ABSTRACT

Immunotherapy is an attractive strategy for cancer treatment. However, self-tolerance is one of the major mechanisms that dampen immune responses against self-tumor antigens. We have demonstrated that Her-2/neu transgenic mice (neu mice) are tolerant to neu antigens and contain only a low avidity repertoire for neu. However, this repertoire has antitumor activity. Immunizations of neu mice are capable of activating the low-avidity T cells that, at best, retard the tumor growth. To increase the efficacy of the antitumor responses in neu mice, we hypothesized that immunotherapy in combination with antiangiogenic therapy would be a more efficient strategy for tumor eradication. The rationale for using this combination was that by decreasing the growth rate of the tumor with an antiangiogenic therapy, the low-avidity repertoire of neu mice stimulated by immunotherapeutic intervention would be more effective in destroying the slow-growing tumor. To test this hypothesis, we stably expressed a soluble form of the Flt-1 vascular endothelial growth factor receptor (sFlt-1) on N202.1A cells, using a retrovirus vector. Expression of sFlt-1 on N202.1A (N202-Flt) cells significantly inhibited the tumor growth compared with N202.1A parental cells. In contrast to the application of immunotherapy alone or antiangiogenic therapy alone, which delayed the tumor growth, the combination of the two therapies provided complete inhibition of tumor growth in Her-2/neu mice. These results indicate that the use of tumor targeting with immunotherapy in simultaneous combination with antiangiogenic therapy provides a more efficient strategy for the treatment of solid tumors.

INTRODUCTION

It is believed that effective T-cell immunity is a critical component of the immune response to a growing tumor. With the identification of TAAs (1, 2), immunotherapeutic strategies designed to induce cellular immune responses have received much attention as a promising approach for the treatment of many types of cancer. However, the induction of protective antitumor immunity with the use of known TAAs is often disappointing. Considering that the majority of TAAs are in fact aberrantly or overly expressed normal gene products (3, 4), mechanisms responsible for self-tolerance have dampened immune responses against these self-tumor antigens. We have evaluated the T-cell responses against self-tumor antigens, using Her-2/neu transgenic mice (5, 6). We demonstrated that Her-2/neu mice were functionally tolerant to neu antigens and that, despite such tolerances, neu mice contain a residual low-avidity T-cell repertoire against neu with antitumor activity. Multiple rounds of immunizations with DCs pulsed with Her-2/neu antigens in conjunction with IL-2 and anti-0X40 mAb significantly enhanced the antitumor responses in these mice and led to substantial tumor reduction. These data demonstrate that it is possible to manipulate immune responses in tolerant hosts. However, the effect of the antitumor immune response is not sufficient for complete eradication of tumors.

Angiogenesis is the ability of preexisting vasculature to send out capillary sprouts leading to the formation of new vasculature (7, 8). It is now well accepted that progression of solid tumors is intrinsically dependent on angiogenesis for growth of both the primary tumor and the metastatic lesions (9). The angiogenic process is promoted by several growth factors, including the VEGF family (10), the angiopeptin family (11), and the ephrin family (12). Among these factors, VEGF maintains its position as the most critical factor for angiogenesis. Tumor cells secrete VEGF, initiating the neovascularization process (13). VEGF regulates vessel formation by signaling through Flt-1 and Flk-1 kinase receptors (14). With the identification of specific biological mediators of angiogenesis, it is now possible to develop antiangiogenic therapies in which molecules can be administered to inhibit the angiogenesis process.

It has been hypothesized that inhibition of tumor angiogenesis blocks tumor growth and decreases the potential for tumor metastases. To disrupt the effect of VEGF, anti-VEGF antibodies have been developed that are able to reduce the growth of a variety of tumors in nude animal models (15). In addition, anti-Flk-1 antibodies (16), soluble Flk-1 (17), and Flt-1 (18) have been demonstrated to inhibit tumor growth. We tested whether the expression of a soluble form of Flt-1 receptor (sFlt-1) in tumor cells would be sufficient for tumor eradication. The sFlt-1 was expressed on N202.1A cells [a cell line generated from a spontaneous tumor from neu mice (19)], using a retroviral vector. At best, inoculation of these cells on neu mice inhibits tumor growth. These results are in accordance with reports from other laboratories (20, 21) showing that antiangiogenic therapy alone is not sufficient to eliminate tumors completely. We hypothesized that the combination of immunotherapy with antiangiogenic therapy could be synergistic, providing a more effective strategy to eliminate the tumor. The rationale for this combination therapy was that decreasing the growth rate of the tumor with an antiangiogenic therapy would make the low-avidity repertoire of neu mice stimulated by immunotherapeutic intervention more effective in destroying the slow-growing tumor. There are a few reports demonstrating that the combination of immunotherapy and antiangiogenic therapy induces stronger antitumor responses (22, 23), validating our rationale for using this combination. However, in these studies we show for the first time that although neu mice are tolerant to the tumor antigen, the combination of immunotherapy and antiangiogenic complemented each other and was far superior than each monotherapy alone, producing a dramatic inhibition of tumor growth.

