Abstract

With a slight asynchronous but consistent progression, all of the mammary glands of female BALB/c mice transgenic for the transforming rat HER-2/neu oncogene progress to atypical hyperplasia and to invasive carcinoma. Previous studies have shown that chronic administration of interleukin (IL) 12 started at the 2nd week of age hampers this progression because of its ability to inhibit tumor angiogenesis and activate a nonspecific immune response. Here we show that a similar inhibition is achieved when 7-week-old mice with fully blown atypical hyperplasia receive a weekly injection of 100 ng IL-12 for 16 times. This lower-dose and later IL-12 administration induces high and sustained levels of serum IFN-γ equivalent to those elicited by more frequent administrations. A lower-dose and less toxic treatment may thus be envisaged as a possible option in the management of preneoplastic mammary lesions.

Introduction

The effectiveness of systemic (1) and local (2) IL-12 in inhibiting mammary tumors seems to rest on its ability to trigger the inhibition of tumor angiogenesis by secondary cytokines and third-level chemokines, to activate CTL and leukocyte subsets capable of producing proinflammatory cytokines, and finally to induce polymorphonuclear cells to destroy neoformed tumor vessels (3, 4). The combination of these activities leads to the rejection of transplantable tumors (1, 4) and the inhibition of chemically induced carcinogenesis (5) and HER-2/neu oncogene-dependent mammary carcinogenesis in transgenic mice (6). Inhibition of HER-2/neu carcinogenesis is particularly effective when IL-12 treatment is begun during the very early stages of carcinogenesis and tapers off when it is started at week 13 (7). In addition to timing, the IL-12 dose is another critical issue. Inhibition was achieved with 5-day injections of 100 ng IL-12, whereas doses 10 and 50 times lower were almost ineffective (7).

Extrapolation of these previous findings to a clinical setting suggested that IL-12 treatment may be a sensible approach for healthy women with a genetic risk of cancer, though it would be very poorly effective in patients with preneoplastic lesions. Moreover, an equivalent of the total dose of IL-12 per body weight and the heavy schedule of administration would hinder the use of IL-12 in the prevention of human mammary tumors (8).

We show here that a much lower dose of IL-12 that is started when adult mice already present full-blown atypical mammary hyperplasia is as efficacious as much earlier and heavier treatments.

Materials and Methods

Mice. Inbred female BALB/c mice overexpressing the transforming rat HER-2/neu oncogene (neuT/neuT) driven by the mouse mammary tumor virus promoter (BALB-neuT) and transgene negative (neuT/neuT+; BALB/c) were bred under specific pathogen-free conditions by Charles River (Calco, Italy), screened for the presence of the transgene as previously described in detail (8), and treated in accordance with European Union and institutional guidelines. Because all of the 10 mammary glands of BALB-neuT females undergo carcinogenic transformation with a definite progression (6, 9), these were inspected weekly, and tumor masses were measured with calipers in the two perpendicular diameters. Progressively growing masses of >3 mm in mean diameter were regarded as tumors. Growth was monitored until all of the mammary glands displayed a palpable tumor or until a tumor exceeded an average diameter of 10 mm, at which time mice were killed for humane reasons. Surviving mice were killed at 33 weeks (6). Because some immunized mice do not display carcinomas in all of the mammary glands, the mean number of palpable mammary carcinomas per mouse was calculated as the cumulative number of incidents tumors/total number of mice.

IL-12 Administration. Recombinant IL-12 (Genetics Institute, Cambridge, MA) in PBS supplemented with 0.01% MSA (Sigma, St. Louis, MO) was administered i.p. Starting from the 7th week of age of BALB-neuT mice received weekly i.p. injections of 0.2 ml of PBS containing MSA only (MSA controls) or MSA plus 100 ng of IL-12, for a period of 4 weeks, followed by a 3-week rest. This course was repeated either once, twice, or three times (Fig. 1). Another group of mice remained untreated. Because no appreciable differences in tumor growth rate and pathological findings were found between the untreated mice and the MSA controls, only the data of the latter group are shown. In another set of experiments, BALB/c mice treated i.p. with IL-12 were killed at various times after treatment to test the IFN-γ titer in sera and the ability of Spc to produce IFN-γ after polyclonal activation. These mice were injected daily for 1–5 days or weekly for 1–3 weeks.

Histological and Immunohistochemical Analysis. Groups of three IL-12-treated and MSA-treated BALB-neuT mice were killed at progressive times. For histological evaluation, tissue samples were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4 μm, and stained with H&E or Giemsa and the trichrome method.

