

Anti-angiogenesis therapy can overcome endothelial cell anergy and promote leukocyte-endothelium interactions and infiltration in tumors

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ABSTRACT Tumor escape from immunity, as well as the failure of several anti-cancer vaccination and cellular immunotherapy approaches, is suggested to be due to the angiogenesis-mediated suppression of endothelial cell (EC) adhesion molecules involved in leukocyte-vessel wall interactions. We hypothesized that inhibition of angiogenesis would overcome this escape from immunity. We investigated this *in vivo* by means of intravital microscopy and *ex vivo* by immunohistochemistry in two mouse tumor models. Angiogenesis inhibitors anginex, endostatin, and angiostatin, and the chemotherapeutic agent paclitaxel were found to significantly stimulate leukocyte-vessel wall interactions by circumvention of EC anergy *in vivo*, i.e., by the up-regulation of endothelial adhesion molecules in tumor vessels. This was confirmed by *in vitro* studies of cultured EC at the protein and mRNA levels. The new angiostatic designer peptide anginex was most potent at overcoming EC anergy; the enhanced leukocyte-vessel interactions led to an increase in the numbers of tumor infiltrating leukocytes. While anginex inhibited tumor growth and microvessel density significantly, the amount of infiltrated leukocytes (CD45), as well as the number of CD8⁺ cytotoxic T lymphocytes, was enhanced markedly. The current results suggest that immunotherapy strategies can be improved by combination with anti-angiogenesis.—Dirkx, A. E. M., oude Egbrink, M. G. A., Castermans, K., van der Schaft, D. W. J., Thijssen, V. L. J. L., Dings, R. P. M., Kwee, L., Mayo, K. H., Wagstaff, J., Bouma-ter Steege, J. C. A., Griffioen, A. W. Anti-angiogenesis therapy can overcome endothelial cell anergy and promote leukocyte-endothelium interactions and infiltration in tumors. *FASEB J.* 20, 621–630 (2006)

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OVER THE LAST DECADES, immuno-directed anti-tumor strategies, based on adoptive or vaccination approaches, have been developed (1, 2). This approach has not been as effective as had been anticipated. Several explanations for this have been put forward. First, most vaccines have been directed toward stimulating cytotoxic T lymphocyte (CD8) responses; the continuous stimulation of these cells without T cell help (CD4) eventually leads to anergy and tumor escape. Second, regulatory host T cell responses may counteract induced immunity. Third, the antigens toward which the immunity is directed are not tumor specific enough. An alternative explanation might be that although immune effector cells are being generated *in vivo*, they are unable to migrate into tumor sites and therefore cannot exert an anti-tumor effect.

Angiogenesis, a pivotal process in the outgrowth and metastasis of tumors (3–5), has been described to be involved in the escape of tumors from immune surveillance. We and others have shown that tumor EC have a suppressed expression of adhesion molecules (EC anergy; ref 6), such as intercellular adhesion molecule-1/2 (ICAM-1/2), vascular endothelial cell adhesion molecule-1 (VCAM-1), and CD34 (7–9), due to exposure to angiogenic factors such as vascular EC growth factors (VEGFs) and fibroblast growth factors (FGFs) (6, 10, 11). We recently demonstrated in several mouse tumor models that tumors indeed inhibit leukocyte-vessel wall interactions by down-regulation of vascular adhesion molecules (12).

The role of angiogenesis in anti-tumor immunity is an emerging theme in both angiogenesis and immunology research fields. This is exemplified by recent

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studies of the major prognostic value of T lymphocyte infiltration in tumors and the relationship with expression of angiogenic factors (13), immunization against angiogenesis-related proteins (14, 15) and ECs as vaccines (16). In this study, we use several angiostatic molecules to demonstrate that angiogenesis inhibition can be a way to increase leukocyte-vessel wall interactions and subsequent infiltration. Anginex is a designed 33-mer peptide angiogenesis inhibitor that inhibits vasculature formation by induction of apoptosis in endothelial cells (17, 18). Angiostatin and endostatin are two other angiogenesis inhibitors with a direct effect on endothelial cells (19–21). TNP-470 was described in the early 1990s as an angiostatic derivative of the fungus antibiotic fumagillin (22, 23). Several chemotherapeutic agents with claimed angiostatic activity (24) such as paclitaxel and cyclophosphamide were included in these studies. It is suggested that the stimulating effect of angiogenesis inhibition on leukocyte-vessel wall interactions may be applicable for the improvement of various immunotherapy approaches.

MATERIALS AND METHODS

Cell culture

Human LSI74T colon carcinoma cells were grown in DMEM (Life Technologies, Paisley, Scotland), supplemented with 10% fetal calf serum (FCS, Bio Whittaker, Verviers, Belgium), 1% glutamine. Mouse B16F10 melanoma cells (kindly provided by dr. J. Fidler, Houston, TX, USA) were cultured using Hank's MEM containing 5% FCS, 1% non-essential amino acids, 1% sodium pyruvate, 1.5% MEM vitamins (Life Technologies), and 2% sodium bicarbonate (all from Life Technologies).

Mouse b.END5 brain endothelioma cells (obtained from ECACC, Salisbury, UK) were cultured in Dulbecco's MEM (Life Technologies) containing 10% FCS, 1% glutamine (Life Technologies), 5 $\mu\text{mol/L}$ 2-mercaptoethanol (Sigma, St. Louis, MO, USA), 1% non-essential amino acids (Life Technologies), and 1 mmol/L sodium pyruvate (Life Technologies). All cells were harvested using 0.25% trypsin (Difco Laboratories, Detroit, MI, USA)/0.02% EDTA (Merck, Darmstadt, Germany) in 0.9% NaCl solution.