MATERIALS AND METHODS

Mice and Cell Lines. The Her-2/neu transgenic mice (line 202) were obtained commercially from The Jackson Laboratory and maintained homozygously. The N202.1A mammary cell line derived from a tumor from the Her-2/neu transgenic mice was obtained from Dr. P. L. Lollini (University of Bologna, Bologna, Italy). These cells express H2D9/H-2L3 and high levels of Her-2/neu molecules; they form tumors in neu mice but not in conventional...
FVB mice. The N202 cells form tumors that are histologically similar to the spontaneous tumors.

The N202-H2B-GFP cell line was generated by infecting the N202.1A cell line with the H2B-GFP-LXRN vector as described previously (24). The N202-Flt cell line was derived by expressing the murine Flt-1 gene in the N202.1A cells. The Flt-1 gene was amplified by reverse transcription-PCR from RNA prepared from kidneys. The amplified product was inserted into the TOPO vector (Invitrogen), and the sequence was verified and then inserted in the LXRN expression vector. The resultant vector was used to produce retroviral supernatant to transduce the N202.1A cell line. Expression of the Flt-1 gene on N202.1A cells was confirmed by reverse transcription-PCR. All cell lines were maintained in complete RPMI medium (RPMI 1640) supplemented with 10% FCS, 2 mM glutamine, 5 × 10⁻² 2-mercaptoethanol, and 50 μg/ml gentamicin. All infected cells were selected in the presence of 1 mg/ml G418. The embryonic 3T3-FVB fibroblast cell line was generated in our laboratory in the same manner as other embryonic 3T3 fibroblast cell lines that had developed previously (25). Recombinant human IL-2 was obtained from the Biological Resource Branch, National Cancer Institute (Bethesda, MD). Anti-OX40 (OX86) mAb was obtained from the European Cell Culture Collection (Wiltshire, United Kingdom).

**Cell Proliferation.** To evaluate whether expression of H2B-GFP or sflt-1 affected the growth of the N202.1A cells in vitro, we compared proliferation among N202.1A, N202-H2B-GFP, and N202-Flt cells. We plated 1 × 10⁴ N202.1A, N202-Flt, and N202-H2B-GFP cells in 96-well plates in set of triplicates and assessed cell proliferation at 24, 48, and 72 h post-plating by (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (26).

**Generation of DCs and Induction of Apoptosis of Tumor Cells.** DCs were derived from bone marrow as described by Inaba et al. (27). Briefly, bone marrow cells were depleted of lymphocytes by use of magnetic beads conjugated with antibodies against CD4, CD8, B220, and class II molecules. The remaining cells were cultured in complete RPMI medium containing 3% GM-CSF (supernatant from J558L cells transfected with murine GM-CSF gene; obtained from Dr. R. Steinman, Rockefeller University, New York, New York). Medium was changed every second day; each time fresh complete RPMI medium containing 3% GM-CSF was added. On day 8, DCs were collected and tested for cell surface expression of class I and II, B7-1-2, and 5896, SE. (8)

