Enhancement of T-cell–Mediated Antitumor Response: Angiostatic Adjuvant to Immunotherapy against Cancer

Ruud P.M. Dings1, Kieng B. Vang2, Karolien Castermans4, Flavia Popescu2, Yan Zhang3, Mirjam G.A. oude Egbrink5, Matthew F. Mescher2, Michael A. Farrar2, Arjan W. Griffioen4, and Kevin H. Mayo1

Abstract

Purpose: Tumor-released proangiogenic factors suppress endothelial adhesion molecule (EAM) expression and prevent leukocyte extravasation into the tumor. This is one reason why immunotherapy has met with limited success in the clinic. We hypothesized that overcoming EAM suppression with angiogenesis inhibitors would increase leukocyte extravasation and subsequently enhance the effectiveness of cellular immunotherapy.

Experimental Design: Intravital microscopy, multiple color flow cytometry, immunohistochemistry, and various tumor mouse (normal and T-cell deficient) models were used to investigate the temporal dynamics of cellular and molecular events that occur in the tumor microenvironment during tumor progression and angiostatic intervention.

Results: We report that while EAM levels and T-cell infiltration are highly attenuated early on in tumor growth, angiostatic therapy modulates these effects. In tumor models with normal and T-cell–deficient mice, we show the active involvement of the adaptive immune system in cancer and differentiate antiangiogenic effects from antiangiogenic mediated enhancement of immunoextravasation. Our results indicate that a compromised immune response in tumors can be obviated by the use of antiangiogenic agents. Finally, with adoptive transfer studies in mice, we show that a phased combination of angiostatic therapy and T-cell transfer significantly \( P < 0.0013 \) improves tumor growth inhibition.

Conclusions: This research contributes to understand the cellular mechanism of action of angiostatic agents and the immune response within the tumor microenvironment, in particular as a consequence of the temporal dynamics of EAM levels. Moreover, our results suggest that adjuvant therapy with angiogenesis inhibitors holds promise for cellular immunotherapy in the clinic.

Clin Cancer Res; 17(10); 3134–45.

Introduction

Although the immune system can recognize and eliminate tumors (1), many cancers escape host immunity, as evidenced annually by the number of cancer-associated deaths (2). In addition, although cellular immunotherapy initially showed great promise in preclinical trials, it has yet to fulfill this promise in the clinic (3). For insight into this apparent conundrum, we investigate endothelial cell (EC) anergy as a novel mechanism by which tumors can escape immunosurveillance and subsequently impede effects from immunotherapy. EC anergy is defined as the down-regulation of EC adhesion molecules [EAM; e.g., intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule (VCAM-1), and E-selectin; ref. 4]. EAMs are crucial for leukocyte extravasation into the tumor, and their expression can be suppressed by proangiogenic factors (5–7), such as VEGF and fibroblast growth factor (FGF). Therefore, we hypothesized that inhibition of angiogenesis (using antiangiogenic agents) will overcome growth factor–induced EAM suppression and lead to normalized EAM levels. This, in turn, should promote vascular rolling, adherence, and transmigration of leukocytes into tumor tissue, making tumors more vulnerable to host immunity. More importantly, because cellular immunotherapy relies heavily on leukocyte extravasation into the tumor, we hypothesized that normal vascular EAM levels in the tumor microenvironment should increase the effectiveness of adoptive immunotherapy.
Anginex, a designer peptide 33mer, has been shown to target galectin-1 (gal-1), a novel player in angiogenesis research (8, 9). Others and we have shown that the interaction between anginex and galectin-1 inhibits tumor-activated endothelial cell proliferation via anoikis (10) and attenuates tumor angiogenesis and tumor growth (11, 12). By weakly binding to “carrier protein” plasma fibronectin (11), anginex is transported through the cardiovascular system to the tumor where the peptide strongly binds gal-1 (Kd = 90 nmol/L; ref. 8). We have previously shown that this peptide can synergistically enhance the effects of chemotherapeutics and radiotherapy in several solid tumor types (13–15). Here we show that anginex and its topomimetic 0118 (15, 16) can counteract proangiogenic factor–induced EAM suppression and promote T-cell–specific antitumor immunity. By treating B16F10 melanoma and Lewis lung carcinoma (LLC) tumor-bearing mice (CD4+/− and CD8+/− vs. wild type) with these angiogenesis inhibitors, we differentiated antangiogenic from immunoe extravasation enhancement effects. In addition, the temporal dynamic changes in EAM levels and T-cell extravasation during tumor progression and therapeutic intervention were simultaneously elucidated, and we revealed that increased immunoe extravasation accounted for up to 70% of tumor growth inhibition at early time points postinitiation of angiogenic therapy. Subsequently, adoptive immunotransfer studies showed that immunotherapy administered within this angiogenic therapy–initiated time window significantly increased tumor growth inhibition. Overall, our work strongly suggests that adjuvant therapy with angiogenesis inhibitors holds promise for immunotherapy in the clinic.

