Interleukin 12-mediated Prevention of Spontaneous Mammary Adenocarcinomas in Two Lines of Her-2/neu Transgenic Mice

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Summary

The ability of interleukin (IL)-12 to prevent tumors when administered to individuals with a genetic risk of cancer was studied in two lines of transgenic mice expressing rat HER-2/neu oncogene in the mammary gland. Female BALB/c (H-2^d) mice carrying the activated HER-2/neu oncogene show no morphological abnormalities of the mammary gland until 3 wk of age. They then progress through atypical hyperplasia to in situ lobular carcinoma and at 33 wk of age all 10 mammary glands display invasive carcinomas. Adult FVB mice (H-2^q) carrying the HER-2/neu protooncogene develop mammary carcinomas with a longer latency (38-49 wk) and a lower multiplicity (mean of 2.6 tumors/mice). Treatment with IL-12 (5 daily intraperitoneal injections, 1 wk on, 3 wk off; the first course with 50 ng IL-12/day, the second with 100 ng IL-12/day) begun at 2 wk of age in BALB/c mice and at 21 wk of age in FVB mice markedly delayed tumor onset and reduced tumor multiplicity. Analogous results were obtained in immunocompetent and permanently CD8^+ T lymphocyte–depleted mice. In both transgenic lines, tumor inhibition was associated with mammary infiltration of reactive cells, production of cytokines and inducible nitric oxide synthase, and reduction in microvessel number, in combination with a high degree of hemorrhagic necrosis.

Key words: interleukin 12 • adenocarcinomas • tumor prevention • angiogenesis • Her-2/neu

Current genetic studies are leading to the identification of gene mutations that predispose to cancer. They thus hold out the hope that not-yet affected patients or “unpatients” with a defined genetic prognosis can be detected (1). This, indeed, is already a reality in breast cancer, one of the most common malignancies in women.

With the paucity of effective preventive options, probing of the human genome is raising new and dramatic ethical, psychological, and cultural issues (1). On the other hand, identification of a mutated gene and its altered or amplified products is providing a new chance of undertaking immunologic maneuvers against oncogene products (2) in an unprecedented setting, where they may be able to prevent the onset or inhibit the initial growth of tumors in healthy individuals with a high risk of cancer.

This paper offers evidence of the efficacy of administration of mouse recombinant IL-12 (rIL-12) in counteracting the occurrence and progression of spontaneous mammary carcinomas in the females of two lines of inbred mice transgenic for the rat HER-2/neu oncogene under the transcriptional control of mouse mammary tumor virus (MMTV)^1LTR (3). BALB–NeuT female mice are from a new line of transgenic BALB/c mice carrying the activated HER-2/neu oncogene (4) and presenting high mammary

**Abbreviations used in this paper: iNOS, inducible NO synthase; IP-10, IFN–γ–inducible protein 10; NO, nitric oxide; MIG, monokine induced by γ-IFN; MMTV, mouse mammary tumor virus; MSA, mouse serum albumin; VCAM, vascular cell adhesion molecule.**
tumor multiplicity and relatively fast tumor growth (5). FVB–NeuN female mice carry the HER-2/neu protooncogene (6) and display lower multiplicity and longer latency. Prolonged administration of low doses of rIL-12 delayed tumor onset and reduced tumor multiplicity in both lines. Analogous results were obtained in BALB–NeuT mice permanently depleted of CD8⁺ T lymphocytes.

Tumor inhibition in both lines was associated with deficient peri- and intratumoral angiogenesis, infiltration of reactive cells, production of proinflammatory cytokines, and inducible nitric oxide synthase (iNOS) activation. Vascular damage resulted in a high degree of hemorrhagic necrosis of established tumor masses.