Human umbilical vein endothelial cells (HUVEC) were cultured in RPMI-1640 (Life Technologies, Breda, the Netherlands) supplemented with 20% heat-inactivated human pooled serum (provided by the University Hospital Maastricht), 2 mM L-glutamine (Life Technologies), 50 ng/mL streptomycin, and 50 U/mL penicillin (ICN Biomedicals, Maidenhead, UK) in 0.2% gelatin-coated tissue culture flasks at 37°C, 5% CO₂.

Intravital microscopy

In vivo experiments were approved by the local Ethical Review Committee on Animal Experiments. Small tumors were grown in the left ear of C57BL/6 mice (25–30 g, obtained from Charles River, Maastricht, the Netherlands), as described before (12). Briefly, 10 μL cell suspension containing 10⁶ B16F10 cells was injected intradermally into the dorsal side of the ear. Within 3–5 days ear tumors grew to a diameter of ~2 mm, at which time the treatment started. Four anti-angiogenic drugs were used, i.e., the designer peptide ang-

inex (17, 25), endostatin, and angiostatin (EntreMed, Rockville, MD, USA) (26), and TNP-470 (Takeda Chemical Industries, Osaka, Japan) (27), as well as two anti-neoplastic drugs with claimed anti-angiogenic properties, i.e., paclitaxel (Taxol, Bristol-Myers Squibb B.V., Woerden, the Netherlands) and cyclophosphamide (Asta Medica, Diemen, the Netherlands).

For treatment of tumor-bearing mice with anginex ($n=6$), Alzet osmotic minipumps (Durect Corporation, Cupertino, CA, USA) were implanted on the flank on day 5 after tumor cell administration. Pumps administered 6 mg/kg/day of anginex, a dose that inhibited tumor growth in this model by ~70–80% (17). Other groups of mice were similarly treated with endostatin (2 mg/kg/day, $n=5$) and angiostatin (2 mg/kg/day, $n=5$) (19, 26). In two additional mice an osmotic minipump was implanted that administered equivalent amounts of control protein (bovine serum albumin). In these groups, intravital microscopic measurements were performed on day 12 after tumor cell inoculation. TNP-470 was administered according to a previously optimized protocol (27), by i.p. injections of 20 mg/kg (in 5% glucose/ethanol solution) on days 5, 8, 11, and 13 after tumor cell administration ($n=4$). In these mice intravital microscopy was performed on day 14. Paclitaxel (6 mg/kg in 0.9% NaCl solution, i.p.; $n=7$) and cyclophosphamide (12.5 mg/kg in sterile water, i.p.; $n=4$) were administered on days 5 and 7 after tumor cell administration. Intravital microscopy was performed on day 8, when tumor growth was already inhibited significantly (Bouma-ter Steege, unpublished results), while no toxic side effects were observed. As a control group, five tumor-bearing mice were used that were not treated with any of the anti-angiogenic or anti-neoplastic compounds, but treated similarly otherwise. To investigate the effect of the drugs in healthy blood vessels, we used mice without a tumor and treated them with anginex ($n=2$), endostatin ($n=2$), angiostatin ($n=2$), TNP-470 ($n=2$), paclitaxel ($n=4$), and cyclophosphamide ($n=2$); as control, five mice were left untreated.

On the day of the experiment, mice received 500 ng recombinant murine TNF α (R&D Systems, Abingdon, UK) i.p. 4 h before intravital microscopy. Administration of TNF α was performed to up-regulate leukocyte-vessel wall interactions; without cytokine pretreatment, adhering leukocytes are hardly present in mouse ear venules, which would preclude the assessment of a possible reduction in leukocyte adhesion (28). Mice were anesthetized by s.c. administration of a mixture of ketamine (0.1 mg/g b.w. Nimatek) and xylazine (0.02 mg/g b.w. Sedamun, Ad Usem Veterinarium, Cuyk, the Netherlands). Intravital microscopy was performed as described before (12). Briefly, body temperature was kept at 37°C by an infrared heating lamp. In all mice, venules (10–40 μm) in both ears were visualized using a Leitz intravital microscope adapted for telescopic imaging (29). To enable intravital microscopic observation of leukocytes, 10–20 μL of a Rhodamine 6G solution (Sigma; 1 mg/mL in sterile 0.9% NaCl) was injected into a tail vein. As many vessels as possible (typically 8–12) were recorded for 3–5 min each in areas within the tumor, outside the tumor (<2.5 mm from the tumor), at the edge of the ear (>5 mm from the tumor), and in the contralateral tumor-free ear. To enable quantification of systemic leukocyte counts, 20 μL blood was sampled via heart puncture at the end of every experiment. Leukocytes were counted and differentiated as polymorphonuclear (PMN) or monomorphonuclear (MMN).

Experimental parameters

Vessel diameters were measured using an in-house built image shearing device. Centerline blood flow velocity was

determined by frame-to-frame analysis, using the fastest passing fluorescent leukocyte as a marker. The level of leukocyte rolling was determined by counting the number of rolling cells passing a vessel segment per minute. Leukocytes were considered as rolling along the vessel wall when their velocity was at least an order of magnitude lower than that of the free flowing blood cells. The level of leukocyte adhesion was assessed in a 100 μm vessel segment and expressed as number of cells per endothelial surface area (assuming the cross section of the venules to be circular). Leukocytes were considered adherent when they remained stationary for at least 30 s. The total number of leukocytes interacting with the vessel wall at a particular moment was determined as well. This parameter includes not only the numbers of adhering and rolling leukocytes, but also the velocity with which the latter roll along the endothelium within a vessel segment of 100 μm length. When leukocytes roll slowly, the number of interacting leukocytes observed in such a vessel segment will be higher than when they roll faster, despite similar numbers of rolling and adhering leukocytes. This information is important, because it tells something about the adhesion molecules involved; leukocytes are known to roll very slowly on E-selectin, whereas rolling on P-selectin is much faster. In addition, slowly rolling leukocytes are the first to become adherent. To determine this parameter, we counted in a frozen video frame the total number of rolling and adhering leukocytes in a 100 μm vessel segment. At each time point this count was performed in four randomly chosen video frames and the data were averaged.