**Materials and Methods**

**Cell culture**

Human umbilical vein–derived endothelial cells (HUVEC), mouse B16F10 melanoma cells (kindly provided by Dr. Isaiah J. Fidler, Houston, TX), LLC (American Type Culture Collection), and the human epithelial ovarian carcinoma cell line MA148 (kindly provided by Prof. Dr Ramakrishnan, Minneapolis, MN) were cultured as previously described (17).

**Real-time quantitative reverse transcriptase PCR**

Total RNA isolation from cultured cells or from tumor tissue, cDNA synthesis and real-time quantitative reverse transcriptase PCR (qRT-PCR) were carried out essentially as described previously using SYBR Green PCR master mix (Eurogentech) spiked with 20 nmol/L fluorescein (BioRad; ref. 18). The expression of each target gene was normalized to the expression of the control gene β-actin. Species-specific primers can be found in Supplementary Table S2.

**Intravital microscopy**

B16F10 tumors were grown in the flank of C57BL/6 mice by s.c. injection of 1 × 10⁶ cells in 100 μL in 0.9% NaCl solution. Mice were treated (10 mg/kg/d i.p. bid) daily for 2 days with anginex (n = 7), 0118 (n = 8), or vehicle (n = 6) when tumors had a volume of about 80 mm³ and imaged the day after. To enable intravital microscopic observations of leukocytes, 10–20 μL of a Rhodamine 6G solution (1 mg/mL in 0.9% NaCl solution) was injected i.v. via tail vein, as described before (19).

**Mice and tumor mouse models**

All mice used in this study [C57BL/6J, CD8−/− null (B6.129S2-Cd8atm1Mak/J); CD4−/− null (B6.129S6-Cd4tm1Kow/J); CD8−/− CD4−/− null (B6.Cg-Foxn1nul/J); or athymic nude nu/nu] were purchased from Jackson Laboratory and allowed to acclimate to local conditions for at least 1 week. Forkhead box N-1 (Foxn1) null mice have a mutation in the nude locus that affects normal thymus development, resulting in T-cell immunodeficiency. Animals were provided water and standard chow ad libitum and were maintained on a 12-hour light/dark cycle. Experiments were approved by the University of Minnesota Research Animal Resources Ethical Committee. For tumor cell inoculation, a 100 μL solution of 2 × 10⁵ of B16F10 or 1 × 10⁵ LLC were injected s.c. in the right rear leg of the mice. For the human ovarian MA148 carcinoma studies, female athymic nude mice were inoculated s.c. with 2 × 10⁵ MA148 cells into the right flank, as described previously (17). Wild-type and null mice (sex- and age-matched littermates) were randomly inoculated and were randomized again prior to the initiation of treatment. Tumor volume was determined by measuring the diameters of tumors with calipers and calculated by the equation for volume of a spheroid: (a² × b × π)/6, were a is the width and b, the length of the tumor. When tumors reached a volume of approximately 100 mm³ (approximately 7 days for B16F10, 10 days for LLC, and 40 days for MA148), treatment was initiated by administering anginex or 0118 (10 mg/kg/d i.p. bid), as described previously (16). In most studies, angiostatic treatment was administered daily for 8 to 10 days up to the end of the study. For adoptive immunotransfer studies (Fig. 6), angiostatic treatment was administered for only 2 days, as described in the text.
Flow cytometry and fluorescence activated cell-sorting analysis—in vitro cultures