Preparation and Activation of Spc. Seven days after the beginning of treatment, Spc from BALB/c mice untreated or injected one to five times with 100 ng of IL-12 were suspended at 2 × 10^7/ml in RPMI 1640 (BioWhittaker Europe, Verviers, Belgium) with 50 μg/ml gentamicin (BioWhittaker Europe) and 10% heat-inactivated FCS (RPMI complete medium; Life Technologies) with or without 2 μg/ml ConA and used for the ELISPOT assay.

ELISPOT Assay. Ninety-six-well MultiScreen Filteration plates (Millipore S.A., Molsheim, France) were coated with capture anti-IFN-γ monoclonal antibody (R46A2; Endogen, Woburn, MA) at 5 μg/ml in PBS overnight at.
4°C. They were then blocked with PBS 5% BSA and washed with PBS. Two hundred thousand Spc or ConA-activated Spc were added to each well in 100 μl of complete RPMI. After 48 h of incubation at 37°C and 5% CO2, the plates were washed with PBS 0.05% Tween 20 (Sigma), and biotinylated detection monoclonal antibody (XMG1.2; Endogen) at 1 μg/ml in PBS 2% BSA was added and incubated overnight at 4°C. One hundred μl of a 1:6000 dilution of streptavidin-horseradish peroxidase (N-200; Endogen) in the appropriate buffer (N-500, Endogen) were then added and incubated for 1 h at room temperature. The spots were developed with the AEC101 kit (Sigma) according to the manufacturer’s instructions and counted on a computer-assisted ELISPORT image analyzer AID (AID ELISPORT Version 2.0; Autoimmun Diagnostika GmbH, Strassberg, Germany).

**IFN-γ Titration in Sera.** Serum samples from BALB/c mice injected daily for 1–5 days or weekly for 1–3 weeks with 100 ng of IL-12 were collected at the indicated time points and assayed for the presence of IFN-γ with a sandwich ELISA kit (PharMingen, San Diego, CA).

**Statistical Analysis.** Differences in tumor incidence were evaluated by the Mantel-Haenszel log-rank test; differences in the number of tumors per mouse and the number of spots in the ELISPORT test were evaluated by Student’s t test.

**Results**

**Delayed Carcinogenesis in Mice Receiving IL-12.** The aggressive mammary carcinogenesis that takes place in all of the mammary glands of BALB-neuT female mice (Fig. 1) was significantly hampered in those receiving 16 i.p. administrations of 100 ng IL-12 divided into four courses of weekly injections for 4 weeks, followed by a 3-week rest (Figs. 1 and 2, A and B). Both a delay in the onset of the first mammary tumor (Fig. 2A) and a reduction in the number of mammary glands with a palpable tumor at 33 weeks were found (Fig. 2B). All of the IL-12-treated mice were free of palpable tumors at 20 weeks, whereas 76% of the control mice were still completely tumor free (Fig. 2A). The number of tumors per mouse was also significantly lower in the IL-12-treated mice (Fig. 2B). Statistical analysis showed that the whole progression of carcinogenesis was significantly delayed ($P < 0.0001$). To assess if all of the four courses of IL-12 treatment were necessary for the effective tumor inhibition, in another set of experiments BALB-neuT mice received only the first two or three courses of IL-12. Although three courses were still effective, though to a lesser extent, two courses delayed the onset of the first mammary tumor (Fig. 2A), but all of the glands had a palpable tumor at 33 weeks (Fig. 2B).

**Pathological Findings.** A histological examination of mammary tissue of 7-week-old mice showed widespread atypical hyperplasia of small lobular ducts and lobules characterized by a proliferation of a relatively uniform population of round epithelial cells that assumed a stratified appearance and a solid, occlusive growth. Numerous enlarged capillaries were present in the loose stroma surrounding the hyperplastic foci (Fig. 3a). At week 33, when all of the 10 glands of MSA-treated mice displayed large, invasive lobular carcinomas (Fig. 3b), ~50% of those of the IL-12-treated animals showed only multiple foci of atypical hyperplasia surrounded by a dense and fibrotic stroma with an evident reactive cell infiltrate (Fig. 3, c and e). A dense stroma was also interposed among the neoplastic alveolar nodules of the invasive lobular carcinomas observed in the other 50% (Fig. 3, d and f). The neoplastic epithelial cells of these carcinomas were often necrotic and necrotic. Tumor growth thus lacked cohesion and was marked by the presence of fissions filled with necrotic and hemorrhagic material.