Tumor growth experiments

At day 0, 10^6 LS174T human colon carcinoma cells or 2×10^5 B16F10 melanoma cells were inoculated subcutaneously on the right flank of 6wk-old Swiss nu/nu or C57BL/6 mice, respectively. On day 5, when established tumors were present ($\sim 50 \text{ mm}^3$), an Alzet osmotic minipump (Durect) was placed on the left flank. For LS174T tumors, treatment was performed for 2 wk with 6 mg/kg/day anginex in 0.9% NaCl solution (saline). In the B16F10 tumors, treatment was performed for 11 days with a dose range of 0, 2, 6, or 12 mg/kg/day of anginex in saline). Tumor volumes were measured daily and calculated as follows: $\text{width}^2 \times \text{length} \times 0.52$. At the end of the experiments, tumors were excised and snap frozen for histochemical analysis. Statistical differences in tumor growth curves were analyzed using the 2-way ANOVA test.

Immunohistochemistry

Five μm sections were made, dried overnight, and fixed in acetone for 15 min at -20°C . After blocking of endogenous peroxidase, sections were blocked with BSA for aspecific staining (in 1%BSA/20%FCS/0.1%Tween-20 in PBS for 30 min) and stained using rat anti-mouse CD31 (PharMingen, San Diego, CA, USA) or CD8 or CD45 (both kind gifts from Dr. A Duijvestijn, Maastricht). CD31 was detected by HRP-labeled goat anti-rat Ig antibody (Chemicon, El Segundo, CA, USA), whereas CD8 and CD45 were detected by biotinylated donkey anti-rat Ig antibody (Jackson Immuno Research Laboratories, Inc., West Grove, PA, USA) and avidin-biotin-HRP complex (Dako, Carpinteria, CA, USA). Microvessel density, CD45⁺ and CD8⁺ cells were counted in four independent areas in each section (using a 0.25 mm^2 grid at a 200 \times magnification) by two independent observers. Statistical analysis was done using the Mann-Whitney U test.

FACS analysis

b.END5 cells were cultured with or without 50 ng/mL bFGF, with or without either 30 or 10 $\mu\text{g}/\text{mL}$ anginex, 500 $\mu\text{g}/\text{mL}$ endostatin, 1 mg/mL angiostatin, 100 ng/mL TNF-470, or 10 ng/mL paclitaxel. TNF α (40 ng/mL) was added 6 h before harvesting. ICAM-1 and VCAM-1 expression was determined by rat anti-mouse ICAM-1 (CD54; R&D Systems, Abingdon, Oxon, UK) or rat anti-mouse VCAM-1 (CD106; PharMingen) monoclonal antibodies, as described previously (12). Similar experiments were done with HUVECs, using 10 ng/mL bFGF. ICAM-1 was detected by MEM111 mouse anti-human ICAM-1 antibody (Monosan, Uden, the Netherlands). Mouse blood leukocytes were isolated by Ficoll density gradient centrifugation (Amersham, Sweden) and incubated with R1-2 rat anti-mouse VLA-4, Mel-14 rat anti-mouse L-selectin, M17.4 rat anti-mouse LFA1 α , or M18.2 rat anti-mouse LFA1 β (PharMingen), as described before (12).

Quantitative real-time RT-PCR (qRT-PCR)

qRT-PCR was performed with murine specific primers (30) detecting β -actin, cyclophilin, ICAM-1, VCAM-1, and E-selectin (Sigma-Genosys, Haverhill, UK) and analyzed with Sequence Detection System software (Applied Biosystems, Foster City, CA, USA). The expression of each target gene was normalized to the control genes.

Statistics

Data obtained from intravital microscopic experiments are presented as medians with interquartile ranges (i.e., the spread from the 25th to 75th percentile). Other data are presented as means with standard errors (SEM). Differences between two independent data groups were tested with the Mann-Whitney U test. Differences between paired data groups were tested with the Friedman test (>two groups), followed by a multiple comparison procedure. Correlation between variables was determined using Spearman's correlation test. In all tests the level of significance was set at 0.05.

RESULTS

Leukocyte-vessel wall interactions are reduced in tumor vessels in vivo; restoration by anti-angiogenesis treatment

It has been shown in different mouse models that proangiogenic factors reduce cytokine (TNF α) -induced leukocyte-vessel wall interactions in tumors and in control tissues (10, 12). To investigate whether angiogenesis inhibitors can revert this process in tumors, tumor-bearing mice were treated with the angiostatic peptide anginex, using a protocol (6 mg/kg/day for 7 days) that inhibits tumor growth by 70–80% (17). This treatment completely restored the total level of cytokine-induced leukocyte-vessel wall interactions inside the tumor ($P < 0.001$), as well as in vessels adjacent to the tumor ($P < 0.05$), to the level observed in healthy control mice (Fig. 1). Discrimination between leukocyte rolling and adhesion revealed that treatment with anginex significantly increased both leukocyte adhesion ($P < 0.05$) and rolling ($P < 0.01$, Fig. 1) in tumor

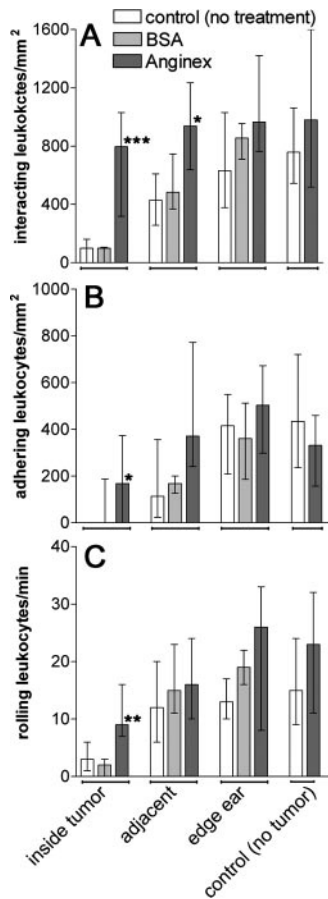


Figure 1. Leukocyte-vessel wall interactions are suppressed in tumor microvessels, normalization by treatment with anginex. C57Bl6 mice with established B16F10 tumors were treated for 7 days with anginex, bovine serum albumin (BSA) as protein control, or received no treatment at all. Leukocyte vessel wall interactions were analyzed using intravital microscopy 4 h after i.v. injection with 500 ng TNF α . Effects of treatment on the total level of interacting leukocytes (A), on leukocyte adhesion (B), and on leukocyte rolling (C). Data are presented as medians and interquartile ranges. Statistical significance was assessed in comparison to values in untreated control mice (* P <0.05, ** P <0.01, *** P <0.001).

