Antibodies to Vascular Endothelial Growth Factor Enhance the Efficacy of Cancer Immunotherapy by Improving Endogenous Dendritic Cell Function

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ABSTRACT
Inadequate function of dendritic cells (DCs) in tumor-bearing hosts is one mechanism of tumor escape from immune system control and may compromise the efficacy of cancer immunotherapy. Vascular endothelial growth factor (VEGF), produced by most tumors, not only plays an important role in tumor angiogenesis but also can inhibit the maturation of DCs from hematopoietic progenitors. Here, we investigate a novel combination of antiangiogenic and immunotherapy based on this dual role of VEGF. Two s.c. mouse tumor models were used: D459 cells, expressing mutant human p53; and MethA sarcoma with point mutations in the endogenous murine p53 gene. Therapy with anti-mouse VEGF antibody (10 μg i.p., twice a week over 4 weeks) was initiated when tumors became palpable. Treatment of established tumors with anti-VEGF antibody alone did not affect the rate of tumor growth. However, anti-VEGF antibody significantly improved the number and function of lymph node and spleen DCs in these tumor-bearing animals. To investigate the possible effects of this antibody on the immunotherapy of established tumors, tumor-bearing mice were immunized with DCs pulsed with the corresponding mutation-specific p53 peptides, together with injections of anti-VEGF antibody. Therapy with peptide-pulsed DCs alone resulted in considerable slowing of tumor growth but only during the period of treatment, and tumor growth resumed after the end of the therapy. Combined treatment with peptide-pulsed DCs and anti-VEGF antibody resulted in a prolonged and much more pronounced antitumor effect. This effect was associated with the induction of significant anti-p53 CTL responses only in this group of mice. These data suggest that inhibition of VEGF may be a valuable adjuvant in the immunotherapy of cancer.

INTRODUCTION
The success of cancer immunotherapy depends not only on the choice of appropriate tumor antigens and methods of antigen delivery but also on the ability of the patient’s immune system to produce an effective and sustained response to immunization. Host professional antigen-presenting cells play a key role in this process by taking up vaccine antigens, processing and presenting them on class I and II MHC molecules, producing cytokines, and stimulating the production of activated antigen-specific cytotoxic effector cells. In addition, because tumor cells are inefficient stimulators of T-cell proliferation, the continuous presence of effective host antigen presentation may be needed to maintain an effective response. The most potent of these antigen-presenting cells is the tissue DC. These cells are responsible for the induction of primary immune responses and are very effective in the stimulation of secondary responses as well (1).

The function of the immune system in tumor-bearing hosts is often seriously compromised (reviewed in Refs. 2 and 3). Defective antigen presentation by DCs is one of the mechanisms of immune dysfunction in cancer and is observed both in tumor-bearing mice and in cancer patients (4–9). We and others have demonstrated previously that defective DC differentiation caused by tumor-derived factors (10–15) contributes to the observed defective DC function in tumor-bearing hosts. One of these factors was found to be a VEGF (11). VEGF is induced by hypoxia and produced constitutively by almost all tumors. It directly stimulates the growth of vascular endothelial cells and the formation of tumor neovasculature (reviewed in Ref. 16). In animal models, we have shown that VEGF infusion results in a dramatic decrease in DC production associated with hyperproduction of immature myeloid cells (17). We have shown previously that neutralizing anti-VEGF antibody is able to abrogate the negative effects of tumor cell supernatants on DC differentiation from progenitors in vitro (11). This suggests that blockade of VEGF signaling may be an approach for improving DC function and hence immune system function and the efficacy of cancer immunotherapy in tumor-bearing hosts in vivo. Here, we tested this hypothesis in two experimental tumor models using mutated p53 as a defined tumor antigen: a poorly immunogenic

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The abbreviations used are: DC, dendritic cell; DCc, DCs pulsed with control peptides; DCsp, DCs pulsed with the D459 mutant p53-specific peptide; VEGF, vascular endothelial growth factor.
Anti-VEGF Antibody in Immunotherapy of Cancer

MATERIALS AND METHODS

Animals

Female BALB/c and CBA mice, 6–8 weeks of age, were purchased from Harlan, Inc. (Indianapolis, IN) and were housed in specific pathogen-free units of the Division of Animal Care at Vanderbilt University Medical Center.

