Tumor Angiogenesis Modulates Leukocyte-Vessel Wall Interactions in Vivo by Reducing Endothelial Adhesion Molecule Expression


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ABSTRACT

The expression of endothelial cell (EC) adhesion molecules involved in leukocyte-vessel wall interactions is suppressed in malignancies. In the present study, we investigated in vivo the regulation of leukocyte-vessel wall interactions by the presence of a tumor. By means of intravital microscopy, tumor necrosis factor α-stimulated leukocyte-vessel wall interactions were studied in ear skin microvessels of nude mice bearing small human LS174T colon carcinomas and in C57Bl/6 mice bearing murine B16F10 melanomas. Leukocyte-vessel wall interactions were studied both within and outside small tumors growing in the ear, and in ear microvessels of mice with a large tumor growing on their flank. Tumor-free mice were used as controls. Compared with values measured at the edge of the ear and in the contralateral ear, leukocyte adhesion was found to be diminished significantly in vessels inside the ear tumor in both mouse models. This reduction disappeared with increasing distance from the tumor. Surprisingly, the level of leukocyte adhesion in ear venules of mice with a large flank tumor was also reduced significantly. Leukocyte rolling, i.e., the step preceding adhesion, was not influenced by the presence of a tumor in nude mice, but was down-regulated in immune-competent C57Bl/6 mice. Treatment of mice bearing a small ear tumor with a humanized antivascular endothelial growth factor antibody prevented the down-regulation of leukocyte-vessel wall interactions inside the tumor vessels compared with the nontreated group. Fluorescence-activated cell sorter analysis showed that isolated tumor ECs have suppressed levels of intercellular adhesion molecule 1 as compared with ECs from normal mouse tissues. In cultured b.END5 cells the tumor necrosis factor α-induced up-regulation of intercellular adhesion molecule 1 and vascular cell adhesion molecule 1 was reduced in ECs that were preincubated with basic fibroblast growth factor or vascular endothelial growth factor. The current results may have an impact on the effectiveness of clinical immunotherapeutic treatment protocols, because immune effector cells may not be able to enter tumor tissue.

INTRODUCTION

It has been known for some time that both immunotherapy (1) and inflammation (2) can contribute to the regression of solid tumors. To infiltrate tissues, including tumors, leukocytes have to interact with the venular vessel wall. These interactions start with leukocytes becoming tethered to and then slowly rolling along the vascular wall. This may result in firm adhesion with subsequent activation of the leukocytes, followed by their diapedesis and emigration into the surrounding tissue (3). ECs can influence the selection of leukocyte subtypes that compose the immune infiltrate by regulating adhesion molecule expression. By interfering with this regulated expression, tumors may have developed a mechanism of escaping from immune infiltration (4–7).

Angiogenesis, the formation of new blood vessels from pre-existing ones, is a prerequisite for outgrowth and metastasis of tumors (8, 9). To achieve this, tumors secrete high levels of angiogenic factors such as VEGF and fibroblast growth factors. We have shown previously that these factors are responsible for down-regulating endothelial adhesion molecules such as ICAM-1/2 and CD34 (4, 10). Angiogenic factors also inhibit inflammatory cytokine induced expression of VCAM-1 and E-selectin (11, 12). In addition, it has been established both in vitro (13) and in vivo (14) that suppressed adhesion molecule expression by angiogenic factors results in diminished leukocyte-vessel wall interactions.

The present study aimed to investigate the regulation of adhesion molecule expression and leukocyte-vessel wall interactions in vivo by the presence of a tumor. To this end, a mouse model was developed that allows the investigation of leukocyte-vessel wall interactions, both inside and outside a tumor. The effects of a small tumor in the ear were compared with those of a larger tumor on the flank. Intravital microscopy was used to quantify the effects of the tumor on leukocyte-vessel wall interactions and on local fluid dynamic conditions in ear skin microvessels. To this purpose, a xenograft model was used in which a human tumor (LS174T) was grown in nude mice. These experiments were repeated using immune-competent mice (C57Bl/6) with a mouse tumor (B16F10). In addition, ex vivo and in vitro experiments were performed to detect ICAM-1 and VCAM-1 expression on ECs isolated from both tumor and healthy tissues (obtained from Swiss/nude mice and C57Bl/6 mice) and on mouse b.END5 brain endothelioma cells using flow cytometry.