Materials and Methods

Mice. A transgenic CD1 random-bred breeder male mouse (no. 1330) carrying the mutated rat HER-2/neu oncogene driven by the MMTV promoter (Tg-NeuT, provided by Dr. L. Clerici, Euratom, Ispra, Italy; reference 5) was mated with BALB/c females (H-2b; Charles River, Calco, Italy). The progeny was screened for the transgene by PCR. Transgene-carrying males were backcrossed with BALB/c females for 12 generations and HER-2/neu-NeuT BALB/c mice (BALB-NeuT) were used in these experiments. Parental FVB–NeuN N202 transgenic mice (6) carrying the rat HER-2/neu protooncogene driven by the MMTV promoter on the H-2b FVB inbred background were provided by Dr. W.J. Muller (McMaster University, Hamilton, Ontario, Canada) and bred in our animal facilities. Females of both transgenic lines show a MMTV-driven overexpression of the transgene in the mammary gland and a definite tumor growth involving the mammary gland epithelium (5–7). Individually tagged virgin females were used in this study. Starting at the age of 5 wk, their mammary glands were inspected once a week, and masses were measured with calipers in the two perpendicular diameters (8). Progressively growing masses >3 mm mean diameter were regarded as tumors. BALB–NeuT mice were killed at wk 33 when these masses were evident in all 10 mammary glands. FVB–NeuN mice were killed when a mammary mass exceeded 2 cm mean diameter, and surviving mice were killed at 61 wk. All mice were evaluated histologically for mammary tumor development and toxicity related to IL-12 administration.

IL-12 Administration. rIL-12 (Genetics Institute, Cambridge, MA) in HBSS supplemented with 0.01% mouse serum albumin (MSA; Sigma Chemical Co., St. Louis, MO) was administered intraperitoneally. MSA control mice received similar injections of MSA only.

Depletion of CD8⁺ Lymphocytes. After thymectomy, a few BALB–NeuT neonatal mice were thymectomized and received 200 µg of purified anti-CD8⁺ cell mAb (33.6.72 hybridoma, anti-Lyt2; American Type Culture Collection, Rockville, MD) injected intraperitoneally 2 d later. Complete removal of thymus lobes and effective CD8⁺ T lymphocyte depletion was checked at 33 wk. Immunofluorescence of lymph node cells was performed by staining with FITC-conjugated rat anti mouse CD3 (clone 145-2C11), CD4 (clone R M 4-4), or CD8 (clone 53-6.72) mAbs (all from Pharmingen, San Diego, CA). Cytofluorometry showed a persistent reduction and depletion of CD8⁺ lymphocytes (<4%) compared with untreated mice.

Morphologic Analysis. Groups of two or three BALB–NeuT mice were killed at wk 2 and 3 and then every other week until wk 33; similar groups of FVB–NeuN were killed every 4 wk from wk 5 to 61. For histologic evaluation, tissue samples were fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned at 4 µm, and stained with hematoxylin and eosin or Giemsa. For immunohistochemistry, astrocyte-fixed cryostat sections were incubated for 30 min with anti-CD4, anti-CD8a (all from Sera-Lab, Crawley Down, Sussex, UK), anti-Mac-1 (anti CD11b/CD18), anti-Mac-3, and anti-1a (all from Boehringer Mannheim, Milan, Italy), antipolyomavirus nuclear leukocytes (Rb6-BC5; provided by Dr. R.L. Coffman, DNAX Inc., Palo Alto, CA), antiendothelial cells (mEC-13.324; reference 9), anti-IL-1α (Genzyme Corp., Cambridge, MA), anti–TNF-α (Immuno Kontakt, Frankfurt, Germany), anti–IFN-γ (10), anti–IL-6 (Pharmingen), and anti–INOS (Transduction Laboratories, Lexington, KY) antibodies. After washing, the cryostat sections were overlaid with biotinylated goat anti-rat, anti–hamster, and anti–rabbit or horse anti–goat IgG (Vector Labs, Burlingame, CA) for 30 min. Unbound IgG was removed by washing and the slides were incubated with avidin–biotin complex (ABC)/alkaline phosphatase (AP) (Dako, Glostrup, Denmark). Quantitative studies of immunohistochemically stained sections were performed independently by three pathologists in a blind fashion. From mice with multiple tumors, 2 or more samples (1/tumor growth area) and 10 randomly chosen fields in each sample were evaluated for each point determination. For microvessel and cell counts, individual microvessels and cells were counted under a microscope ×400 field (>40 objective and ×10 ocular lens; 0.180 mm² per field). The expression of adhesion molecules, cytokines, and mediators was defined as absent (−) or scarcely (+/−), moderately (+), or frequently (+++) present on cryostat sections tested with the corresponding antibodies.