HUVECs were cultured for 3 days with or without growth factors bFGF, VEGF, and different concentrations of angiogenesis inhibitors anginex, or topomimetics 0118, 1049, and 1097, which were synthesized and purified as described previously (16). For the detection of VCAM-1 and E-selectin, 4 ng/mL TNF-α (PeproTech, Inc.) was added 6 hours before harvesting. Cells were trypsinized and fixed with 1% paraformaldehyde for 30 minutes at room temperature. The expression of ICAM-1, VCAM-1, and E-selectin was detected by anti-human ICAM-1 (MEM111; Monosan), VCAM-1 (1G11; HBT), or E-selectin (ENA-1, a kind gift from Dr. W.A. Buurman, Maastricht University, the Netherlands). Followed by incubation with biotin-conjugated rabbit anti-mouse IgG and Strep-PE (both DAKO).

In vivo studies

Tumors were harvested and were nonenzymatically disrupted by shear force to yield single-cell suspensions (20) on the days indicated. Treatment with 0118 (10 mg/kg, i.p. bid) was initiated on day 10, and from that day on, size-matched tumors were excised at time points indicated. Anti-mouse antibodies CD54-PE, CD106-FTC, CD31-PE, CD31-FTC, CD31-PE-Cy7, CD34-Pacific blue, aSMA-FTC, CD45-Pecy5.5, CD45-FTC, CD3-PE, CD8α-biotin, CD8α-APC Alexa Fluor 750, CD4-biotin, CD4-Alexa Fluor 700, CD69-biotin, streptavidin-APC, and isotype controls were purchased from eBioscience and used for either fluorescence activated cell-sorting (FACS) analysis or immunofluorescence. Intracellular Foxp3 and granzyme B staining was done, as described before (20). FITC-labeled anti-mouse antibodies (eBioscience) for macrophages/monocytes/granulocytes (CD11b), NK cells (NKG1.1), erythrocytes (Ter119), macrophages (Gr-1), and B cells (CD19) were used to create an exclusion channel. Samples were analyzed by multiparameter flow cytometry on a LSR II flow cytometer (BD Biosciences) using Flowjo software (Tree Star, Inc.; ref. 20).

T-cell adoptive immunotherapy in mice

When B16F10 tumors reached an approximate size of 75 mm³ (in either C57BL/6 or Foxn1−/− mice), 0118 (10 mg/kg/d, i.p. bid) treatment was initiated for 2 days. On day 7, a total of 2 × 10⁷ T cells (16.8% CD4, 23.5% CD8, and 56.2% double positives) were transferred i.p. per mouse. The T cells were derived from thymi and spleens of age- and sex-matched C57BL/6 wild-type littermates by MACS beads purification (Miltenyi Biotech), according to the manufacturer’s instructions. Because reports have shown that transfers of activated T cells can actually impair the anti-tumor efficacy (21), T-cell transfers in our model were executed with nonstimulated/nonactivated T cells. Because of the aggressive growth rate of B16F10, only one adoptive transfer is possible (21). Moreover, in our case, the transfer had to be preceded by 2 days of angiostatic treatment without interfering with tumor establishment.

Immunofluorescence

Immunofluorescence stainings were carried out on acetone-fixed cryosections (3 μm). Images of the sections were acquired on Olympus BX-60 microscope at 200× magnification and digitally analyzed and differentially quantified by morphometric analysis, as described earlier (16).

