups of BALB/c Her2/neu pfp<sup>+/+</sup> mice were depleted of NK cells in vivo by treatment with rabbit anti–asialo-GM1 antibody (Wako Chemicals) or treated i.p. with anti-CD8 mAb (53-6.7, rat IgG<sub>2a</sub>) or rabbit control immunoglobulin weekly (100 μg) from days 42 to 112 after birth. Effective depletions were monitored as previously described (20, 21).

Histopathology and whole mount preparation of mammary glands. A full autopsy was done at sacrifice and tumors (macroscopically detected) were routinely examined by H&E staining of histology of formalin-fixed tissues by Dr. Duncan MacGregor at the Department of Anatomical Pathology, Austin and Repatriation Medical Centre, Heidelberg, Australia. Whole mount preparations and immunohistochemical analyses were done as briefly described. The mouse skin was removed and fixed overnight in 10% buffered formalin. Mammary fat pads were scored into quarters and gently scraped from the skin. These were immersed in acetone overnight and then rehydrated and stained in ferric hematoxylin (Sigma-Aldrich), dehydrated in increasing concentrations of alcohol, cleared with histolemon, and stored in methyl salicylate (Sigma-Aldrich). Digital pictures were taken with a Nikon Coolpix 995 (Nital) mounted on a stereoscopic microscope (MZ6; Leica Microsystems).

Generation of mammary carcinoma cell lines. Cell lines were generated as previously described (22). Briefly, nonmetastasized, lobular breast carcinomas were excised from 20- to 23-week-old BALB/c Her2/neu and BALB/c Her2/neu pfp<sup>−/−</sup> mice. The tumors were cut into small pieces and digested with DNase I and collagenase for 45 min at 37°C with agitation. Cells were washed several times with RPMI medium (RPMI 1640 + 10% FCS, L-glutamine, Pen/Strep, nonessential amino acids, and sodium pyruvate) and incubated for 2 to 3 days in 10% CO<sub>2</sub> at 37°C. Cell lines were analyzed from two to five in vitro passages before a new aliquot was used.

Flow cytometry. Mammary carcinomas derived from mice were assessed for surface phenotype by multiparameter flow cytometric analysis. The following reagents used for flow cytometry were purchased from BD PharMingen: anti–H-2D<sup>d</sup>-PE (34-5-8S), anti–H-2K<sup>d</sup>-biotin (SF1-1.1), anti–CD1d-PE (1B1), anti-pan Rae-1 (clone 186107, rat IgG<sub>2a</sub> isotype reacts with Rae-1<sub>α, β, γ, δ</sub> and ε), anti–H60-biotin, and streptavidin-allophycocyanin antibodies. Isotype controls included mouse IgG<sub>2a</sub>, PK136, rat IgG<sub>2a</sub>, R35-95, hamster IgG (H57-97), and rat IgG<sub>1</sub> (R3-34). Anti-FcR (2.4G2) was used to prevent nonspecific binding by mAb. Analysis was conducted on a LSR II using FACSDIVA software (BD Biosciences).
Cytotoxicity assay. The sensitivity of mammary carcinoma cell lines to recombinant mouse perforin and/or human granzyme B was assessed in a 4 h 51Cr-release assay as previously described (23, 24). 2PK3 parental and 2PK3-mTRAIL transfectants were used as effector cells to determine tumor necrosis factor–related apoptosis inducing ligand (TRAIL) sensitivity of Her2/neu mammary carcinomas by an 18 h 51Cr-release assay as described (9). 4T1.2 mouse mammary carcinoma cells, originally derived from the 4T1 mouse mammary epithelial cell line, and kindly provided by Dr. R. Anderson (Peter MacCallum Cancer Centre) were used as a positive control for perforin and TRAIL sensitivity. The sensitivity of mammary carcinoma cell lines to alloreactive (H-2b anti-d) CTL was assessed at various effector/target (E/T) ratios in a 4-h 51Cr-release assay in which effector cells were popliteal lymph nodes from BALB/c mice immunized twice with DBA/2 splenocytes as previously described (25) and the mouse H-2b RMA lymphoma was a negative control target. The NK cell sensitivity of the mammary carcinomas was assessed at various E/T ratios in a 4-h 51Cr release assay using mouse Yac-1 lymphoma as a positive control target.