vessels. Interactions in the contralateral ears of tumor-bearing mice were highly similar to results observed in healthy control mice (data not shown). In ear skin venules of mice without a tumor, anginex did not significantly influence the total level of leukocyte-vessel wall interactions. Administration of bovine serum albumin as a protein control had no significant effect on leukocyte-vessel wall interactions (Fig. 1). Intravital microscopic images of normal tissue, B16F10 tumor tissue and anginex-treated tumor tissue are shown in Fig. 2. Video images of these experiments can be viewed at <http://www.fdg.unimaas.nl/Angiogenesis-Lab>. These experiments were performed in a suboptimal TNF α -induced situation in which both rolling and adhering leukocytes are present, at least in the control situation. In pilot experiments, in which no TNF α was used, leukocyte-vessel wall interactions appeared to be reduced inside a tumor as well, although the difference

was smaller, and anginex up-regulated these interactions (unpublished data).

To investigate whether the effect of anginex is common among angiogenesis inhibitors, three other angiostatic drugs were tested (Fig. 2A). Treatment of mice with endostatin (2 mg/kg/day; ref 19) induced an increase in leukocyte-vessel wall interactions in tumor vessels as well (P <0.01). Similarly, treatment of mice with angiostatin (2 mg/kg/day; ref 19) increased leukocyte-vessel wall interactions in tumor vessels (P <0.05), although to a lesser extent than anginex and endostatin. The effects of both inhibitors were due to an increase in leukocyte adhesion in tumor vessels, whereas leukocyte rolling remained unchanged (Table 1). As with anginex, endostatin and angiostatin did not influence leukocyte-vessel wall interactions in the absence of a tumor (Table 1). TNP-470 did not induce an increase in the total level of leukocyte-vessel wall interactions in tumor vessels (Fig. 2A). Instead, this compound tended to decrease both leukocyte adhesion and rolling and, hence, the total level of interactions (Table 1). This effect was seen especially in tumor-free mice, where TNP-470 significantly decreased the total level of leukocyte-vessel wall interactions (P <0.001) as well as leukocyte rolling and adhesion (P <0.01; Table 1).

To test whether chemotherapeutic agents with claimed angiostatic properties (24) share the capacity to overcome tumor EC anergy with specific angiogenesis inhibitors, tumor-bearing mice were treated with either paclitaxel or cyclophosphamide. Paclitaxel increased the total level of leukocyte-vessel wall interac-

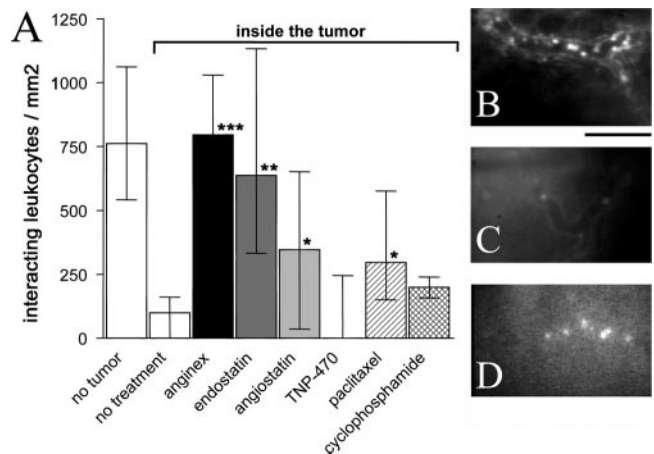


Figure 2. Suppressed leukocyte-vessel wall interactions in tumor vessels can be normalized by angiostatic and chemotherapeutic compounds. A) Normalization of the total number of interacting leukocytes in mouse tumor vessels to numbers observed in vessels of mice without a tumor. Data are presented as medians and interquartile ranges. Statistical significance was assessed in comparison to values in tumor vessels of untreated mice (* P <0.05, ** P <0.01, *** P <0.001). B–D) Typical intravital fluorescence microscopy images of a normal ear skin vessel (B), an untreated tumor vessel (C), and an anginex-treated tumor vessel (D); leukocytes are fluorescently labeled with Rhodamin 6G. The bar between panels B and C represents 45 μ m. Video images can be viewed at <http://www.fdg.unimaas.nl/AngiogenesisLab>.

TABLE 1. Effects of anti-angiogenesis or anti-neoplastic drugs on leukocyte-vessel wall interactions in ear skin venules of TNF α -treated C57BL/6 mice^a