Statistical analysis

Tumor volume data were first transformed into a natural log scale and then those related to Supplementary Figure S3 were analyzed by ANOVA using factorial design and those related to Figures 5 and 7 and Supplementary Figure 4 were analyzed using general linear mixed models for repeated measures as described previously (16). For the latter type of analysis, the likelihood ratio test was used to determine variance–covariance structures and model components, and only the linear time trend was considered.

Raw data of tumor volume related to Figures 5 and 6 and Supplementary Figure 4 were also analyzed in their original scale by using the general linear mixed model for repeated measures, in which both linear and quadratic time trends were considered. Using the estimates of model parameters, model-based tumor growth curves for raw data were drawn and the area under the curve was calculated via integration. This integral provided estimates of percentage inhibition in tumor growth for treatment versus reference groups over the time course of the study, rather than just on a single day. Student’s t test was used where indicated to determine the validity of differences between control and treatment data sets. A P value of 0.05 or less was considered significant.

Online supplementary material

Supplementary Figure S1 shows quantifications of images from intravital fluorescence microscopy using rhodamine-labeled leukocytes in healthy tissue or in treated or untreated B16F10 tumors. Supplementary Figure S2 shows the effects of angiostatic treatment on circulating leukocytes. Supplementary Figure S3 shows tumor progression in different strains of T-cell–deficient mice. Supplementary Figure S4 shows tumor progression in different strains of T-cell–deficient mice with and without angiostatic treatment. Supplementary Figure S5 shows the reduction in tumor endothelial cells by 0118 treatment. Supplementary Table S1 shows the fluid dynamic parameters in tumor vessels of treated and nontreated mice. Supplementary Table S2 shows the murine-specific sequences of primers used in the real-time qRT-PCR.

Results

Overcoming EC anergy promotes leukocyte–vessel interactions in tumors

Here we show that the gal-1–targeted designer peptide angiex, partial peptide 6DBF7, and its calixarene-based topomimetic 0118 (12), as well as tyrosine kinase inhibitors Gleevec (imatinib) and Tarceva (erlotinib), but not Avastin, could normalize ICAM-1 expression on cultured human ECs (Fig. 1A).
On the other hand, nonangiostatic calixarene analogues of 0118 (1049 and 1097) showed no significant effect on ICAM-1 normalization in this assay. Furthermore, an increase in ICAM-1 protein was also revealed at the transcription level, as evidenced by mRNA levels for ICAM-1, VCAM-1, and E-selectin, using real-time qRT-PCR with anginex and 0118 (Fig. 1B).

To assess real-time leukocyte–endothelium interactions in vivo, we used intravital microscopy on B16F10 tumor–bearing mice. Exemplary still shots are provided

Figure 1. EAM expression and leukocyte–vessel wall interactions. A, FACS analysis of ICAM-1 protein expression on HUVECs, with or without bFGF treatment (10 ng/mL), 3 days posttreatment with 10 µmol/L of anginex, 6DBF7, 0118, 1049, and 1097 or 10 µg/mL of Gleevec or Tarceva. Results shown are mean fluorescence intensity [MFI] (SEM) of 4 independent experiments normalized to the control (*, P < 0.05 vs. no bFGF; *, P < 0.05 vs. control). B, mRNA expression of ICAM-1, VCAM-1, and E-selectin (analyzed using real-time qRT-PCR) in HUVECs treated with and without 10 µmol/L anginex or 0118. Results shown are mean (SEM) values of 4 independent experiments, as fold changes compared with no bFGF (*, P < 0.05 vs. no bFGF; *, P < 0.05 vs. control). C, images from intravital fluorescence microscopy showing rhodamine-labeled leukocytes (arrows) in microvessels (dotted lines) of B16F10 tumors from mice treated with vehicle, anginex, and 0118. D, interacting leukocytes per mm² vessel surface in B16F10 tumor vessels in angiostatically treated and untreated mice. Number of adhering per mm² (E) and rolling per minute (F) leukocytes in tumor vessels of angiostatically treated and untreated mice. Angiostatic treatment was administered for 2 days at a dose of 10 mg/kg i.p. bid and imaged on day 3. For D–F, data are presented as medians and interquartile ranges (*, P < 0.05).
in Figure 1C.1 While leukocyte–vessel wall interactions in tumors of control mice are significantly reduced (P < 0.01) compared with those in normal tissue (Supplementary Fig. S1; Supplementary Information), leukocyte–vessel wall interactions are increased by about 3-fold treatment in tumors of mice treated with angiex or 0118 (Fig. 1D), without inducing such changes in normal tissue of the ear (Supplementary Fig. S1). Quantification of the interactions allows differentiation of adhered and rolling leukocytes, both of which are increased in tumor vessels as the result of angiostatic therapy (Fig. 1E and F). These observations were not the result from leukocyte activation or mobilization (Supplementary Fig. S2), altered expression of leukocyte adhesion molecules LFA-1α, LFA-1β, VLA-4, or L-selectin on peripheral blood leukocytes (data not shown) or reduced blood flow velocity (Supplementary Table S1).