MATERIALS AND METHODS

Animals. The experiments were approved by the local Ethical Review Committee on Animal Experiments. We used Swiss/nude mice (23–31 g; Charles River, Maastricht, the Netherlands), housed under sterile conditions, and C57Bl/6 mice (25–30 g; Charles River), housed under standard conditions.

Cell Culture. Tumor cells (LS174T) were cultured in DMEM (Life Technologies, Inc.) containing 10% FCS. Experimental Protocol and Intravital Microscopy. In vivo experiments were performed in four groups of Swiss/nude mice. After anesthetizing these mice briefly (i.p.) using a mixture of ketamine (0.1 mg/g b.w. Nimetak; Ad Usem Veterinarium, Cuijk, the Netherlands) and xylazine (0.05 mg/g b.w. in

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3 The abbreviations used are: EC, endothelial cell; VEGF, vascular endothelial growth factor; ICAM, intercellular adhesion molecule; VCAM, vascular cell adhesion molecule; b.w., body weight; TNF, tumor necrosis factor; PMN, polymorphonuclear; MMN, monomorpho...
nude mice and 0.02 mg/g b.w. in C57Bl/6 mice, Sedanum; Ad Usen Veterinariaum, the first group (ear tumor group; n = 6) received 10 μl of a dense tumor cell suspension (10^6 cells) intradermally in the dorsal side of the left ear. Within 3–5 days the ear tumor grew to a diameter of ~2 mm, at which time point the experiment was performed. In the second group (flank tumor group; n = 4) 100 μl of a tumor cell suspension (10^6 cells) was injected s.c. into the flank. In this group experiments were performed after 2–3 weeks, at which time the flank tumor had reached a diameter of ~1.5 cm. The third group served as control (n = 4). These mice were not injected with tumor cells, but were otherwise treated similarly. Two additional mice bearing a small LS174T ear tumor (n = 2) were treated with HuMV833 (a humanized anti-VEGF antibody, 3.0 mg/kg, i.p.; kindly provided by Protein Design Labs, Fremont, CA) on the days 5 and 7 after injection of the LS174T cells in the left ear. On day 8, intravital microscopy was performed. The experiments were repeated with three groups of C57Bl/6 mice carrying B16F10 murine melanoma tumors: an ear tumor group (n = 7), a flank tumor group (n = 4), and a control group (n = 4).

Four hours before the start of the intravital microscopic observations, Swiss/nude mice received 750 ng of recombinant murine TNF-α (R&D Systems, Abingdon, United Kingdom) whereas C57Bl/6 mice received 500 ng of TNF-α. Two additional Swiss/nude mice as well as two C57Bl/6 mice were investigated that did not receive TNF-α, to serve as control for the TNF-α effects in the present setup.

After TNF-α administration (3.5 h), mice were anesthetized by s.c. administration of the ketamine/xylazine mixture (see above). Body temperature was kept at 37°C by an infrared heating lamp. To enable intravital microscopic observation of leukocytes, 10–20 μl of a Rhodamine 6G solution (1 mg/ml in 0.9% NaCl solution) was injected into the tail vein when needed. In all of the mice, venules (10–40 μm) in both ears were visualized using a Leitz intravital microscope adapted for telescopic imaging (15). Images were recorded on videotape for offline analysis.

To enable quantification of systemic leukocyte counts, 20 μl of blood were sampled from the vena cava at the end of each experiment and added to Turks solution (Merck) in a 1:10 dilution. Leukocytes were counted and differentiated as PMN or MMN in a counting chamber (Clay Adams, Parsippany, NJ).

Experimental Parameters. Venular diameters were determined with a home-built image-shearing device (16). Centerline blood flow velocity was measured 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 min. Leukocytes were considered as rolling when their velocity along the vessel wall 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; at each time point, this count was performed in 4 randomly chosen video frames, and the data were averaged. In the tumor-bearing ears, leukocyte-venule wall interactions were quantified in venules in three different regions: (a) within the tumor; (b) adjacent to the tumor (within a radius of 2.5 mm around the tumor); and (c) at the edge of the ear. In ears without a tumor the venules were chosen at random.