Mammary tumor transplantation. Mammary carcinomas derived from mice were assessed for their s.c. growth when transplanted (5 x 105 cells) into female BALB/c Her2/neu pfp+/+ mice. Tumor growth was measured every 2 days with a caliper square as the product of two perpendicular diameters and represented as the mean ± SE of five mice per group.

Results
Perforin delays the onset of breast carcinogenesis. Because there is little evidence for immune surveillance of epithelial malignancies with nonviral etiologies, we sought to investigate this possibility by comparing mammary carcinogenesis in BALB/c Her2/neu pfp+/+ mice, BALB/c Her2/neu pfp+/− mice, and BALB/c Her2/neu pfp−/− mice. There was a significant delay in the onset of mammary carcinogenesis when the mice expressed at least one wild-type allele of perforin (Fig. 1). The onset of breast tumors was later, and the number of tumors in each mammary gland lower, for BALB/c Her2/neu pfp+/+ mice or BALB/c Her2/neu pfp+/− mice compared with BALB/c Her2/neu pfp−/− mice (Fig. 1A). Indeed, the average age of first tumor onset was significantly earlier in BALB/c Her2/neu pfp+/+ mice compared with either BALB/c Her2/neu pfp−/− mice or BALB/c Her2/neu pfp+/− mice (117 ± 15 days versus 138 ± 12 and 136 ± 17 days, respectively; P ≤ 0.0005, Mann-Whitney; Fig. 1B). This finding contrasted with the similar tumor multiplicity and average age of first tumor onset among BALB/c Her2/neu IFN-γ−/− mice, BALB/c Her2/neu TRAIL−/− mice, and BALB/c Her2/neu wild-type control mice (ref. 22; and data not shown).

Perforin deficiency does not affect mortality caused by epithelial carcinomas. Despite the earlier tumor appearance and greater tumor multiplicity in BALB/c Her2/neu pfp+/− mice, perforin deficiency did not significantly affect the mortality rate of BALB/c Her2/neu pfp−/− mice with breast cancer (Fig. 2A) and there was no significant difference in the average age at death within the three groups (Fig. 2B). Once the mammary tumors appeared, there was no significant difference in tumor growth rate between tumors arising in BALB/c Her2/neu pfp−/− mice or BALB/c Her2/neu pfp+/− mice (Fig. 2C), thus indicating that once neoplasia had developed, perforin had little role in its control.

Perforin affects early phases of in situ tumorgenesis. Because there was an earlier onset of mammary carcinogenesis in BALB/c Her2/neu pfp+/− mice compared with BALB/c Her2/neu pfp+/+ mice, mammary whole mounts of groups of three to five mice, between 3 and 18 weeks of age, were prepared to evaluate lesion progression from hyperplasia to neoplasia. Importantly, the progression from the hyperplastic lesions to carcinoma in situ and lobular carcinomas occurred earlier in BALB/c Her2/neu pfp−/− mice than in BALB/c Her2/neu pfp+/+ mice (14–16 weeks versus 17–20 weeks, respectively; Fig. 3; Supplementary Fig. S1). Hyperplastic lesions (at 3–6 weeks) and mammary carcinomas (at 15 weeks) arising in BALB/c Her2/neu pfp+/+ mice and BALB/c Her2/neu pfp−/− mice both expressed similar levels of Her2/neu, and were dividing as determined by staining for proliferating cell nuclear antigens (data not shown). This data indicated that any differences observed were not due to a variation in the level of Her2/neu expression between the lesions. Critically, the difference between BALB/c Her2/neu pfp+/− and BALB/c Her2/neu pfp+/+ strains with respect to the early stages of in situ carcinoma (between weeks 14 and 18) suggested that there was a brief period in which lymphocytes may recognize and kill precancerous or neocancerous cells via perforin. This represents possibly the first illustration of a defined time period when an effector molecule was controlling spontaneous tumor development.