EAM levels during tumor growth

Because of the plasticity of the tumor microenvironment during cancer progression (22), we hypothesized that EAM levels, and consequently T-cell infiltration, have a dynamic temporal component. As shown by flow cytometry, ICAM-1 (Fig. 2A) and PECAM (Fig. 2C) levels were reduced during tumor growth whereas average VCAM levels (Fig. 2B) seemed unchanged.

To assess the temporal effect of antiangiogenic therapy on EAM levels, we treated a randomized subset of mice with angiostatic compound 0118 (10 mg/kg i.p. bid) starting on day 10 postinoculation when the average tumor size was 100 mm³ to prevent concerns with tumor take. Angiostatic compound 0118 (10 mg/kg i.p. bid) allows differentiation of adhered and rolling leukocytes, without inducing such changes in normal tissue of the ear. To assess the temporal effect of antiangiogenic therapy on peripheral blood leukocytes (data not shown) or reduced blood flow velocity (Supplementary Table S1).

Quantifying immune enhancement during angiostatic therapy

So far, our data show that angiostatic therapy with angiex or 0118 has a dual mechanism of action. Although its traditionally accepted mechanism of action is to reduce vessel density in tumors (Supplementary Fig. S5; refs. 15, 16), we now know that it also promotes T-cell infiltration into tumors by counteracting EC anergy. To differentiate the effects on tumor growth from an angiogenesis inhibitor and angiogenesis inhibitor–mediated immunomodulation, we used CD8+/− and CD4+/− null mice with B16F10 or LLC tumors. Because tumor growth is delayed in wild-type compared with null mice (Supplementary Fig. S3), we initiated angiostatic treatment in either cohort when tumors were about 100 mm³ (i.e., to have size-matched tumors and not to interfere with tumor take). Tumor growth curves are shown for B16F10 in CD8+/− and wild-type mice (Fig. 5A). Tumor growth curves for B16F10 in CD4+/− and wild-type mice and for LLC in CD8+/− and wild-type mice can be found in the Supplemental Information (Supplementary Fig. S4).

Statistical analysis of tumor volume data by using general linear mixed models showed that B16F10 tumor growth was significantly inhibited up to 2.5-fold in
CD8−/− mice treated with anginex (P = 0.0316) or 0118 (P = 0.0222) on the last day of treatment (Fig. 5A). Whereas in wild-type mice, tumor growth was more significantly inhibited [anginex (P = 0.0009) and 0118 (P = 0.0018)]. Comparable results were obtained for B16F10 tumors by 0118 in CD4−/− mice (P = 0.0553) compared with wild-type mice (P = 0.03; Supplementary Fig. S4) and in the LLC tumor model, in which on the last day of treatment LLC tumor growth was significantly inhibited in CD8−/− mice treated with anginex (P = 0.0367) or 0118 (P = 0.0118; Supplementary Fig. S4), as compared with the tumor growth inhibition of anginex (P < 0.0001) or 0118 (P = 0.0062) in wild-type mice (Supplementary Fig. S4).
Using the area under the curve approach (9), we calculated tumor growth inhibition over the entire time of treatment, based on the estimated tumor growth curves of raw data. Taken together, angiostatic treatment inhibited tumor growth on average by 65% (±5.4%) in wild-type mice compared with 44% (±3.6%) in null mice (P = 0.01). These results indicate that the angiostatic mediated increase in leukocyte infiltration into tumors accounts for a time-averaged tumor growth inhibitory effect of 32% (21 of 65).