Cell Lines and Antibodies

Two tumor cell lines were used. The tumor cell line D459 was constructed by transfection of BALB/c 3T3 cells with EJ ras and a mutant human p53 expression vector. Details of this cell line were described elsewhere (10, 18). MethA sarcoma cells were obtained from Dr. L. J. Old. This is a transplantable 3-methylcholanthrene-induced sarcoma of BALB/c origin passages as an ascitic tumor (21). The following antibody-producing hybridomas were obtained from American Type Culture Collection (Rockville, MD) and used as culture supernatants: anti-CD4 (L3T4, TIB-207), anti-CD8 (Lyt-2.2, TIB-210), and anti-MHC class II (1-A	extsubscript{bb}, HB-120). Goat anti-mouse immunoglobulin was purchased from Sigma Chemical Co. (St. Louis, MO). FITC- and phycoerythrin-labeled antibodies used in flow cytometry were purchased from Pharmingen (San Diego, CA): anti-CD11c, anti-CD86 (B7–2), and anti-I-Ad. FITC- and phycoerythrin-conjugated IgG was used in control. Neutralizing goat anti-mouse VEGF antibody was purchased from R&D Systems (Minneapolis, MN). Control goat immunoglobulin was obtained from Sigma.

Cell Preparation

Cells were prepared as described earlier (4). Briefly, a single-cell lymph node suspension was prepared by inguinal, axillary, and brachial lymph nodes by pressing the tissues through wire mesh. Cells were washed and then layered onto a metrizamide (Nygaard, Oslo, Norway) gradient (14.5 g plus 100 ml of RPMI 1640) and centrifuged for 10 min at 600 × g. Cells at the interface were washed once and resuspended in complete culture medium (RPMI 1640; Life Technologies, Inc., Gaithersburg, MD with 100 IU/ml penicillin, 0.1 mg/ml streptomycin, 1 × 10\textsuperscript{-5} M 2-mercaptoethanol, and 10% FCS; HyClone, Logan, UT). DCs were identified by their distinctive morphology and by labeling with N418 antibody. Purity of DCs was >90%. For functional tests, DCs were additionally purified as described above to obtain >80% purity.

For immunization, DCs were generated from bone marrow as described earlier (10). Briefly, bone marrow cells obtained from femurs and tibias were placed in six-well plates (Falcon, Becton Dickinson) at a concentration of 5–8 × 10\textsuperscript{5} cells/ml in complete culture medium supplemented with 3 ng/ml recombinant murine granulocyte/macrophage-colony stimulating factor and 5 ng/ml recombinant murine interleukin 4 (R&D, Systems, Minneapolis, MN). After 4 days, one-half of the medium was removed after gentle swirling and replenished with an equivalent volume of fresh granulocyte/macrophage-colony stimulating factor and interleukin 4 supplemented media. Four to 5 days later, clusters of DCs were dislodged. Purity of DCs was >60% with a viability >90%.

Tumor Induction and Immunization

Two hundred thousand D459 cells or 6 × 10\textsuperscript{5} MethA sarcoma cells were injected s.c. into the shaved backs of BALB/c mice. These doses of tumor cells were selected after preliminary experiments to result in tumor formation in 100% of the mice. Palpable tumors were usually formed 7–10 days after tumor inoculation. DCs generated from the bone marrow of control healthy mice were pulsed for 2 h at 37°C with 10 μg of the appropriate peptides and then washed in PBS. DCs (1–2 × 10\textsuperscript{5}) were injected i.v. Immunizations with peptide-pulsed DCs were started when tumors reached 5 mm in diameter (usually 12–14 days after tumor inoculation).

For the D459 tumor model, T1272 mutant p53 peptide (TYSPALNKMFYQLACTPVQL) was used. This peptide contains a mutation of cysteine to tyrosine in position 135 corresponding to the mutant p53 gene expressed in D459 cells. An irrelevant p53 peptide STPPGTRFRMAIKYQKS was used as a control. Immunization of control BALB/c mice with T1272, but not control peptide, elicits specific antimitant p53 CTL responses (4, 18, 19).