		Ear tumor			
		Inside tumor	Adjacent to tumor	Edge ear	No tumor
Control (no treatment)	Interacting/mm ²	99 (0-161)	429 (257-610)	632 (378-1030)	758 (544-1061)
	Adhering/mm ²	0 (0-0)	114 (24-356)	415 (209-549)	434 (236-720)
	Rolling/min	3 (1-6)	12 (6-20)	13 (10-17)	15 (9-24)
Anginex	Interacting/mm ²	796*** (318-1030)	937* (638-1235)	965 (764-1421)	979 (518-1655)
	Adhering/mm ²	168* (0-374)	371 (241-772)	503 (298-672)	332 (157-459)
	Rolling/min	9** (7-16)	16 (10-24)	26 (8-33)	23 (11-32)
Endostatin	Interacting/mm ²	637*** (332-1133)	693 (354-1038)	796 (474-1384)	819 (503-1222)
	Adhering/mm ²	282** (0-700)	157 (0-321)	490 (163-621)	233* (92-479)
	Rolling/min	6 (2-12)	25 (10-47)	20 (8-37)	18 (11-23)
Angiostatin	Interacting/mm ²	346* (35-651)	758 (355-908)	1038* (902-1664)	803 (549-973)
	Adhering/mm ²	142* (0-374)	227 (138-365)	472 (101-638)	274 (99-557)
	Rolling/min	3 (1-8)	12 (5-27)	19 (13-31)	18 (15-32)
TNP-470	Interacting/mm ²	0 (0-245)	249 (109-484)	442 (265-872)	152*** (84-345)
	Adhering/mm ²	0 (0-0)	0* (0-0)	277 (141-408)	205*** (55-236)
	Rolling/min	1 (1-1)	7 (3-13)	9* (7-11)	5** (3-8)
Paclitaxel	Interacting/mm ²	296* (150-576)	332 (263-454)	438* (290-648)	571 (434-860)
	Adhering/mm ²	155* (0-297)	138 (109-297)	367 (216-463)	199*** (94-354)
	Rolling/min	8** (5-13)	30** (24-40)	25*** (21-39)	22** (12-42)
Cyclophosphamide	Interacting/mm ²	199 (158-239)	265 (166-435)	364* (315-583)	303*** (133-411)
	Adhering/mm ²	187* (0-239)	138 (0-466)	364 (233-599)	277* (133-407)
	Rolling/min	0* (0-1)	1*** (1-3)	1*** (1-4)	1*** (0-1)

^a Data are presented as median values and interquartile ranges in parentheses; * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ as compared to the control group; number of vessels per category: control 10-47, anginex 12-24, endostatin 12-24, angiostatin 14-24, TNP-470 4-10, paclitaxel 11-49, cyclophosphamide 11-23.

tions in tumor vessels significantly ($P < 0.05$), although interactions were still clearly lower than in healthy control vessels (Fig. 2A). The effect of paclitaxel appeared to be due to an increase in both leukocyte adherence and leukocyte rolling (Table 1). In tumor-free mice, paclitaxel exhibited a dual effect by inducing an increase in leukocyte rolling ($P < 0.01$) and, at the same time, decreasing leukocyte adherence ($P < 0.001$). This resulted in a nearly significant decrease ($P < 0.07$) in the total level of leukocyte-vessel wall interactions (Table 1). The increase in interactions observed inside a tumor is therefore particularly striking.

Treatment of mice with cyclophosphamide induced an increase in leukocyte-vessel wall interactions in tumor vessels (Fig. 2A), but this effect did not reach the level of significance ($P < 0.095$). This effect of cyclophosphamide resulted from an increase in leukocyte adherence ($P < 0.05$), whereas leukocyte rolling appeared to be decreased ($P < 0.05$; Table 1). In tumor-free mice, treatment with this compound clearly reduced both leukocyte rolling ($P < 0.001$) and adhesion ($P < 0.05$) and, hence, the total level of interactions ($P < 0.001$; Table 1). Therefore, as with paclitaxel, the non-significant increase in leukocyte-vessel wall interactions in tumor vessels after treatment with cyclophosphamide is remarkable.

Leukocyte adhesion molecule expression, systemic leukocyte counts, and fluid dynamic parameters

The decrease in leukocyte-vessel wall interactions in tumor vessels cannot be explained by altered adhesion

molecule expression on leukocytes (12). We found that the effects of the angiogenesis inhibitors were not due to changes in leukocyte adhesion molecule expression. Differences in expression of VLA-4, L-selectin, LFA-1 α , and LFA-1 β on the circulating leukocytes were not observed between the different treatment groups (data not shown).

The number of leukocyte-vessel wall interactions can be influenced by the level of leukocyte delivery to the observed blood vessels. This parameter depends on leukocyte concentration in the passing blood and on local blood flow. Treatment with anginex, endostatin, angiostatin, and paclitaxel did not influence the number of circulating leukocytes or the percentage of PMNs and MMNs (Table 2). TNP-470 had no effect on the systemic leukocyte count as well, although the percentage of PMNs was increased. Treatment with cyclophosphamide, however, induced a significant decrease in systemic leukocyte count ($P < 0.01$) with a concomitant decrease in the amount of PMNs ($P < 0.01$).

In all treatment groups, local vessel diameters and blood flow were not influenced (data not shown). Therefore, it is suggested that the observed effects of all angiogenesis inhibitors except cyclophosphamide on leukocyte-vessel wall interactions in and around the tumor cannot be explained by changes in leukocyte delivery to the vessels. In the present mouse model, cyclophosphamide treatment appears to be associated with a decrease in leukocyte delivery to vessels due to a significant reduction in systemic leukocyte concentration.

TABLE 2. Effect of treatment with anti-angiogenesis or anti-neoplastic drugs on systemic leukocyte concentration and percentage of polymorphonuclear (PMN) and monomorphonuclear cells (MMN) in blood of TNF α -treated C57BL/6 mice^a

	Systemic leukocyte count ($\times 10^6$ /mL)	PMNs (%)	MMNs (%)
Control (no treatment)	6.6 \pm 0.9	16 \pm 2	84 \pm 2
Anginex	9.0 \pm 0.8	17 \pm 1	83 \pm 1
Endostatin	6.0 \pm 0.2	12 \pm 2	88 \pm 2
Angiostatin	7.7 \pm 1.4	16 \pm 3	84 \pm 3
TNP-470	6.5 \pm 1.0	33 \pm 4*	67 \pm 4*
Paclitaxel	5.5 \pm 0.9	22 \pm 3	78 \pm 3
Cyclophosphamide	2.1 \pm 0.3*	5 \pm 2*	95 \pm 2*

^a Data are presented as means \pm SE; * P < 0.01 as compared to control.