Figure 3. Changes in T-cell populations during tumor growth. Multicolor FACS analysis was used to simultaneously measure changes in T-cell populations in B16F10 tumors during growth. Data are shown as points for individual mice, and lines connect the mean values for CD4+ and CD4+CD69+ cells (A) and CD8+ and CD8+CD69+ cells (B). Relative enhancement in the number of CD4+ (C), CD8+ (D), CD4+CD69+ (E), and CD8+CD69+ (F) cells in tumors treated with 0118. To correctly compare different size tumors throughout the course of the experiment, results are depicted as cells/mm³. G, exemplary FACS analysis dot plots for control and 0118-treated B16F10 tumor-derived cell suspensions on day 13, stained for CD4, CD8, and CD69.
minus this ratio times 100 yields the percentage enhancement, which is plotted versus time in Figure 5B for all tumor models investigated. The immunoextravasation effect is greatest at the onset of treatment and then declines up to the end of the study. We found essentially the same trend in each tumor model, and linear fits to these data indicate highly significant correlations with a regression coefficient $R = 0.92$ for all data with 0118 and $R = 0.90$ for all data with anginex.

**Enhancement of adoptive immunotherapy**

To show feasibility, we conducted an adoptive immunotherapy study in which we treated B16F10 tumor–bearing T-cell–deficient (Foxn1$^{-/-}$) and wild-type control mice with 0118 for only 2 days, followed by adoptive transfer of isolated T cells. Because reports have shown that transfers of effector T cells can actually impair the antitumor efficacy (21), so T-cell transfers in our model were executed with nonstimulated/nonactivated T cells. Angiostatic treatment (10 mg/kg i.p. bid) was initiated when tumors reached approximately 70 mm$^3$ and was administered only for 2 days, days 5 and 6 (in contrast to the 8 days of treatment in Fig. 6). The adoptive transfer of $2 \times 10^7$ T cells (16.8% CD4, 23.5% CD8, and 56.2% double positives) was administered on day 7.

The 2 days of angiostatic therapy alone showed no significant effect on tumor growth in both the Foxn1$^{-/-}$ and wild-type mice, whereas the adoptive transfer alone inhibited tumor growth by about 50% on the last day in Foxn1$^{-/-}$ mice (Fig. 6A). However, the greatest effect resulted from the staggered combination of both therapies, wherein tumor growth was inhibited by about 70% in Foxn1$^{-/-}$ mice ($P = 0.0015$), with significant improvement over 0118 ($P = 0.0144$) and marginally significant improvement over T cells alone ($P = 0.089$) on the last day of treatment (Fig. 6A), and by around 90% in wild-type mice ($P < 0.0001$), with significant improvement over the monotherapies ($P < 0.0001$ vs. 0118, and $P = 0.0013$ vs. T cells) on the last day of treatment (Fig. 6B).

As proof that T cells from the adoptive transfer indeed infiltrated into tumors of Foxn1$^{-/-}$ mice, we observed a significant ($P < 0.01$) increase in T cells in the combination-treated mice, compared with adoptive T-cell transfer alone (Fig. 6C and E). No CD45$^+$ CD3$^+$ cells were detected in vehicle-treated or 0118-treated mice, as these mice did not receive a T-cell transfer (Fig. 6E), and the CD45$^+$ cells present in vehicle and 0118-treated mice (Fig. 6D), likely arising from other types of leukocytes (e.g., macrophages) present in Foxn1$^{-/-}$ mice.