For the MethA sarcoma model, two peptides have been used. The peptide 234CM (KYICNSSCM), which contains isoleucine at position 3, corresponds to the sequence that results from a point mutation in codon 234 of p53. The peptide 234CW (KYMCNSSCM) is the wild-type counterpart of 234CM. Immunization of mice with 234CM, but not 234CW, resulted in potent specific CTL and antitumor immune responses (21). All peptides were purchased from QCB, Inc. (Hopkinton, MA).

T-Cell Proliferation Assay

Allogeneic Mixed Leukocyte Reaction. DCs were obtained from lymph nodes or spleens as described above. Cells were irradiated (2500 cGy) and incubated in triplicate for 3 days with 5 × 10\textsuperscript{5} T cells obtained from allogeneic CBA mice. Different DCs:T cell ratios (from 1:50 to 1:400) were used. The
cultures were pulsed overnight with 1 μCi of \(^{3}H\)thymidine (Amersham, Arlington Heights, IL). Cells were harvested using a cell harvester (Skatron Instruments, Sterling, VA). \(^{3}H\)Thymidine uptake was counted using a liquid scintillation counter.

**Antigen-specific T-Cell Proliferation.** DCs were infected with 250 HAU/ml influenza virus (strain PR8: A/Puerto Rico/8/34; American Type Culture Collection, Rockville, MD) for 2 h in serum-free medium. Cells were washed and incubated in triplicate with 5 \(\times\) 10^5 T cells obtained from syngeneic BALB/c mice for 4 days. Different DCs:T cell ratios (from 1:10 to 1:80) have been used. The cultures were pulsed overnight with 1 μCi of \(^{3}H\)thymidine (Amersham) and counted as described above.

**Statistical Analysis**

Statistical analysis was performed with parametric and nonparametric methods using JMP statistical software (SAS Institute, Cary, NC).

**RESULTS**

To estimate the in vivo level of VEGF production, mice were inoculated s.c. with 2 \(\times\) 10^5 D459 cells or 6 \(\times\) 10^5 MethA cells. The level of VEGF in plasma was measured using ELISA (R&D Systems) when tumors reached 1.5 cm in diameter. The plasma concentrations of VEGF in five mice bearing D459 tumors ranged from 100 to 300 pg/ml and in five mice with MethA sarcomas from 150 to 600 pg/ml. These concentrations are similar to those reported for other tumor models and in patients with cancer (23–25).

Anti-VEGF antibody was injected i.p. twice per week for 4 weeks. This schedule was selected based on data reported previously with anti-VEGF antibody (26, 27). Because direct anti-tumor effects of VEGF blockade could possibly complicate the interpretation of our data and tumor regression by any means may improve DC function, we tried to select a dose of antibody that was not able to directly affect the growth of the tumors used in these studies. Three doses of the antibody were tested: 2 μg, 5 μg, and 10 μg per mouse. We tested whether anti-VEGF antibody was able to directly inhibit tumor growth at these doses. Mice were injected with D459 cells or MethA sarcoma cells, and when tumors reached 4–5 mm in diameter, treatment with anti-VEGF antibody was initiated. A control group of mice was treated with the same doses of goat IgG. In five independently performed experiments (four mice per group), anti-VEGF antibody at these doses did not measurably affect the growth of these established tumors (data not shown). The 10-μg dose was used in all subsequent experiments. Four-week treatment of MethA sarcoma-bearing mice with anti-VEGF antibody significantly reduced VEGF levels in plasma to 88.6 ± 11.4 pg/ml from the 422.7 ± 140.4 pg/ml detected in tumor-bearing mice treated with goat IgG (P < 0.01). This level was still almost 10-fold higher than the VEGF level in control, tumor-free animals (8.6 ± 4.5 pg/ml; P < 0.001).