Real-Time Quantitative RT-PCR. To detect VEGF and bFGF gene transcription, RNA was extracted from frozen LS174T tumor tissue using a RNaseasy mini kit (Qiagen, Valencia, CA) and from proliferating LS174T cells using TRIzol reagent (Invitrogen, Breda, the Netherlands). Subsequently, a real-time quantitative RT-PCR was performed to quantitate transcription levels of VEGF and bFGF as described by Meulemans et al.4

Adhesion Molecule Expression on Isolated ECs and b.END5. To culture mouse microvascular ECs, normal (lung, heart, and kidney) and tumor tissues obtained from both Swiss/nude and C57Bl/6 mice were mechanically and enzymatically digested during 1 h at 37°C, using a mixture of 1 mg/ml collagenase and 2.5 IU/ml dispase (Life Technologies, Inc.). After 30 min of incubation, 75 μg DNase (Sigma Chemical Co.) was added for another 30 min. The single-cell suspensions were allowed to adhere for 3 h to gelatin-coated tissue culture flasks (Costar, Corning, NY). The remaining adherent cell population consisted of 2–15% ECs as determined by CD31 expression. Cells were cultured for 3 days in DMEM (with 1000 mg/liter glucose) containing 20% FCS. In one experiment, cells obtained from normal C57Bl/6 tissues were cultured for 3 days in the presence of 0, 10, 25, 50, or 100 ng/ml bFGF (Sanvertech, Heerhugowaard, the Netherlands). b.END5 cells were cultured for 3 days with 50 ng/ml bFGF or 50 ng/ml VEGF. When applied, TNF-α was added 6 h before harvesting. Finally, the expression of ICAM-1 and VCAM-1 was determined flow cytometrically (see below).

FACS Analysis of Endothelial and Leukocyte Adhesion Molecules. Cells were harvested and fixed for 30 min in 1% paraformaldehyde (Merck) at room temperature. Afterward, cells were resuspended in 20 μl of appropriately diluted rat anti-ICAM-1 monoclonal antibody (R&D Systems) or rat anti-VCAM-1 monoclonal antibody (PharMingen, San Diego, CA) and incubated for 1 h on ice. Subsequently, the cells were incubated for 1 h with goat-antirat IgG conjugated to FITC (Pickell Laboratories, Amsterdam, the Netherlands). Finally, the cells were incubated with phycoerythrin-conjugated rat anti-mouse CD31 (Dako, Glostrup, Denmark). Stained cells were analyzed on a FACScan caliber flow cytometer. Data analysis was performed using Cellquest software (Becton Dickinson, Mountain View, CA).

Mouse blood leukocytes were isolated by Ficoll density gradient centrifugation (Amersham, Uppsala, Sweden). Cells were fixed for 30 min in 1% paraformaldehyde (Merck) at room temperature. Cells were incubated with the supernatant of the following hybridoma cell lines: R1-2 (rat anti-ICAM-1), Mei14 (rat anti-endothelial cell-selectin), M17.4 (rat anti-LFA-1 α chain), or M18.2 (rat anti-LFA-1 β chain; all obtained from PharMingen) for 1 h at 4°C. Subsequently, the cells were incubated with FITC-conjugated goat anti-rat IgG (Pickell Laboratories) for 1 h at 4°C.

Statistics. Because of their nonsymmetrical distribution, data obtained by intravital microscopic experiments are presented as medians with interquartile ranges (i.e., the spread from the 25th to 75th percentile). Data obtained by FACS analysis are presented as means with corresponding SE. 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 (more than two groups), followed by a multiple-comparison procedure. Correlation between variables was determined using Spearman’s correlation test. In all of the tests the level of significance was set at 0.05.