**Discussion**

The causal relationship between inflammation, immune response, and cancer has now been widely accepted (22). However, the precise involvement of the tumor microenvironment, in particular tumor ECs, remains largely unclear. Various types of immunosuppression and tolerance have been associated with the tumor microenvironment, which
can imped the adequate effector function of T cells, such as coinhibitory effects of cytokines on activation, proliferation, and survival of T cells (24), interference with migration of activated T cells to the tumor due to lack of tumor chemokine or T-cell chemokine receptor expression (3), or suppression of the effector functions of T cells by Tregs, TGF-β, or iNOS (inducible nitric oxide synthase; ref. 23). These immunosuppressive mechanisms occur at different stages of the T-cell response and have been suggested as possible reasons for the limited success of T-cell–based immunotherapy (3). Here, we investigate the downregulation of EAM levels as a novel mechanism of immunosuppression through which tumors can escape immunosurveillance and attenuate effects from immunotherapy.

Angiogenesis is postulated to contribute to the escape of tumors from host immunity by modulating EAMs and thereby reducing leukocyte–vessel wall interactions and subsequent infiltration (5, 6). For example, in response to angiogenic growth factors, EAMs display an acute temporal upregulation within hours (25), after which the expression levels decrease below the initial baseline around day 2 (6, 7, 25–27). The exposure to proangiogenic stimuli for an extended period of time, such as less than 24 hours in vitro or as we show here in an in vivo setting such as a tumor, results in the downregulation of EAMs and subsequent reduced leukocyte–endothelium interactions. In our tumor mouse models, we show that these dynamic changes in EAM levels are most pronounced on ICAM-1 and PECAM levels, which are decreased by about 50% during tumor growth, whereas VCAM levels appear on average to remain constant. Mechanistically, it has been shown that angiogenic growth factor–mediated suppression of ICAM-1 results from inhibition of phosphorylation and degradation of IκB, the natural inhibitor of NFκB (28), and that upregulation of ICAM-1 in tumor ECs occurs via epigenetic mechanisms, primarily at the level of histone deacetylation of tumor endothelial genes (29). Angiogenesis inhibitors such as anginex, angiostatin, and endostatin have been shown to upregulate NFκB and subsequent ICAM-1 levels, whereas Avastin (bevacizumab) failed to do so (30).

In addition, we show that tumor progression occurs more rapidly in T-cell–compromised mice, indicating that
the adaptive immune system should be important in cancer. Nevertheless, this is apparently insufficient, as the tumor still progresses and the amount of activated T cells decreases over time. This suggests that the T-cell–mediated response is hampered by tumor-induced counter measures, such as downregulation of EAMs (i.e., EC anergy). We show here that angiostatic treatment abrogates growth factor–induced suppression of EAMs and specifically facilitates host immunity by promoting T-cell adhesion to tumor ECs and subsequent infiltration into the tumor. Furthermore, we found that the number of activated effector T cells with specific antitumor activity is significantly increased on angiostatic therapy. The tumor growth inhibition differential effect of angiostatic treatment in tumor-bearing CD8 and CD4 null mice compared with wild type also argues for specific antitumor immunity.

Whereas innate immune cells have been shown to promote tumorigenesis in certain instances (31), the adaptive immune system generally exhibits antitumor effects. Most studies conclude that an increase in T lymphocytes in tumor tissue improves patient survival (32, 33), yet some studies report that substantial lymphocyte infiltration promotes tumor progression (34). This apparent contradiction may be explained by considering the state of T-cell activation and the cytolytic capacity of tumor-infiltrated lymphocytes (35). In addition, tumor-specific CD4 T cells appear to be functionally manifold because they help or hinder antitumor immune responses (23). For example, in a MMIV-PyMT model, CD4<sup>+</sup> T cells had no effect on primary mammary tumor growth or angiogenesis, but they did enhance the number of pulmonary metastasis through the innate involvement of macrophages (36). Moreover, CD4<sup>+</sup> T cells can give rise to Tregs. In this regard, Tregs are undesirable, because Tregs prevent induction of tumor-associated, antigen-specific immunity and inhibit the effector function of cytotoxic T cells and NK cells (3, 37). In addition, Tregs express ectoenzymes CD39/ENTPD1 and CD73/ecto-5’-nucleotidase, which generate pericellular immunosuppressive adenosine from extracellular nucleotides via degradation of ATP to AMP (38). This is supported by the observation that the inactivation of the A2A adenosine receptor rescues endogenous antitumor T-cell...
responses and induces successful tumor rejection (39). Therefore, the coordinated expression and cross-talk of CD39/CD73 on Tregs and the adenosine A2A receptor on activated effector T cells generates immunosuppressive loops, inhibiting optimal antitumor immune responses. Although the amount of Tregs in our tumor models is low (1%), blocking Treg function would likely further improve the antitumor effects because studies have shown that antitumor therapy can be improved by removal of this immunoregulatory host mechanism to permit a robust, persistent immune response in the tumor (40, 41).