**Anti-VEGF Antibody Improves the Number and Function of DCs in Tumor-bearing Mice.** To investigate the effect of anti-VEGF antibody on DC function, mice were inoculated with 2 \(\times\) 10^5 D459 cells. Treatment with anti-VEGF antibody was initiated when tumors reached 4–5 mm in diameter (usually 10–12 days after tumor inoculation). Mice were treated with goat IgG or goat anti-mouse VEGF antibody, i.p. twice per week for 4 weeks. After that time (when tumors had reached a size of 300–400 mm^3) mice were sacrificed, DC fractions were isolated from lymph nodes and spleen, and cells were analyzed morphologically, phenotypically, and functionally. DC fractions isolated from tumor-bearing mice expressed lower levels of MHC class II^+ and B7–2^+ cells. This was in agreement with observations published previously (4, 6, 8). To quantify the presence of typical DCs in lymph nodes and spleen, cells were labeled with CD11c and B7–2 antibodies. Double-positive cells represent a population of relatively mature DCs. The proportion of morphologically distinct and CD11c^+ B7–2^+ DCs were analyzed. Tumor-bearing mice treated with IgG had a 3-fold reduced fraction of DC in lymph nodes and an almost 5-fold reduction in the spleen compared with control animals. This decrease was much less pronounced in tumor-bearing mice treated with anti-VEGF antibody (Fig. 1A). This decrease in the proportion of DCs in tissues resulted in a dramatically reduced ability of these cells to stimulate allogeneic T cells (Fig. 1B) and antigen-specific primary T-cell response (Fig. 1C). Treatment of mice with anti-VEGF antibodies significantly improved these functions (Fig. 1, B and C). Although the level of T-cell stimulation in these mice was still lower than in control animals, those differences were not statistically significant.

**Anti-VEGF Antibody Improves the Efficacy of Cancer Immunotherapy.** We then asked whether improved DC function resulted in more effective immunotherapy. We tested the effect of anti-VEGF antibody on the effectiveness of immunotherapy of the poorly immunogeneic D459 tumor. Tumor cells (2 \(\times\) 10^5) were injected s.c. into the shaved back of mice. When tumors reached 4–5 mm in diameter, mice were split into four groups. Each group included 10 mice (two independent experiments with five mice per group). A control group was treated with DCc and treated with goat IgG. The second group had mice treated with DCc and anti-VEGF antibody. The third group was immunized with T1272 (DCsp), along with goat IgG. The fourth group was treated with DCsp and anti-VEGF antibody. DCs (1–2 \(\times\) 10^5 cells) were injected i.v. on days 0, 4, and 7 after the start of the treatment. Anti-VEGF antibody or goat IgG (10 μg/mouse) was injected i.p. twice per week for 4 weeks. Tumor growth was significantly (P < 0.05) slower in mice immunized with the specific peptide (DCsp) during the first 2 weeks of the treatment (Fig. 2A). However, in mice treated with DCsp and control IgG, it resumed quickly after that. These results are consistent with previously reported data that the effects of immunotherapy of poorly immunogeneic tumors is often transient (10, 19, 28). Combined treatment of DCsp and anti-VEGF
antibody resulted in a more pronounced and much longer lasting decrease in tumor growth rate. This effect persisted during the entire period of observation (32 days; Fig. 2A).

We asked whether this effect was associated with an improved p53-specific CTL response. Spleen cells obtained from mice on day 28 of the treatment were restimulated with the mutant p53-specific T1272 peptide, and CTL activity was measured against unpulsed or T1272 peptide-pulsed P815 target cells. CTL activity was measured in four mice/group. The level of nonspecific killing (nonpulsed targets) was subtracted in each case. As expected, mice from the control group (DCc+IgG) did not demonstrate specific CTL activity (Fig. 2B). Very little CTL activity was found in immunized mice treated with control IgG (DCsp+IgG). However, three of four mice in the group immunized with the specific peptide and treated with anti-VEGF antibody (DCsp+aC-VEGF) demonstrated significant levels of CTL activity (Fig. 2B).