Anti-angiogenesis treatment prevents down-regulation of endothelial ICAM-1 and VCAM-1 expression

To investigate whether the effects of the angiogenesis inhibitors are due to a normalization of the suppressed adhesion molecule expression on tumor ECs, in vitro experiments were performed using cultured b.END5 mouse EC. When cultured in the presence of 50 ng/mL bFGF, the proliferation rate of b.END5 cells is enhanced and ICAM-1 and VCAM-1 expression is down-regulated by 60% and 81%, respectively (Fig. 3A, B). Comparable but less pronounced results were observed by culturing in the presence of VEGF (data not shown). The bFGF-mediated down-regulation of ICAM-1 was observed in HUVEC (Fig. 3C), which is in agreement with previous observations (6, 7). Culture in the presence of anginex completely prevented the bFGF-mediated down-regulation of both ICAM-1 and VCAM-1 at the protein level (Fig. 3A–C) and at the RNA level (Fig. 3C–E, for ICAM-1, VCAM-1 and E-selectin). Endostatin treatment increased adhesion molecule expression at the RNA level (Fig. 3D–F). Angiostatin and TNP-470, the compounds with less and no activity in vivo (see Fig. 2), did not significantly influence adhesion molecule expression in b.END5 mouse EC in this in vitro bFGF-driven model. The latter compounds did have an effect on ICAM-1 expression in HUVEC, suggesting a difference in reactivity between mouse and human endothelial cells. Paclitaxel did prevent the down-regulation of ICAM-1, but not VCAM-1.

To investigate adhesion molecule levels in vivo, B16F10 tumor tissues were analyzed. In untreated B16F10 tumors, low mRNA expression levels of ICAM-1 and VCAM-1 were detectable whereas the expression of E-selectin was virtually absent (Fig. 4A). In lung, the expression of all three adhesion molecules was >100-fold higher than expression in tumors (data not shown). In tumors of mice treated with anginex the expression of the EC specific molecules VCAM-1 and E-selectin was significantly up-regulated by the treatment [2.38-fold (P < 0.05) and 2.43-fold (P < 0.05), respectively; Fig. 4B]. When the

change in microvessel density is taken into account (a 3.3-fold decrease compared with control, see Fig. 5F), the relative increase in expression of both VCAM-1 and E-selectin is >8-fold. The enhancement of ICAM-1 expression was not detectable using this method, most likely because this adhesion molecule is also expressed by tumor and/or stromal cells, which are not affected by specific anti-angiogenesis treatment.

Leukocyte infiltration is enhanced by angiogenesis inhibition

We investigated in two different mouse tumor models whether the enhancement of endothelial adhesion molecules and leukocyte-vessel wall interactions contributes to an increased leukocyte infiltration in a tumor. After 2 wk of treatment of human LS174T-bearing Swiss nu/nu mice with anginex, tumor growth (P < 0.05, Fig. 5A) and microvessel density (P < 0.0005, Fig. 5B) were significantly reduced. At the same time, anginex treatment enhanced the number of infiltrating

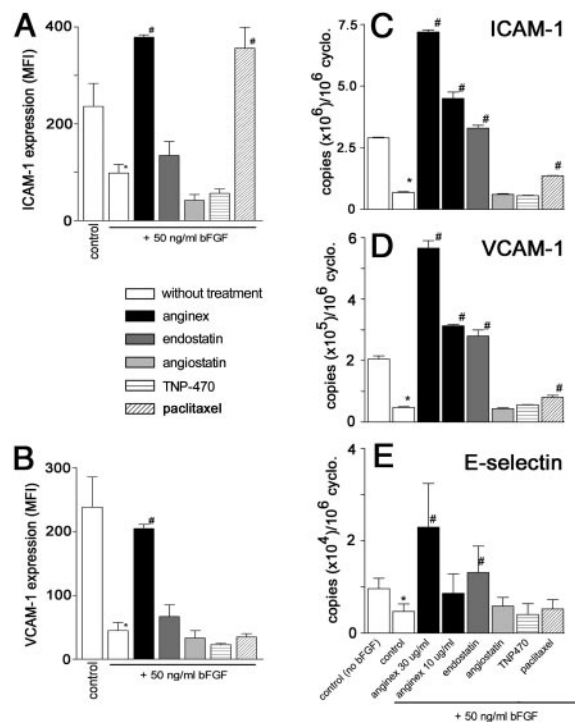


Figure 3. Growth factor (bFGF) induced down-regulation of adhesion molecule expression on mouse EC; normalization by angiostatic and chemotherapeutic agents. A, B) FACS analysis of ICAM-1 and VCAM-1 expression in b.END5 mouse EC, with or without bFGF, and after treatment with angiostatic and chemotherapeutic agents. Data are expressed as mean fluorescence intensity (MFI). C) FACS analysis of ICAM-1 expression in HUVEC. D, E, F) Quantitative real-time RT-PCR analysis of the effect of treatment on expression of ICAM-1 (D), VCAM-1 (E), and E-selectin (F). Data are presented as mean number of RNA copies per 10^6 copies of cyclophilin RNA. For all panels, data are means (\pm SEM) of 4 independent experiments. * P < 0.05 vs. cells without bFGF, # P < 0.05 vs. untreated cells.