mRNA for Cytokine. Total RNA was prepared from carcinoma masses of mice treated or not with rIL-12 by using Ultraspec (Biotech Lab. Inc., Houston, TX). 2 µg of RNA were reverse transcribed with Moloney murine leukemia virus reverse transcriptase (200 U) in 50 µl of reaction mixture with oligo dT and dNTP (GIBCO BRL, Paisley, UK). The cDNA were tested for the presence of murine glucose 3-phosphate dehydrogenase, IL-1α, IL-6, IFN-γ, TNF-α, GM-CSF, monokine induced by IFN-γ (MIG), and IFN-γ-inducible protein (IP-10) sequences in PCR reactions (Gene Amp Kit; Perkin Elmer Cetus, Norwalk, CT) performed in 50 µl volumes, by using specific primer pairs prepared by us (IP-10 and MIG) or from Clontech (Palo Alto, CA). The results are arbitrarily scored from − to +++ based on the intensity of UV fluorescence of the ethidium bromide-stained gels as independently evaluated by two operators in a blind fashion.

Figure 1. Progression of HER-2/neu neocarcinogenesis in untreated BALB–NeuT and FVB–NeuN female mice. (A) Successive alterations of the mammary gland in untreated BALB–NeuT mice and indications of the time of 5–d courses of rIL-12 administration (●, 50 ng/day; ■, 100 ng/day). Histology shows that ductular atypical hyperplasia (a) is already evident and widely distributed in all mammary glands at wk 3 of age. This hyperplasia progresses to carcinoma in situ (b) between wk 13 and 17, and then to an invading lobular carcinoma (c). (B) Successive alterations of the mammary gland in FVB–NeuN mice during the 61-wk follow-up. Squares (●, ■, as in A). Histology shows that the mammary glands are normal (a) until at least wk 29 of age. Thereafter the neoplastic process begins in one mammary gland with focal hyperplasia and carcinoma in situ (b) and progresses by giving rise to an invasive lobular carcinoma (c) between wk 39 and 48. Original magnification: ×630.
A

BALB-NeuT female mice

WEEKS OF AGE

0 5 10 15 20 25 30 33

atypical mammary hyperplasia
cancer in situ one palpable mass ten palpable masses

B

FVB-NeuN female mice

WEEKS OF AGE

0 20 30 40 50 61

no hyperplasia one palpable mass
cancer in situ multiple masses (median 2.5 tumor/mouse)
Results

At week 33, lobular carcinomas were palpable in all 10 mammary glands of BALB-N euT females carrying the activated HER-2/neu oncogene (Fig. 1 A). FVB-N euN females carrying the HER-2/neu protooncogene display one or more mammary glands with tumor at 49 wk, and the mean number affected at 61 wk is still very low (Fig. 1 B). This rapid onset and total gland involvement suggests that expression of activated HER-2/neu in BALB-N euT mice requires few, if any, additional genetic events to transform the mammary epithelial cell (7), whereas the delayed onset and asynchronous progression observed in the other line requires few, if any, additional genetic events to transform the mammary epithelial cell (7), whereas the delayed onset and asynchronous progression observed in the other line indicates that overexpression of HER-2/neu protooncogene results in stochastic tumor development.