We then studied the effect of anti-VEGF antibodies in another tumor model (the relatively immunogenic MethA sarcoma). We tested the effect of a large number of tumor cells ($6 \times 10^5$). At this dose, tumor was developed in 100% of mice without any episodes of spontaneous tumor regression. The treatment was initiated when tumors reached $4–5$ mm in diameter (within $6–8$ days after tumor inoculation). Mice were divided into four groups. The treatment was essentially the same as for the D459 tumor model, except that the MethA mutant 234CM p53 peptide was used in DCsp group, and the corresponding wild-type 234CW p53 peptide was used in the DCc group. Tumor growth was almost blocked in mice immunized with the specific peptide (DCsp) during the first 2 weeks of treatment. However, 10 days after the end of therapy, tumor growth resumed (Fig. 3A). Tumor growth in mice receiving combined therapy with anti-VEGF antibody was significantly slower than in the DCsp+IgG group, and survival was significantly prolonged ($P < 0.05$). The experiments described previously were repeated with $2.5 \times 10^5$ MethA sarcoma cells. Tumor growth in mice immunized with specific peptide was significantly slower than in DCc-immunized animals. By day 22, the average tumor size in the group treated with DC CM234+IgG was only one-third of that in groups treated with DCc+IgG or anti-VEGF antibody alone (Fig. 3B). The addition of anti-VEGF antibody dramatically improved the effectiveness of this immunotherapy. In three of five mice, the tumor completely regressed, and tumor growth in the fourth mouse was considerably slowed. Only one mouse from this group exhibited a tumor similar in size to those in the DC CM234+IgG group (Fig. 3B).

Fig. 1 Effects of anti-VEGF antibody on the presence and function of DCs in tumor-bearing mice. A, effects of anti-VEGF antibody on the presence of DCs in lymph nodes and spleens. Lymph nodes and spleens were obtained from control tumor-free mice (Control), D459 tumor-bearing mice after 4 weeks of treatment with either goat IgG (IgG), or anti-VEGF antibody (Anti-VEGF). The presence of DCs was determined based on morphological criteria and the surface expression of CD11c and B7–2 markers. The cumulative results of three experiments are shown. Ps demonstrate differences from control (tumor-free) group. B, effects of anti-VEGF antibody on the ability of DCs from tumor-bearing mice to stimulate allogeneic T-cell proliferation. Purified DCs were obtained from lymph nodes or spleens as described in “Materials and Methods.” Cells were irradiated (2500 cGy) and incubated in triplicates for 3 days with $5 \times 10^4$ T cells obtained from allogeneic CBA mice. The cultures were pulsed overnight with $1 \mu$Ci of $[^{3}H]$thymidine and harvested using a cell harvester. $[^{3}H]$Thymidine uptake was counted using a liquid scintillation counter. Typical results of one of three performed experiments are shown. C, effects of anti-VEGF antibody on the ability of DCs to stimulate antigen-specific T-cell proliferation. DCs were infected with influenza virus for 2 hi serum-free medium. Cells were washed and incubated in triplicate with $5 \times 10^4$ T cells obtained from syngeneic BALB/c mice for 4 days. Data from a DC:T cell ratio of 1:20 is shown. The cultures were pulsed overnight with $1 \mu$Ci of $[^{3}H]$thymidine (Amersham) and counted as described above. Typical results of one of three experiments are shown. *, statistically significant differences ($P < 0.05$) from control. A–C: bars, SE.
DISCUSSION

Defects in the immune system in tumor-bearing hosts contribute greatly to immune nonresponsiveness to tumors. Defective function of professional antigen-presenting cells, DCs, is one of the factors responsible for this immune nonresponsiveness. We and others have previously demonstrated that tumor-derived factors may affect the normal process of DC differentiation from progenitor cells, resulting in the production of immature cells that are not able to effectively stimulate antitumor immune responses (10–15). VEGF is one of the factors implicated in these defects (11, 17, 29). In vitro data suggested that blockade of VEGF receptors on hematopoietic progenitors may improve the function of DCs in tumor-bearing hosts. In this study, we for the first time address the question whether these in vitro findings can be reproduced in vivo and whether blockade of VEGF effects may, in turn, result in enhanced antitumor responses to immunotherapy. To test this hypothesis, we selected two animal tumor models: the poorly immunogenic D459 tumor and the relatively immunogenic MethA sarcoma. Both of these tumors produce moderate levels of VEGF.