**Analysis of Tumor Growth Inhibition by Intravitral Microscopy.** Dorsal skin chambers in the mouse were prepared as described previously (25). Female Her-2/neu mice (25–30 g body weight; around 10–12 weeks of age) were anesthetized (7.3 mg ketamine hydrochloride and 2.3 mg xylazine/100 g body weight; i.p.) and placed on a heating pad. Two symmetrical titanium frames were implanted into a dorsal skinfold so as to sandwich the extended double layer of skin. A 15-mm full-thickness layer was excised. The underlying muscle (M. cutaneous max) and s.c. tissues were covered with a glass coverslip incorporated in one of the frames. After a recovery period of 3–4 days, tumor spheroids were carefully placed in the chamber. For spheroid formation, N202-H2B-GFP or a mixture of N202-H2B-GFP and N202-Flt cells at 1:1 ratio were plated at 5 × 10⁴ cells/well in 100 μl/well in 96-well round-bottomed plates coated with 50 μl of 1% agarose. The spheroids were allowed to compact for 48 h and then were washed in serum-free medium for implantation into the chambers. Vaccination with pulsed DCs was performed as described above.

Fluorescence microscopy was performed using a Mikron Instrument Microscope (Mikron Instrument, San Diego, CA) equipped with an epilluminaator and video-triggered stroboscopic illumination from a xenon arc (MV-7600; EG&G, Salem, MA). A silicon-intensified camera (ST16; Dage-MTI, Michigan City, IN) was attached to the microscope. A Hamamatsu image processor (Argus 20) with firmware version 2.5 (Hamamatsu Photon System, USA) was used for image enhancement and for capturing images to a computer. A Leitz PL100 objective was used to obtain an overview of the chamber and to determine tumor size. A Zeiss long-distance objective 100/22 was used to capture images for calculation of vascular parameters. The tumor spheroid was analyzed off-line from the captured images with use of photodensitometric computer software to calculate tumor area and relative photometric density of the tumor (Image-Pro Plus; Media Cybernetics, Silver Spring, MD). For each spheroid, video recordings were used to calculate vascular density of the neovascularature being induced by the implanted spheroids (Image-Pro Plus.).

**In Vivo Tumor Model.** To evaluate the antitumor effect of N202-Flt or the combination of immunotherapy and antiangiogenic therapy in vivo, nude mice were implanted with the transplantable N202-Flt cell line. On day 0, animals received s.c. implants of 10⁵ tumor cells. Tumors were allowed to grow for 7 days before treatment was initiated. On day 7 after tumor inoculation, animals were randomly divided into groups of five and vaccinated three times s.c. with DCs pulsed with apoptotic N202.1A cell (with intervals of 10 days between each vaccination) in the presence of rIL-2 and anti-OX40 mAb. The rIL-2 was injected daily for 3 weeks (10⁴ IU/day), and anti-OX40 mAb was injected once a week for 3 weeks (100 μg/injection). Tumor growth was monitored every 5 days, and growth rates were determined by caliper measurements in two diameters. Tumor volume was expressed as: (minor diameter)² × major diameter/2. Statistical analysis was performed with Student’s t test. In each experiment at least five animals were included per group.

**RESULTS**

**In Vitro and in Vivo Analysis of Cell Growth of Transduced N202.1A Cells.** To determine whether the expression of sflt-1 or H2B-GFP affected the growth of stably transduced N202.1A cells, we first analyzed the cell growth in vitro. These cell clones are microscopically indistinguishable from one and another and, as shown in Fig. 1, N202-Flt and N202-H2B-GFP had similar growth rates compared with N202.1A parental cells. To examine the effect of sflt-1 expression in vivo, we used a dorsal skinfold chamber tumor model and a s.c. tumor model. The dorsal skinfold chamber tumor model allowed us to (a) micro implant tumors (5 × 10⁵ cells) and study tumor growth in real time, making it possible to do a kinetic analysis; (b) evaluate, in a short period of time, the effect of therapeutic intervention by measuring the tumor size; and (c) observe the angiogenic process. The s.c. tumor model allowed us to test therapies with a larger tumor burden, implanting 10⁶ cells.
To examine the effect of sFlt-1 expressed on N202.1A cells at the level of angiogenesis and tumor growth, we implanted N202-Flt tumor spheroids in dorsal skinfold chambers in neu mice. Because the N202-Flt cells are not fluorescently labeled, to visualize the tumor in the chamber, we mixed N202-H2B-GFP and N202-Flt cells at a 1:1 ratio to form the spheroids. As illustrated by the photomicrographs in Fig. 2A, tumor spheroids that secreted the sFlt-1 (mixture of N202-H2B-GFP and N202-Flt cells) grew more slowly than the tumor spheroids composed of the N202-H2B-GFP cells. Measurements of the tumor growth (Fig. 2B) indicated that there was a significant delay in tumor growth in animals implanted with the mixture of N202-H2B-GFP and N202-Flt cells compared with animals implanted with only the N202-H2B-GFP cells. In addition, vascular density measurements (Fig. 2C) demonstrated that the sFlt-1 secreted by the tumor substantially inhibited the neovascularization of the tumor. Although new vasculature was generated in the presence of the N202-Flt cells, the angiogenic activity was significantly lower compared with N202-H2B-GFP tumors. We confirmed the antiangiogenic activity of the N202-H2B-GFP cells.
sFlt-1 secreted by the transduced N202.1A cells by use of a recombinant sFlt-1 protein prepared in a baculovirus expression vector. This recombinant Flt-1 protein was able to inhibit the neovascularization of the tumor and delayed tumor growth in the same manner that tumor cells secreting the Flt-1 did (data not shown). In addition, our data indicated that sFlt-1 secreted from N202.1A or the recombinant sFlt-1 did not affect the existing vasculature, and no overt effects such as hemorrhage or disruption of the already formed vasculature in the chamber were observed. These data indicate that the sFlt-1 secreted by the N202-Flt cells is bioactive with antiangiogenic activity inhibiting the neovascularization process of the tumor.