These kinetic patterns provide two distinct models of HER-2/neu mammary oncogenesis with which to test the effect of prolonged administration of low doses of rIL-12. Since histologic examination revealed that widespread atypical hyperplasia was already evident in the mammary glands of 3-wk-old BALB-N euT mice, and HER-2/neu mammary oncogenesis quickly progressed to overt carcinoma, these mice first received a 5-d course of 50 ng of rIL-12 plus MSA, injected intraperitoneally at 2 wk of age. The dose was then increased to 100 ng, and 5-d courses followed by 3 wk off were run from wk 5 to 29 (Fig. 1 A). MSA control mice received similar courses of MSA only. Mice were inspected weekly. Those displaying progressively growing masses in all 10 mammary glands and those that reached 33 wk of age were killed and morphologically examined.

A significant delay in the onset of the first mammary tumor was evident in the treated mice as compared with the MSA controls. Moreover, at wk 33 the number of mammary glands with a palpable tumor was much lower (Fig. 2, top). Histologic examination of the mammary glands at progressive time points in a fashion blind to the treatment revealed a general delay in the progression of the HER-2/neu oncogenesis in the treated mice. Atypical hyperplasia was less vigorous and less widely distributed than in the MSA controls, and no carcinoma in situ was found before wk 15 (data not shown). This delay in tumor progression was associated with deficient neovascularization and a marked increase in infiltrating CD8+ and to a lesser extent CD4+, lymphocytes (Table 1). Lymphocyte infiltration was concomitant with enhanced vascular cell adhesion molecule (VCAM)-1 expression (Fig. 3), as well as expression of mRNA for proinflammatory cytokines and their production (Table 1). Extensive expression of iNOS on tumors from treated mice was shown immunohistochemically (Fig. 3). This suggests that IL-12-primed NO exerted a cytotoxic and antitumor activity directly or through its reactive products (11, 12). Moreover, strong expression of IP-10 and MIG mRNA was revealed by reverse transcriptase PCR in the tumor area from treated mice (Table 1). These two chemokines mediate the angiogenic activity of IL-12, and, in combination, chemoattract activated T cells (13–15). Their modulation is probably a consequence of IL-12-induced expression of IFN-γ and TNF-α in NK and T cells. IFN-γ and TNF-α also amplify the production of proinflammatory cytokines, angiogenic inhibitors, and NO by reactive cells (15). This multifactorial scenario offers an explanation for both the inhibition of tumor neoangiogenesis and vascular damage leading to the impressive ischemic and hemorrhagic necrosis of advanced carcinomas in rIL-12-treated BALB-N euT mice (Fig. 4).

Our previous studies have shown that CD8+ lymphocytes play a pivotal role in the antitumor reaction elicited by IL-12, whereas CD4+ cells play an inhibitory role (8, 16, 17). To evaluate the weight of CD8+ cells in rIL-12-delayed oncogenesis, BALB-N euT mice treated with rIL-12 or MSA only were thymectomized at 4 wk and received
200 μg of anti-CD8 mAb intraperitoneally. Both the progression of mammary oncogenesis and the protective effect of IL-12 were unaffected by this permanent CD8+ depletion (Fig. 2, middle).

The protective effect of rIL-12 was also evident in FVB–NeuN mice. Here, because of the slower tumor progression and the virtual absence of atypical mammary hyperplasia foci before wk 29, the initial 5-d course was administered to 21-wk-old mice (Fig. 1 B), followed by the same schedule as for the BALB–NeuT mice until wk 57. Mice displaying a tumor mass >2 cm mean diameter and those that reached the wk 61 of age were killed: 50% of the treated mice were tumor-free, whereas all of the MSA controls displayed palpable tumors. The mean number of mammary glands with a palpable tumor was 2.6 per mouse in the MSA controls compared with only 0.9 in the IL-12–treated mice (Fig. 2, bottom). Deficient peri- and intratumoral vascularization, enhanced expression of adhesion molecules, enhanced CD8+ infiltration, cytokine production, and iNOS activation (Table 1 and Fig. 5), and large areas of hemorrhagic necrosis in established tumors (Fig. 4) were evident, as in the IL-12–treated BALB–NeuT mice.