Hyperplastic nodules were observed from 3 weeks of age in both BALB/c Her2/neu pfp+/+ mice and BALB/c Her2/neu pfp−/− mice, and there was no discernible difference in the onset or appearance of hyperplastic foci within BALB/c Her2/neu pfp+/+ and BALB/c Her2/neu pfp−/− mammary glands between the ages of 3 and 9 weeks (data not shown). The lack of differences in hyperplasticity between BALB/c Her2/neu pfp+/− mice and BALB/c Her2/neu...
pfp" mice was indicative that immune lymphocytes simply failed to recognize mammary epithelial hyperplasia or that perforin was not an important host mechanism at the very earliest stages of tumor development. Similar data have also been obtained with BALB/c Her2/neu TRAIL" mice (22) and BALB/c Her2/neu IFN-γ" transgenic mice (data not shown).

**NK cells delay the onset of breast carcinogenesis.** It is likely that immune effector cells expressing perforin were engaged in an early immune reaction to tumor and thus it was important to additionally assess mammary tumor development in mice depleted of, or lacking, subsets of lymphocytes. Both NK cells and CD8" T cells are major populations expressing the perforin effector molecule (26). Thus, we sought to investigate this possibility by comparing mammary carcinogenesis in BALB/c Her2/neu mice treated with control immunoglobulins, compared with those depleted of NK cells or CD8" T cells. The anti-asialo-GM1 treatment protocol effectively reduced NK cell numbers in these mice without affecting NT cells, other T cells, and monocytes (ref. 21; Supplementary Fig. S2). There was a significantly faster onset of mammary carcinogenesis in mice depleted of NK cells (Fig. 4A and B), which correlated with the effect seen in BALB/c Her2/neu pfp" mice. The onset of breast tumors was later, and the number of tumors in each mammary gland lower, for control immunoglobulin- or anti-CD8"-treated BALB/c Her2/neu mice compared with NK cell–depleted BALB/c Her2/neu mice (Fig. 4A). Indeed, the average age of first tumor onset was significantly earlier in NK cell–depleted BALB/c Her2/neu mice compared with either control immunoglobulin- or anti-CD8"-treated BALB/c Her2/neu mice (119 ± 3 days versus 136 ± 2 and 136 ± 3 days, respectively; P ≤ 0.0007, Mann-Whitney; Fig. 4B). In an independent study, it was observed that BALB/c Her2/neu transgenic mice on a NK T cell–deficient background (Jo18" or CD1d" ) did not have a significantly different mammary tumor development from their wild-type littermates. Thus, these data imply that NK cells are likely the key perforin-expressing effector cells that delay mammary carcinoma development in this model. Proving this point may only be possible when perforin can be selectively gene-targeted in different lymphocyte subsets.