In a second series of experiments, we performed a dose titration by mixing N202.1A and N202-Flt cells at different ratios. We implanted $10^6$ N202.1A or N202-Flt cells or a mixture of N202.1A and N202-Flt cells at 1:1 and 9:1 ratios, respectively. Tumor volume was measured every 5 days by caliper in two dimensions. Data are means of five animals per group ± SE (bars). Data are representative of one of two experiments. A significant ($P < 0.001$, Student’s $t$ test) difference was found between the mice that received N202.1A cells and mice that received N202-Flt cells.

DISCUSSION

Antiangiogenic therapy is a promising approach to controlling tumor growth by inhibiting the formation of tumor vasculature. VEGF regulates vessel formation by signaling through Flt-1 and Flk-1 receptors (20, 21). In these studies we established a tumor model using Her-2/neu transgenic mice in which the syngeneic N202.1A tumor cell line stably expressed the soluble form of Flt-1 (sFlt-1). It has been demonstrated that sFlt-1 binds VEGF with high affinity and selectively sequesters VEGF, impeding tumor growth (7). Our data showed that animals inoculated with N202.1A cells expressing sFlt-1 had significantly slower tumor growth and prolonged survival times compared with animals that had received implanted parental cells. With the use of intravitral microscopy, we demonstrated that animals inoculated with N202-Flt cells displayed substantially lower tumor neovascularization compared with animals with implanted parental cells, indicating that the sFlt-1 secreted by the tumor cells was the factor responsible for the inhibition of tumor vasculization. It is important to note that the presence of sFlt-1 secreted by the tumor cells did not have any adverse effects on the existing vasculature. Experiments performed with recombinant sFlt-1 also showed the capability to inhibit the tumor neovascularization, and we did not

![Graph showing tumor volume over time](https://example.com/graph.png)
observe any adverse effects on the existing vasculature. Therefore, the use of sFlt-1 shows potential as a therapy to control tumor growth. In this regard, Kuo et al. (23) demonstrated that adenoviruses expressing different angiostatic molecules, such as angiostatin, endostatin, Flk-1, Flt-1, and neuropilin, could inhibit 50–70% of preexisting tumor growth in vivo. Interestingly, adenovirus expressing Flt-1 had the strongest antitumor effect.

We showed that higher levels of sFlt-1 in the tumor microenvironment contributed to stronger tumor growth inhibition. Although the inoculation of N202-Flt cells prolonged the survival times of the animals compared with control animals, these animals eventually developed tumors with a slow growth rate. It is possible that the levels of sFlt-1 produced by the tumor cells were not sufficient to completely sequester all of the VEGF produced. In addition, we should not discard the possibility that other angiogenic factors were secreted that might contribute to the neovascularization of the tumor. Our data and data from others (28–30) suggest that antiangiogenic therapy is capable of retarding tumor growth; however, it is not sufficient to completely eliminate the tumor.