Increased spleen and liver extramedullary hematopoiesis and a slight exacerbation of hepatic perportal mononuclear

Table 1. Effects of rIL-12 Treatment on Neovascularization, the Expression of Adhesion Molecules, the Secretion of Mediators, and the Infiltration by Reactive Cells in 7-mm Mean Diameter Tumors Growing in BALB–NeuT and FVB–NeuN Female Mice

| Pathologic findings (immunohistochemistry) | MSA | MSA + IL-12 | MSA | MSA + IL-12 |
|-------------------------------------------|-----|-------------|-----|-------------|
| Microvessel count                          | 15.9 ± 2.1 | 9.5 ± 1.3* | 19.7 ± 3.7 | 10.5 ± 2.4* |
| Endothelial adhesion molecules             |
| ICAM-1                                     | +   | +     | +   | +     |
| VCAM-1                                     | −   | +/−   | −   | +/−   |
| ELAM-1                                     | −   | +/-   | −   | −     |
| Infiltrating cells                         |
| M acrophages                               | 12.5 ± 4.1 | 13.4 ± 3.8 | 16.1 ± 4.2 | 14.2 ± 5.2 |
| Granulocytes                               | 5.2 ± 1.9  | 7.6 ± 2.3  | 5.0 ± 2.8  | 6.9 ± 3.0  |
| CD8+ lymphocytes                           | 2.9 ± 0.8  | 42.0 ± 83* | 4.8 ± 2.1  | 14.6 ± 61* |
| CD4+ lymphocytes                           | 3.1 ± 1.7  | 15.2 ± 51* | 3.6 ± 1.9  | 5.7 ± 2.6  |
| Cytokines and mediators                    |
| IL-1β                                      | −   | +/−   | −   | +/−   |
| TNF-α                                      | +/− | +     | +/− | +     |
| IFN-γ                                      | −   | +     | −   | +     |
| IL-6                                       | +/− | +     | +/− | +     |
| iNOS                                       | −   | +     | −   | +     |
| Reverse transcriptase PCR (mRNA)           |
| Cytokines and chemokines                   |
| IL-1β                                      | ++  | +++  | NT† | NT   |
| TNF-α                                      | −   | +    | NT  | NT   |
| IFN-γ                                      | −   | +/−  | NT  | NT   |
| IL-6                                       | ++  | +++  | NT  | NT   |
| GM-CSF                                     | +/− | +    | NT  | NT   |
| MIG                                         | +   | +    | NT  | NT   |
| IP-10                                       | +   | +    | NT  | NT   |

Microvessel counts were performed on cryostat sections tested with an antiendothelial (CD31) mAb as described in Materials and Methods. The expression of adhesion molecules, cytokines, and mediators on cryostat sections was probed with the corresponding mAb. From mice with multiple tumors, two or more samples (one sample per tumor) were evaluated. At least 10 fields were counted per sample. Values are expressed as mean ± SD of five Balb–NeuT and four FVB–NeuN mice. An arbitrary score (from − to ++++ ) was used to indicate the amount of detected signal in a semi-quantitative reverse transcriptase PCR analysis, as described in Materials and Methods. At least three independent tumors from Balb–NeuT mice were evaluated, and a representative expression pattern is shown.

*Value significantly different (P < 0.001) than that in MSA-treated mice.
†NT, not tested.
infiltration were the only signs of chronic rIL-12–related lesions in both lines of mice, showing that this cytokine is effective at well-tolerated doses.