RESULTS

Leukocyte-Vessel Wall Interactions Are Inhibited in Tumor Vessels. We showed previously that angiogenic factors down-regulate leukocyte-venule wall interactions in vivo (14). To investigate whether this observation also holds in the presence of a growing tumor, we developed a noninvasive mouse tumor model that can be used for intravital microscopic determination of leukocyte interactions with the microvascular endothelium.

LS174T human colon carcinoma tumor cells were injected in the ears of nude mice (Fig. 1, A and B), and after the outgrowth of small tumors, TNF-α-induced leukocyte-venule wall interactions as well as local fluid dynamic parameters were examined in ear skin vessels. In the tumor-free control group, the median level of leukocytes interacting with the vascular endothelium after treatment with TNF-α was 812 leukocytes/mm². Inside the ear tumor, this level was significantly lower (382 leukocytes/mm²; P < 0.05). The total level of leukocyte-venule wall interactions increased as a function of distance from the tumor [significant correlation of 0.35 (Rs); P < 0.01], being 728 leukocytes/mm² in areas adjacent to the tumor and 953 leukocytes/mm² at the outer edge of the ear. The latter level is comparable with the one measured in the contralateral ears (885 leukocytes/mm²) and with the level measured in the control group (Fig. 1C). Examples of intravital microscopic images of control and tumor vessels are presented in Fig. 1, D and E. Without TNF-α pretreatment, the total level

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4 Els V. Meulemans, et al., Expression of VEGF, bFGF and VEGF receptors in diffuse large B cell lymphoma, submitted for publication.
of interacting leukocytes in ear skin venules was 318 cells/mm² (data not shown), which indicates that the tumor microenvironment completely eradicated the TNF-α-induced inflammatory response. These results demonstrate a local effect of a small tumor on leukocyte interactions with the vascular endothelium.

The total level of leukocyte-vessel wall interactions includes both leukocyte rolling and adhesion. Discrimination between these two processes revealed that the reduction in leukocyte-vessel wall interactions inside a tumor is because of an effect on leukocyte adhesion (Fig. 2A). Whereas the level of leukocyte adhesion in tumor vessels was drastically diminished to almost undetectable levels as compared with venules in the contralateral ear (375 leukocytes/mm²) and in the control group (331 leukocytes/mm²), such differences were not found at the level of leukocyte rolling (Fig. 2B).

We showed previously that angiogenic factors are able to down-regulate leukocyte-vessel wall interactions (14). Using TaqMan real-time quantitative RT-PCR we were able to show that LS174T tumors, grown in vivo as well as cultured in vitro, show mRNA expression of VEGF. mRNA expression of bFGF was below detection level. To investigate whether VEGF contributed to the effect of a tumor on leukocyte-vessel wall interactions, mice bearing a small LS174T ear tumor were treated with an anti-VEGF antibody. In these mice we did find a significant inhibition in the level of rolling leukocytes inside the tumor (3 leukocytes/min) as compared with the control mice (331 leukocytes/min; P < 0.05; Fig. 2B).

A Large Tumor Has a Systemic Effect on Leukocyte-Vessel Wall Interactions. Injection of LS174T tumor cells on the flank of a nude mouse resulted in a large (~1500 mm³), noninfiltrating, and nonmetastasizing tumor within ~3 weeks. Analysis of the ear venules of this group lead to the surprising finding of a significantly suppressed level of leukocyte-vessel wall interactions (509 leukocytes/mm²; P < 0.05) as compared with the control group without a tumor (813 leukocytes/mm²; P < 0.05; Fig. 4A). Again, comparable with the situation in the small ear tumors, this reduced level of leukocyte-vessel wall interactions can be attributed to a diminished level of leukocyte adhesion (Fig. 4B). The level of leukocyte rolling was not found different from control (Fig. 4C).

These experiments were also performed in the B16F10 mouse melanoma model in C57BL/6 mice. These experiments revealed comparable results, showing reductions in total level of interacting and adhering leukocytes in tumor vessels as compared with vessels outside the tumor [significant correlation of 0.55 (Rs) between leukocyte-vessel wall interactions and distance to the tumor; P < 0.01] and in tumor-free control mice (Fig. 3, A and B). Interestingly, in the immunocompetent mice we did find a significant inhibition in the level of rolling leukocytes inside the tumor (3 leukocytes/min) as compared with the edge of the ear (15 leukocytes/min; P < 0.05; Fig. 3C).