Adoptive immunotherapy has been around for some time but has yet to deliver on its promise to the clinic. So far, this has been explained in part by the generally unaccommodating nature of the tumor microenvironment, such as an aberrant vascular bed, oxidative stress, high lactate levels, low pH, and high interstitial fluid pressure. Here, we show that antiangiogenic agents can improve certain conditions in the tumor microenvironment by upregulating EAMs and promoting leukocyte infiltration into tumor tissue. Because we found that angiostatic induced increase in leukocyte infiltration into tumors accounts for a time-averaged tumor growth inhibitory effect of about one-third, the remaining two-thirds should be attributable to the direct antiangiogenic effect. These effects occur in a time-dependent manner, with the immunoevasion effect being greater (up to 70%) at early time points following initiation of treatment and becoming less as the antiangiogenic effect increases. The timeline of this effect and its peak occurrence within several days postinitiation of angiostatic treatment essentially parallels that observed in our earlier study that showed anginex- and 0118-induced tumor vessel maturation (17). With this in mind, one might argue that angiostatic treatment, which can temporally improve overall tumor physiology, may be responsible for the increase in tumor infiltrate. Yet, the increase in the number of activated T cells argues for an active rather than for a passive effect, such as vessel normalization. In addition, no reduced blood flow velocity or blood pressure was noted on treatment (14). Also, our results cannot be explained by a direct effect on leukocytes themselves, as angiostatic therapy did not change the level of activation, nor did it change the ratio of infiltrating activated to nonactivated leukocytes. Furthermore, the number of circulating leukocytes in peripheral blood was unaffected by angiostatic treatment and the expression of leukocyte adhesion molecules was also not altered. Overall, our work strongly suggests that overcoming EC energy by adjuvant therapy with angiogenesis inhibitors holds promise for immunotherapy in the clinic.

Disclosure of Potential Conflicts of Interest

K.H. Mayo has a financial interest in a pharmaceutical company (PepTix) that holds license to commercialize agents (anginex and 0118) investigated in this article. The other authors disclosed no potential conflicts of interest.

Acknowledgments

We thank Dr. Paul Champoux for the assistance with flow cytometry and Dr. Xianghua Luo and Ms. Xiao Liu for assistance with statistical analysis.

Author Contributions

R.P.M. Dings, K. Castermans, M.G.A. oude Egbrink, M.F. Mescher, M.A. Farrar, A.W. Griffioen, and K.H. Mayo designed the research and analyzed the data. R.P.M. Dings, K.B. Yang, K. Castermans, and F. Popescu executed the research. Y. Zhang statistically analyzed the data.

Grant Support

This research was supported by research grants to K.H. Mayo from the National Cancer Institute (NIH CA-096090) and a research supplement to promote diversity in health-related research (K.B. Vang). This work was also supported in part by NIH P30 CA77598 utilizing the Bioinformatics and Bioinformatics Core, and the Flow Cytometry Core, shared resources of the Masonic Cancer Center at the University of Minnesota.

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Received September 10, 2010; revised December 16, 2010; accepted December 29, 2010; published OnlineFirst January 20, 2011.

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