There are several possible approaches to blocking VEGF interaction with its receptors. One of these is via neutralizing anti-VEGF antibody. Treatment with anti-human VEGF antibody is able to significantly delay the growth of several human tumor xenografts in nude mice (30). It was especially effective against microtumors and metastases (26, 27, 31). Studying DC function in nude mice bearing human tumors is difficult, because DC function in nude mice might be abnormal (32). Therefore, we used syngeneic mouse tumors and anti-mouse VEGF neutralizing antibody. This antibody, at a dose of 10 mg/mouse i.p. twice a week over 4 weeks, decreased the VEGF level in the plasma of tumor-bearing mice almost 5-fold. However, at this dose, soluble ligand was not completely eliminated. We observed that the level of VEGF in our tumor-bearing mice was still almost 10-fold higher than in non-tumor-bearing control mice at the selected dose of antibody. At this dose, anti-VEGF antibody did not affect the growth of established tumors. We...
deliberately did not escalate the dose of the antibody and did not try to achieve therapeutic effect of antibody treatment alone. The absence of a direct effect on the tumor growth rate eliminated the possible confounding effects of other factors related to tumor size on DC function. We reasoned, however, that if the plasma level of VEGF was closely associated with DC defects, a 5-fold drop in VEGF concentration would be sufficient to see improvement in DC function and improve the effectiveness of immunotherapy.

A 5–6-week exposure to tumor resulted in a significant decrease in the fraction of DCs in spleen and lymph nodes. This was accompanied by a reduced ability of these cells to stimulate allogeneic and antigen-specific T-cell proliferation. This is in agreement with previously reported data about defective DC function in tumor-bearing hosts (4, 9). We have shown previously that a decreased proportion of mature DCs in tissues, but not decreased function of mature cells, was responsible for the defects in the total population of DCs (15). Although anti-VEGF antibodies were not able to completely abrogate this effect, they significantly reduced it. Thus, the results of these experiments confirmed the hypothesis that blocking VEGF would improve the differentiation of DCs and therefore increase their function.

In this study, the systemic effects of improved DC function alone were not associated with evident spontaneous immunity induction or direct antitumor effects of anti-VEGF antibody. The reason for this is unknown but may be explained by higher local concentrations of tumor-derived VEGF at the tumor, the site of natural antigen uptake by DC. When functional antigen-loaded DC are introduced i.v., immunity induction may be more efficient.

We asked in this study whether these observed effects of anti-VEGF antibody on endogenous DC function would result in a more effective antitumor immune response to tumor-specific immunotherapy. Mice with established poorly immunogenic D459 tumors were treated with DCs pulsed with specific p53 peptide. In our previous experiments, this treatment resulted in a significant delay of tumor growth. However, the growth delay lasted only for a short time, and tumor growth resumed 1
week after the end of the treatment (4, 19). This indicated that the host immune system was not able to sustain the induced antitumor immune response. The same results were observed in this study in mice immunized with specific peptide-pulsed DCs and control IgG. In contrast, in immunized mice treated with concurrent anti-VEGF antibody, the tumor size was smaller, and tumor growth was suppressed much longer than in immunized mice treated with control IgG. This effect was associated with sustained CTL responses only in this group of mice. Almost exactly the same effect was observed with MethA sarcoma tumors when a large number of tumor cells ($6 \times 10^5$) were used, and a significant percentage of cures were observed with a lower dose of MethA sarcoma cells.

The exact mechanism of the positive effect of anti-VEGF antibody on immune response in tumor-bearing hosts is not clear. As discussed above, we have shown that VEGF inhibits the maturation of DC hematopoietic progenitors. It is possible that VEGF directly inhibits the function of mature immune cells or those of other lineages (macrophages, DCs, or T or B cells), but there is no evidence in the scientific literature for this. It is also possible that VEGF may inhibit the function of immune cells indirectly, via soluble factors released by activated endothelial cells or macrophages. All of these mechanisms are presently under investigation.

These data strongly suggest that inhibition of VEGF signaling may not only be effective in blocking tumor angiogenesis mediated by VEGF but also can improve the function of DCs in tumor-bearing hosts and the effectiveness of specific immune therapy.

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