Similarly influenced in mice with an intact immune system, similar experiments were performed in the B16F10 mouse melanoma model in C57BL/6 mice. These experiments revealed comparable results, showing reductions in total level of interacting and adhering leukocytes in tumor vessels as compared with vessels outside the tumor [significant correlation of 0.55 (Rs) between leukocyte-vessel wall interactions and distance to the tumor; P < 0.01] and in tumor-free control mice (Fig. 3, A and B). Interestingly, in the immunocompetent mice we did find a significant inhibition in the level of rolling leukocytes inside the tumor (3 leukocytes/min) as compared with the edge of the ear (15 leukocytes/min; P < 0.05; Fig. 3C).

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No significant differences were found in leukocyte rolling (Fig. 5C).

**Leukocyte Adhesion Molecule Expression, Systemic Leukocyte Counts, and Fluid Dynamic Parameters.** One explanation for the observed tumor effect on leukocyte-vessel wall interactions could be that leukocytes themselves are affected by the presence of a tumor. To exclude this effect, we investigated adhesion molecule expression on circulating leukocytes in mice bearing a small ear tumor and in healthy controls. We found no difference in expression of LFA-1α, LFA-1β, VLA-4 or β2-selectin between these two groups (data not shown), suggesting that the decreased level of interacting leukocytes we observe inside an ear tumor is not because of altered adhesion molecule expression on leukocytes.

TNF-α induced a decrease in the number of circulating leukocytes from $2.4 \times 10^6$ leukocytes/ml to $0.9 \times 10^6$ leukocytes/ml ($P = 0.05$) in nude mice. This is probably because TNF-α induces leukocyte adhesion systemically. This effect was evident for both MMNs and PMNs, but was most prominent for MMNs (80% reduction versus 55% for PMNs; $P < 0.01$). In contrast to a small ear tumor ($1.3 \times 10^6$ leukocytes/ml), the presence of a large flank tumor tended to mask these TNF-α induced changes ($1.8 \times 10^6$ leukocytes/ml), also suggesting a systemic effect of the tumor.

No significant differences in blood flow velocities were detected between the vessels of the various experimental groups of both Swiss/nude and C57Bl/6 mice (Table 1), except for deviating centerline velocities in the vessels inside the tumor in both mouse models. However, no correlation between centerline velocity values and any of the leukocyte-vessel wall interaction related parameters could be found in any of the groups. Therefore, the observed differences in leukocyte-vessel wall interactions cannot be explained by differences in fluid dynamic parameters.

**Diminished Leukocyte Adhesion in Tumors Is the Result of Suppressed Endothelial Adhesion Molecule Expression.** In previous studies, it was demonstrated that human tumor ECs express suppressed levels of adhesion molecules that are involved in leukocyte-vessel wall interactions (4, 11). In the present study we investigated the expression of ICAM-1 (an important adhesion molecule involved in leukocyte emigration) on tumor ECs. To this purpose, ECs were freshly isolated from LS174T and B16F10 tumors, and from normal tissues. Adhesion molecule expression on ECs was determined...
by double staining, using CD31 and ICAM-1 antibodies, and flow cytometry (4). ICAM-1 expression was found to be down-regulated in tumor-associated endothelium as compared with ECs obtained from normal tissue (Fig. 6, A and B). Whereas ICAM-1 levels in the tumor were low (mean fluorescence intensity of 2 in LS174T tumors and 1.5 in B16F10 tumors), in normal tissues these values ranged between 40 and 57. To investigate whether angiogenic factors produced by the tumor are responsible for this down-regulation of ICAM-1, normal tissue ECs were cultured for 3 days in the presence of various concentrations of bFGF (Fig. 6C). bFGF was observed to down-regulate ICAM-1 on freshly isolated ECs in a dose-dependent manner. In separate experiments, the mouse EC line b.END5 was cultured with or without bFGF or VEGF, and in presence or absence of TNF-α (Fig. 6D). In the absence of TNF-α, bFGF was observed to down-regulate ICAM-1 expression (P < 0.05). On the contrary, TNF-α was able to significantly up-regulate both ICAM-1 (P < 0.01) and VCAM-1 (P < 0.01). The TNF-α-induced up-regulation of ICAM-1 and VCAM-1 was reduced in cells that were preincubated with bFGF (ICAM-1: P < 0.05; VCAM-1: P = 0.07) or VEGF (ICAM-1: P = 0.06; VCAM-1: P = 0.06). These results suggest angiogenic factors as regulators of leukocyte-vascular wall interactions.