Discussion

rIL-12 administration in young BALB–NeuT mice carrying the activated Her-2/neu oncogene delays the appearance of tumor and reduces the number of mammary glands involved. In adult FVB–NeuN mice carrying the HER-2/neu protooncogene, neoplastic progression is less impetuous and 50% of IL-12–treated mice are tumor-free at 1 yr. Since pathologic examinations suggest that rIL-12 does not lead to an appreciable failure in normal mammary tissue development, the increased percentage of FVB–NeuN–protected mice may mean that progression was delayed by rIL-12 beyond the 61 wk of the experiment.

The protection offered by rIL-12 could, of course, be ascribable to its triggered killing of neoplastic cells, thus offsetting the continuous generation of new transformed cells by the transgenic mammary tissue. However, in contrast with the rIL-12–induced immune reaction against established carcinomas (8), the results obtained with thymectomy...
mized BALB-N euT mice suggest that CD8⁺ lymphocytes do not play a major role here, even if they are the domin-
ant population of reactive cells infiltrating the tumor area in rIL-12-treated mice. It is possible that NK cells are the lead-
ing actors in this IL-12–provoked delay in oncogenesis (18). Since the NK1.1 mAb recognizes an allelic form of an
NK cell antigen expressed by C57BL/6 but not BALB/c mice, the type and function of lymphocytes first involved are currently under investigation in (BALB/c × C57BL/6) N euT⁺ F1 mice.
The nonessential role of cytotoxic CD8⁺ lymphocytes in vivo fits in well with both the marginal cytotoxic activity and the lymphokine release observed when T lymphocytes from rIL-12-treated mice were restimulated in vitro with APCs pulsed with the product of the rat HER-2/neu onco-
genep(185), a self-protein in these transgenic mice (data not shown). These findings, along with morphologic data, rather suggest that rIL-12 essentially protects by halting an-
giogenesis (19) through the induction of several secondary cytokines and mediators, thus slowing down the transition from hyperplasia to overt carcinoma (20, 21). Moreover, the secondary cytokines and the tertiary chemokines and monokines elicited by IL-12 are apparently responsible for later vessel endothelial wall injury and hemorrhagic necro-
sis of established tumors (12, 13, 22–24). The endothelial vessel alteration and hemorrhagic necrosis observed in both lines of rIL-12-treated mice are reminiscent of those ob-
served during rejection of an established transplantable mammary adenocarcinoma in BALB/c mice treated with systemic rIL-12 or adenocarcinoma cells engineered to re-
lease IL-12 (8).

Whether immunological maneuvers in healthy individu-
als at risk prevent the development of cancer is a question
that has rarely been examined. Most studies have evaluated immunization before transplantation with frank tumor
cells. Immunoprevention of cancer has been attempted in a few models of chemical carcinogenesis. Interpretation of these experiments may be confounded by the fact that car-
cinogens may be immunosuppressive.

Noguchi et al. have shown that similar doses of rIL-12 in-
hibit 3-methylcholanthrene carcinogenesis in mice through the release of cytokines and mediators and nonspecific im-
mune mechanisms (25). Our findings extend these observations by underscoring the potential of nonspecific immune mechanisms and their cross-talk with the endothelial cells of tumor vessels in the inhibition of HER-2/neu oncogen-
esis.
The HER-2/neu oncogene is expressed in a substantial propor-
tion of human mammmary carcinomas and its product
seems to be a promising target for specific immune inter-
vention (26). However, its expression in humans is not the
same as the expression of a xenogenic oncogene in trans-
genic mice, and this issue may somewhat impact specific immunization rather than nonspecific immune mecha-
nisms. The close resemblance of the progression of mam-
mary carcinogenesis in HER-2/neu transgenic mice to that
in women suggests that chronic administration of nontoxic
rIL-12 regimens may be a significant prophylactic strategy. The direct proportionality between the length of carci-
ogenesis progression and the efficacy of rIL-12 suggest that a
"soft" immunologic alternative to controversial and dis-
tasteful mastectomy may be envisaged.

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