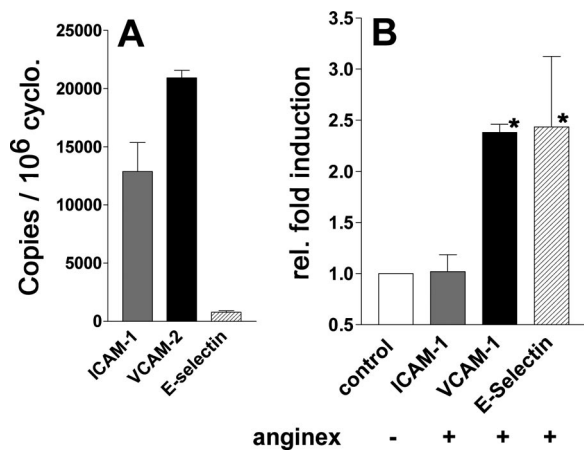


Figure 4. Effect of treatment with anginex on adhesion molecule expression in tumor tissue in vivo. A) Expression levels of ICAM-1, VCAM-1, and E-selectin in untreated tumor tissues by quantitative real-time RT-PCR analysis, normalized to cyclophilin expression. B) Effect of anginex on ICAM-1, VCAM-1, and E-selectin expression in tumor tissue. Data are presented as average relative fold induction. Expression in untreated tumors was set to 1. * $P < 0.05$ compared with untreated control tumors.

leukocytes in the tumor >2 -fold ($P < 0.01$), as determined using the pan-leukocyte marker CD45 (Fig. 5C). Similar but more pronounced results were observed in the B16F10 melanoma model in immunocompetent C57BL6 mice (Fig. 5D–G). Treatment of mice with anginex at 6 mg/kg/day resulted in a dose-dependent inhibitory effect on tumor growth. The inhibition of angiogenesis, resulting in a 3.3-fold decrease in microvessel density, caused a 6.7-fold increase in infiltrated leukocytes (Fig. 5D, G). It was interesting to see that in this immunocompetent model cognate immunity (cytotoxic T lymphocytes, CD8) was directed to the tumor as well (4.4-fold increase, Fig. 5D, H).

DISCUSSION

The present study demonstrates in an orthotopic syngeneic mouse model and in a human xenograft model that anti-angiogenesis treatment can overcome the suppression of endothelial adhesion molecules in tumors, leading to the amelioration of leukocyte-vessel wall interactions and to an increased inflammatory infiltrate in tumors.

It is known that tumors have the capacity to prevent the formation of a proper anti-tumor immune response by down-regulation of endothelial adhesion molecules that are necessary for leukocyte-vessel wall interactions (11, 12, 31). This regulation is mediated by angiogenic growth factors (6, 7). It was recently demonstrated in ovarian carcinoma patients that the expression of VEGF in tumor tissue is negatively correlated with the presence of infiltrating T lymphocytes, which was found to be the strongest prognostic factor in ovarian carcinoma (13). Similarly, we showed that the characteristic intense leukocyte infiltration in medullary breast carcinoma compared with ductal breast carcinoma can be explained by their difference in angiogenic potential (32). We hypothesized that when angiogenesis eradicates the infiltration of leukocytes, inhibition of angiogenesis would circumvent tumor escape from immunity and would lead to increased leukocyte infiltration. Previously, we showed in vitro that the angiogenesis inhibitors platelet factor-4 (PF4) and thrombospondin-1 not only prevent the down-regulation of adhesion molecules by bFGF or VEGF, but also induce re-expression of these molecules in cultured human umbilical vein-derived endothelial cells (33). Similarly, angiostatin was found to enhance the expression of E-selectin (34), while the synthetic angiogenesis inhibitor SU6668 reversed the bFGF-mediated down-regulation of ICAM-1, VCAM-1, and E-selectin (31). Here, we have demonstrated that this phenomenon is shared by more angiogenesis inhibitors and that affects in vivo

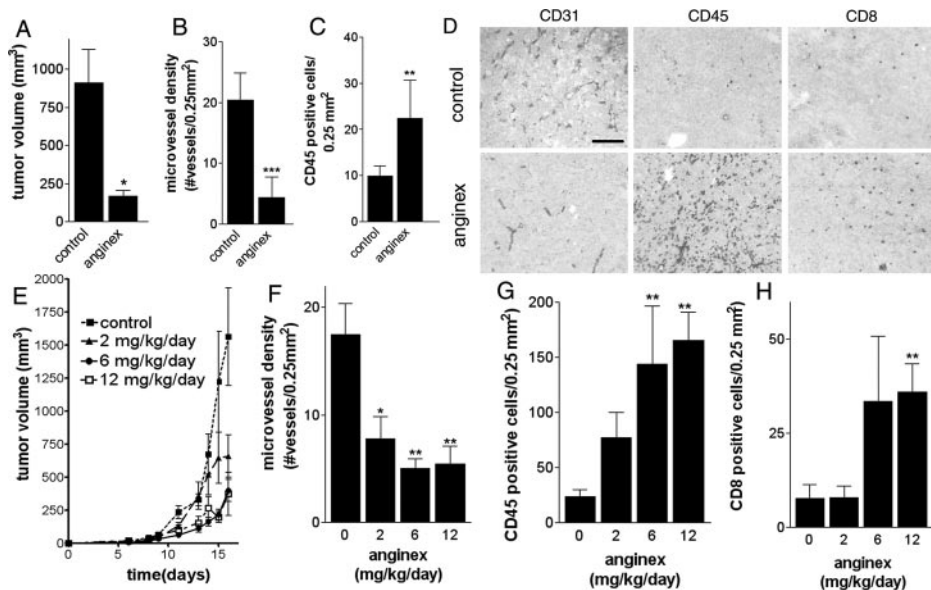


Figure 5. Relationship between angiogenesis inhibition and leukocyte infiltration. Tumor size (A), microvessel density assessment by CD31 staining (B), and infiltration by CD45⁺ leukocytes (C) of LS174T human colon carcinoma with and without anginex treatment. D) Immunohistochemical images of microvessel density (CD31), and infiltration by CD45⁺ leukocytes and CD8⁺ T lymphocytes in B16F10 melanoma of control and anginex-treated mice. Bar in upper left panel represents 50 μ m. E–H) Effect of increasing doses of anginex (2, 6, 12 mg/kg/day) on tumor size, microvessel density, total leukocyte (CD45), and cytotoxic T lymphocyte (CD8) infiltration in B16F10 melanoma. * $P < 0.01$, ** $P < 0.001$, and *** $P < 0.0001$ compared with control.

leukocyte-vessel wall interactions and the formation of an immune infiltrate.