**Tumors emerging in perforin-deficient mice do not seem to be immunoedited.** We and others have provided considerable evidence that the immune system sometimes sculpts the developing tumor in a process termed immunoediting (2, 3, 27). There are many possible reasons for the rapid growth of the mammary carcinomas in the face of host perforin-mediated cytotoxicity, such as escape mutants that are resistant to cytotoxic mechanisms. However, mammary carcinoma cell lines derived from BALB/c Her2/neu pfp" or BALB/c Her/neu pfp" transgenic mice were equivalently sensitive in vitro to recombinant perforin at increasing concentrations (from 2.5 nmol/L upwards; Fig. 5A) or a sublethal dose of perforin (5 nmol/L) and granzyme B (Fig. 5B). Clearly, our assessment of mammary tumor cell lines derived from perforin-sufficient mice showed they were not more inherently resistant to perforin-lytic activity or perforin/granzyme B–mediated apoptosis. It remains possible that greater resistance to perforin or perforin/granzyme B is only manifested when the epithelial cells are at a premalignant/neomalignant phase of development. In addition, mammary carcinoma cell lines derived from BALB/c Her2/neu pfp" or BALB/c Her/neu pfp" transgenic mice were equivalently sensitive to TRAIL-mediated apoptosis (data not shown). Further analysis of sensitivity to whole effector cells such as allogeneic CTL or resting NK cells (data not shown) suggested that mammary carcinoma cell lines derived from BALB/c Her2/neu pfp" or BALB/c Her/neu pfp" transgenic mice were equivalently sensitive in vitro. Despite our best efforts, we were never able to successfully generate mammary cell lines from hyperplastic/dysplastic mammary tissues in BALB/c Her2/neu transgenic mice from 14 to 18 weeks of age. Thus, it seems that emerging mammary carcinomas from BALB/c Her2/neu pfp" mice did not escape immune suppression by becoming inherently resistant to the major cytotoxic effector pathways of lymphocytes.

Another possible escape mechanism for the emerging mammary carcinomas might involve loss of immunogenicity. The NKGD2 activation receptor expressed by NK cells, γδ T cells, activated T cells, and macrophages has been shown to recognize stress ligands on epithelial tumors (28, 29) and perforin seems to be the important effector mechanism engaged by the NKGD2-NKG2D ligand interaction (30, 31). Although expression of the MHC class I molecule, H-2Dd, was variable between individual tumors, a ligand interaction (30, 31). Although expression of the MHC class I molecule, H-2Dd, was variable between individual tumors, a point may only be possible when perforin can be selectively gene-targeted in different lymphocyte subsets.
Her2/neu pfp−/− mammary carcinomas examining the expression of ligands that are important for immune recognition did not reveal any significant differences between wild-type and pfp−/− tumors (Fig. 6A). Additionally, these carcinomas did not express the NKG2D ligands Rae-1 and H60 (Fig. 6A). Furthermore, transplantation of these cell lines into BALB/c Her2/neu pfp−/+ mice did not reveal any significant differences in tumor immunogenicity (Fig. 6B). These data clearly illustrate that tumors emerging in BALB/c Her2/neu pfp−/+ mice were not growing in these hosts because they simply became less immunogenic. The availability of neutralizing antimouse NKG2D mAbs will now allow the importance of this pathway in host protection from Her2/neu transformation to be assessed. Perhaps determining the expression and regulation of MHC class I and NKG2D ligands during the progression of carcinogenesis may also be informative because the importance of these recognition pathways may be transient.

Discussion

No previous studies have directly addressed whether and when lymphocyte-mediated cytotoxicity might be playing a role in controlling the oncogenesis of common primary carcinomas. Importantly, here we have shown that both NK cells and perforin delayed the onset of mammary carcinoma and reduced the number of tumors initially growing in Her2/neu transgenic mice. Clearly, various therapies that mobilize immunity can suppress tumor development in the Her2/neu model, when treatment is commenced at an early stage (32). In general, protection has been predominantly dependent on either perforin, IFN-γ, and/or the production of antibodies by B cells that were reactive with Her2/neu. Notably, however, even when mobilizing such immunity, it is typical for most mice to succumb to tumor development and growth, simply because the tumor mass overwhelms any immune reaction. However, this is the first report of innate immune surveillance against a recognized, clinically relevant epithelial tumor model for one of the most prevalent cancers affecting women today. Despite the extreme oncogenicity of this model and reported immune tolerance mechanisms, NK cells and perforin-mediated cytotoxicity have still been shown to significantly affect tumor onset. Given our results, when considering early adjuvant therapy, NK cells might be a better target than CD8+ T cells, to recognize and destroy precancerous cells in the mammary gland.