Previously we have shown that neu mice were tolerant to neu antigens. However, these animals have a residual low-avidity repertoire for neu antigens with antitumor activity.4 We demonstrated that to induce an antitumor immune response in neu mice it was necessary to apply multiple vaccinations with DCs pulsed with apoptotic N202.1A cells or DCs pulsed with apoptotic 3T3 FVB cells. Animals that received rIL-2 (10^4 IU/injection) received injections from day 5 to day 15. Animal that received anti-OX40 mAb (100 μg/injection) received injections on days 6 and 13. Animals were evaluated every second or third day by intravitral microscopy. Panels represent fluorescent and light images taken at the indicated times. B, percentiles of tumor growth of all animals per group. One hundred percent represents tumor size at the time that tumor measurements were initiated (day 4 after implantation). Data are means of percentile of five animals per group ± SE (bars). A significant (P < 0.001, Student’s t test) difference was found between mice receiving N202-H2B-GFP cells and mice receiving N202-H2B-GFP+N202-Flt cells + Vacc-DCs-Apop-N202.1A. Data are representative of one of two experiments.

Fig. 4. Effect of antiangiogenic therapy and immunotherapy in the chamber model. A, photomicrographs illustrating the effect of combining immunotherapy and antiangiogenesis therapy. The neu mice with surgically implanted chambers were inoculated with N202-H2B-GFP or a mixture of N202-Flt/N202-H2B-GFP spheroids (5 × 10^4 cells total) on day 0. On day 4 animals were immunized with 10^6 DCs pulsed with apoptotic N202.1A cells or DCs pulsed with apoptotic 3T3 FVB cells. Animals that received rIL-2 (10^4 IU/injection) received injections from day 5 to day 15. Animal that received anti-OX40 mAb (100 μg/injection) received injections on days 6 and 13. Animals were evaluated every second or third day by intravitral microscopy. Panels represent fluorescent and light images taken at the indicated times. B, percentiles of tumor growth of all animals per group. One hundred percent represents tumor size at the time that tumor measurements were initiated (day 4 after implantation). Data are means of percentile of five animals per group ± SE (bars). A significant (P < 0.001, Student’s t test) difference was found between mice receiving N202-H2B-GFP cells and mice receiving N202-H2B-GFP+N202-Flt cells + Vacc-DCs-Apop-N202.1A. Data are representative of one of two experiments.
the dorsal skin chamber model, tumors were completely eliminated. It is important to note that at the end of the experiment no neovascularization was observed and all of the tumor cells were eliminated. In the s.c. tumor model, with a larger tumor burden, it was possible to control the tumor growth. It is worth noting the difference in tumor size at different times among the animals. For example, animals that received the combination therapy had very slow tumor growth, with a size at different times among the animals. For example, animals that were immunized three times (5) or four times (4). Animals were immunized s.c. with 10^6 DCs pulsed with apoptotic N202.1A cells or DCs pulsed with apoptotic 3T3 FVB cells on days 7, 17, and 27. Animals that were immunized four times received an additional immunization on day 37. Animals that were immunized three times received i.p. injections of rIL-2 (10^6 IU/injection) from day 7 to day 37, and animals that were immunized four times received rIL-2 until day 47. Anti-OX40 mAb (100 μg/injection) was injected on days 9, 19, and 29 into animals immunized three times, with an additional injection on day 39 for animals that received four immunizations. Tumors were measured every 5 days with a caliper in two dimensions. Data are means of five animals per group ± SE (bars). A significant (P < 0.001, Student’s t test) difference was found between mice treated with immunotherapy or antiangiogenic therapy alone and mice treated with the combination of immunotherapy and antiangiogenic therapy.

and that they recognize and kill tumor cells offers a window of opportunity to exploit these cells for the induction of antitumor responses as described in these studies.

It is well established that tumor cells secrete VEGFs and other angiogenic factors (13). Another advantage of using this combination therapy could be that the elimination of tumors with the use of immunotherapy would reduce the angiogenic activity of the tumor, thereby making the antiangiogenic therapy more effective. Taken together, our results show that there is a cooperative effect once immunotherapy and antiangiogenic therapy are combined and that this combination is superior to each monotherapy alone and can serve as a novel strategy for the treatment of solid tumors.

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Cooperative Effect between Immunotherapy and Antiangiogenic Therapy Leads to Effective Tumor Rejection in Tolerant Her-2/neu Mice

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