In the present study, we show *in vivo* as well as *in vitro*, both at the mRNA and protein level, that angiostasis leads to re-expression of adhesion molecules on endothelial cells. The major role of ICAM-1 in leukocyte extravasation (35) and the marked up-regulation of ICAM-1, VCAM-1 and E-selectin in response to angiogenesis inhibition do not rule out a role for other adhesion structures. CD31, CD34, and ICAM-2, molecules with important functions in leukocyte-vessel wall interactions, are also described to be suppressed in tumor vessels (7, 36). The same holds true for P-selectin, a molecule involved in leukocyte and platelet binding (37). It is expected, but remains to be proved, that these molecules are part of angiogenesis-associated regulation.

The present results indicate that not all angiogenesis inhibitors tested showed the same inflammatory response. For treatment of cancer, the agent of choice would presumably combine the strongest vessel growth inhibitory activity with the most potent inflammatory properties. Apparently these properties are not interdependent; e.g., TNP-470 and anginex differ largely in immune modulatory activity (see Figs. 2, 3), whereas they are equipotent angiogenesis and tumor growth inhibitors in the B16F10 melanoma model (17), which suggests that the mechanisms of these effects are different. Anginex increased the expression of endothelial adhesion molecules on tumor endothelial cells at both the mRNA and protein level. As a consequence, anginex significantly increased leukocyte-vessel wall interactions in tumor vessels to levels found in healthy control animals. This in turn led to increased infiltration of leukocytes into the tumor. Angiogenesis inhibition is suggested to be a promising approach for the treatment of diseases with a chronic inflammatory component, such as rheumatoid arthritis and psoriasis (38), in which a concomitant proinflammatory activity is undesirable. It is tempting to speculate that the good results with TNP-470 in arthritis models are due to the lack of proinflammatory activity. It might be important, therefore, to screen a larger array of compounds for this activity in order to better judge applicability in various diseases.

The inflammatory activity of angiostatic compounds is shared with certain chemotherapeutic agents. It has been observed that chemotherapeutic compounds such as cyclophosphamide and paclitaxel can induce leukocyte infiltration in tumors (39). Currently, there is an ongoing discussion concerning the intrinsic angiostatic activity of several chemotherapeutic drugs (24). We and others have demonstrated for the tubulin binding compounds (taxanes and the vascular targeting agent combrestastatin) that an intrinsic anti-angiogenesis activity exists at the level of endothelial migration and tube formation, at concentrations that do not affect cell growth (40). The presently shown shared activity of paclitaxel and angiogenesis inhibitors at the level of adhesion molecule regulation and leukocyte-vessel wall

interactions may support the theory that certain chemotherapeutics are angiostatic and may be used at ultra-low dosages for prolonged periods as treatment of cancer.

The effects of the angiogenesis inhibitor TNP-470 on leukocyte-vessel wall interactions and adhesion molecule expression contrasted with the results obtained with the specific angiogenesis inhibitors anginex, endostatin, and angiostatin. This may be explained by the fact that TNP-470 has other indirect effects, which is suggested by the systemic decrease in leukocyte-vessel wall interactions in tumor-free mice (Table 1) and the change of leukocyte composition in peripheral blood (Table 2). Like TNP-470, cyclophosphamide decreased leukocyte-vessel wall interactions in healthy microvessels *in vivo*, whereas paclitaxel only reduced the level of leukocyte adhesion in these vessels. This decrease in leukocyte-vessel wall interactions in a tumor-free environment may mask the up-regulating effect of these agents on leukocyte-vessel wall interactions in tumor vessels.

Here, we used recently developed tumor models (12) in which leukocyte-vessel wall interactions can be measured noninvasively, allowing longitudinal analysis during tumor development and over the course of anti-cancer treatment. The combined assessment of leukocyte-vessel wall interactions inside the vascular system and leukocyte infiltration into the tissue provides a functional meaning to changes in leukocyte adhesion. Our findings indicate that the increase in leukocyte-vessel wall interactions in tumor vessels is followed by increased infiltration of leukocytes into the tumor tissue, where they can help to suppress tumor growth. It is an interesting observation that both angiogenesis inhibitors and chemotherapeutic compounds can elucidate this effect. It is tempting to speculate that the reported, sometimes very good, results from combining both treatment strategies (18, 41) are explained by this enhancement of inflammatory infiltrate. In a preliminary pilot study we found that a combination of paclitaxel and anginex leads to an enhanced leukocyte infiltrate compared with either paclitaxel or anginex alone (unpublished results). It remains to be elucidated, however, which leukocyte subtypes are involved in increased homing to the tumor.

The present study was focused on investigating the escape of tumors from immunity and how the leukocyte infiltrate can be augmented in tumors. Although the beneficial effect of cognate anti-tumor immunity is not debated at all, it should be kept in mind that several studies have shown that leukocytes, by their inflammatory cytokine/chemokine production and by their release of matrix metalloproteinases, can contribute to angiogenesis programs and thereby to tumor progression as well (42, 43). Tumor-associated macrophages have been mostly recognized for this phenomenon (44). We, however, favor the view that leukocyte infiltration should be promoted in order to drive anti-tumor immunity.

In conclusion, the present study demonstrates that

angiogenesis inhibitors increase leukocyte-vessel wall interactions in tumor vessels, which leads to increased leukocyte infiltration into tumor tissue. This anti-tumor inflammatory effect of angiogenesis inhibitors is mediated by increased expression of adhesion molecules on tumor endothelial cells and not by altered expression of leukocyte adhesion molecules, effects on the amount of circulating leukocytes, or other direct or indirect vascular effects modulating fluid dynamic parameters. These data strongly support the concept of combining modern immunotherapeutic approaches with angiostatic therapies. At least additive and hopefully synergistic anti-tumor responses would be expected from this combination. **[E]**

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