Why then do NK cells and perforin fail to prevent Her2/neu-driven tumors in this model? First, it must be noted that there is a complete penetrance of the activated rat Her2/neu oncogene in every mammary gland of these transgenic mice, and thus, it is likely that the cytotoxic cells were simply overwhelmed by the number of tumor cells emerging (both by division and de novo carcinogenesis) within the mammary glands. Second, regulatory
mechanisms may be masking a clearer role for the host innate immune system in preventing tumor development. These rat Her2/neu transgenic mice have a T cell repertoire tolerant to the rat Her2/neu expressed from birth, and we have recently shown that CD4+CD25Foxp3+ regulatory T cells suppress both CTL and antibody responses to mammary tumors emerging in these Her2/neu mice (33). Loss of regulatory T cells during anti-CD25 treatment could extend tumor-free survival and reduce carcinoma multiplicity in this model. Therefore, it is very clear that regulatory mechanisms might negate NK cell, CTL, and antibody responses in these mice. Consistent with this idea, we did not see any effect of depleting CD8+ T cells on mammary carcinoma onset. Interestingly, however, despite the recently reported capacity of regulatory T cells to suppress NK cell–mediated tumor immunity (34, 35), we did see an effect of depleting NK cells, thus suggesting the balance between NK cell effector function and regulatory T cell suppression in this tumor model. Alternatively, it remains unclear whether regulatory mechanisms may be perturbed in perforin-deficient mice, but at least one study has indicated that regulatory T cell–mediated suppression is perforin-independent (36). Lastly, tumor growth itself often involves leukocytes that promote inflammation, and mammary tumors are one of the settings in which this has become well accepted (37). Consequently, a number of factors may mask the innate immune surveillance of the Her2/neu-induced tumors. Despite all these factors, this study has shown for the first time an early role for NK cells and perforin in delaying in situ carcinoma formation.

The importance of perforin in tumor immunity has been reviewed extensively (38). Most studies have focused on the inability of perforin-deficient mice to reject a variety of experimental tumors or spontaneous metastases (20, 38), and comparatively few studies have evaluated host immune response to spontaneous tumors in these gene-targeted mice. An initial study by van den Broek and colleagues indicated that perforin played a broad role in protecting the host from tumors initiated by carcinogen or murine leukemia virus (5). Direct evidence for immune surveillance by cytotoxic lymphocytes has also been provided by simply aging various strains of perforin-deficient mice (10, 13, 14). These studies highlighted an important role for perforin-expressing cells including CTL, NK cells, NK T cells, and γδ T cells in preventing spontaneous lymphomas of B cell origin, thus supporting the role of perforin in mechanisms of immune surveillance that prevent lymphomagenesis. Despite this prior work, studies that illustrate the role of host perforin in protecting epithelial tissues from cancer have been very sparse. We reported the late onset of lung adenocarcinomas in 15% to 20% of aging perforin- or IFN-γ–deficient mice (13). Herein, we have shown evidence of a natural perforin-mediated effector mechanism delaying the development of spontaneous mammary carcinomas with a defined oncogene and antigens of clinical significance. Existing methods of depleting NK cells such as using anti–asialo-GM1 and anti-NK1.1 are not perfect, and very new strategies in which NK cells may be conditionally depleted might provide more compelling results (39).

Perhaps in a model in which only a limited amount of mammary epithelium becomes transformed, the effect of perforin would be far more obvious. Towards this end, it may be possible to further attenuate the oncogenicity of the Her2/neu or to reduce the tolerogenic mechanisms employed by the tumor (e.g., by suppressing the response to tolerogenic cytokines) such that extrinsic tumor suppression by the immune system may be more easily revealed. Similar types of approaches should be assessed in a variety of mouse models of epithelial neoplasia. These types of studies will be instrumental in designing more effective immune-logic approaches that could prevent and treat common human cancers.

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