**IFN-γ Production by IL-12-treated Mice.** These data indicate that inhibition of carcinogenesis after weekly IL-12 injections is no less marked than previously observed with five injections per week (7). Because the ability of IL-12 to elicit high levels of IFN-γ correlates with the clinical response (9), serum IFN-γ levels after a single and multiple IL-12 injections were compared. To avoid possible immuno-suppressive activities related to the progression of mammary carcinogenesis, neuT/neuT BALB/c mice were used. Within 6 to 120 h after a single IL-12 injection, the IFN-γ titers were much higher than for the same period after the fifth IL-12 injection (Fig. 4, A and C). Moreover, although 6 h after one or two IL-12 injections high titers of IFN-γ were found, these dropped progressively by further increasing the number of IL-12 injections (Fig. 4B). However,
when mice that received one IL-12 injection were boosted 7 and 14 days later, the levels of IFN-γ decreased (Fig. 4D).

The IFN-γ production was evaluated in an ELISPOT assay by a collection of Spc 7 days after the last IL-12 injection and stimulating them with ConA (Fig. 5). A large number of spots were displayed in Spc from mice injected only once, whereas that displayed by Spc from mice injected five times differed only slightly from that of Spc from control untreated mice (Fig. 5).

Discussion

Present data show that 16 weekly injections of IL-12 significantly hamper the progression of the very aggressive mammary carcinogenesis driven by the activated rat HER-2/neu oncogene, which results in the establishment of a large, fast-growing, metastasizing lobular carcinoma in all of the 10 mammary glands of BALB-neuT mice by the 33rd week of age (6, 10). These mice, in fact, are not simply at risk but are genetically predestined to develop multiple tumors (11).
The path of this carcinogenesis leads through an initial oncogene product expression leading to hyperplasia. The second stage consists in the induction of angiogenesis. A close connection between atypical hyperplasia and the activation of angiogenesis is evident: at 7 weeks, widespread atypical hyperplasia accompanies flourishing neovascularization (10). The success of IL-12, indeed, may well be partly attributable to its commencement at the time of this close connection and hence before overt tumor formation (7). At 33 weeks, ~50% of the glands of IL-12-treated mice were tumor free and showed a fibrosis around the persisting but impoverished hyperplastic foci. This fibrosis was the consequence of the chronic and persistent inflammatory, antiangiogenic reaction induced by IL-12. The necrotic bioappearance of the neoplastic epithelial cells and the numerous foci of ischemic necrosis found in the invasive lobular carcinomas of IL-12-treated mice are equally attributable to the several activities induced by IL-12. These and previous data on HER-2/neu transgenic mice (6), along with those concerning 3-methylcholanthrene carcinogenesis (5), emphasize the significance in tumor prevention (8) of the concurrence of nonspecific immunity and antiangiogenesis elicited by IL-12.

The HER-2/neu oncogene is overexpressed in a substantial proportion of human mammary carcinomas (12). The close resemblance of the progression of mammary carcinogenesis in HER-2/neu transgenic mice to that in women (10) suggests that administration of IL-12 may be a significant prophylactic strategy. Early IL-12 administration would seem unnecessary, because the present findings show that it is still very effective if commenced when widespread atypical hyperplasia is already evident in all of the 10 mammary glands. In a human setting, it might thus be possible to start IL-12 administration when an overt preneoplastic lesion is evident and not to confine it to healthy persons with a genetic risk (13). Moreover, the total dose of IL-12 injected and the frequency of these administrations can be greatly reduced from the levels used in previous studies (6, 7) with no loss of efficacy. The present IL-12 administration schedule seems to escape the dose-dependent transient suppression of the immune response (14, 15) that accompanies chronic administration of more frequent injections (7). It also induces equivalent high, sustained serum IFN-γ levels. This is an important issue, because the ability to maintain IFN-γ induction appears to be associated with the clinical response (9). The use of IL-12 in humans is complicated by schedule- and dose-dependent toxicity (9, 16). Weekly administration of 500 ng/kg is well tolerated by melanoma patients. It is effect clinically (9) and transiently boosts tumor-specific T lymphocytes (17). Unfortunately, even with the present light schedule, the single dose effective in mice is still 8–10 times higher than the 500–700 ng/kg tolerated by humans, and lower single doses are almost ineffective (7). For other cytokines (e.g., IL-2), increased experience and refinements in patient selection and administration schedules have greatly increased the safety of antitumor regimens (18). In any event, cancer prevention is another matter, and comparison with the doses used in cancer therapy may be inappropriate. For example, in the case of estrogen receptor modulators such as tamoxifen or raloxifene, the doses used in tumor-free individuals for prevention or for adjuvant therapy of breast carcinoma are about 10 times lower than those used to treat advanced or metastatic tumors (19, 20).

References

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