**DISCUSSION**

The present study demonstrates, in two separate mouse models, that leukocyte-vascular wall interactions are locally suppressed within a tumor. Surprisingly, large tumor burdens were observed to generate a systemic down-regulation of leukocyte-vascular wall interactions. The reduction in leukocyte-vascular wall interactions in tumor vessels appeared to be caused by a decreased expression of adhesion molecules on ECs and not by suppression of adhesion molecules on leukocytes. Our finding that pretreatment with bFGF or VEGF reduces the TNF-α-induced expression of ICAM-1 and VCAM-1 on cultured mouse ECs strongly suggests that tumor-derived growth factors are involved. A reduced level of leukocyte-vascular wall interactions is likely to result in impaired extravasation and infiltration of leukocytes into the tumor. Through this mechanism a tumor has the opportunity to escape the immune system.

We developed a new tumor model in which leukocyte-vascular wall interactions in and outside a tumor can be measured noninvasively, allowing longitudinal analysis during tumor development and/or in the course of an anticancer treatment. Several other investigators have also used animal models to examine leukocyte-vascular wall interactions in the presence of a tumor (17–23). However, these studies used invasive techniques such as dorsal skin fold chambers, cranial windows, or skin flaps. These techniques are more time consuming, carry a higher risk of failure, and may induce an inflammatory reaction by surgical procedures at the site of interest. In the present study, athymic nude mice were selected to enable implantation of a tumor of human origin. These mice have large, transparent, hairless ears, which is a clear advantage for intravital microscopic measurements. The use of athymic mice to study immune functions may be problematic, attributable to limited cognate immunity because of the lack of mature T lymphocytes. However, innate immunity (i.e., granulocytes, monocytes, and natural killer cells) and B lymphocytes are present and functional in this model. Nevertheless, we repeated the experiments using syngeneic B16F10 melanoma cells in immune-competent C57Bl/6 mice. This model was chosen because of the availability of a large body of literature on immunological antitumor responses in these mice (23–26).

It has been demonstrated previously that the expression of adhesion molecules is suppressed in human tumor ECs (6, 27, 28). Furthermore, we have shown earlier in vitro (4) and in vivo (14) that this is most probably because of differences in metabolic state and phenotype of tumor EC by exposure to tumor-derived angiogenic factors such as bFGF and VEGF. Other studies have shown that bFGF reduces monocyte and natural killer cell adhesion, and migration by down-regulating adhesion molecules on HUVECs (7, 29). Here, we show that in our mouse models tumor ECs also have suppressed ICAM-1 expression. In addition, TNF-α-induced up-regulation of ICAM-1 and VCAM-1 was reduced in cultured b.END5 cells (mouse endothelioma cells) pretreated with either bFGF or VEGF. Because we also show that LS174T tumor cells contain mRNA for VEGF, whereas it is known from literature that B16F10 tumor cells express high levels of bFGF and VEGF (30, 31), our data strongly suggest that the down-regulation of leukocyte-vascular wall interactions in tumor vessels, as observed in the present study, is a result of exposure of ECs to these angiogenic factors. This hypothesis is supported by our observation that treatment of mice bearing a small ear tumor with humanized anti-VEGF antibody prevents the down-regulation of leukocyte-vascular wall interactions inside the tumor.

Our finding that leukocyte-vascular wall interactions are decreased inside a tumor was confirmed in several previous studies (17–22).
However, in literature there is some dispute with regard to the effects of VEGF on the expression of adhesion molecules on ECs. Whereas the results of some studies are in agreement with our data and show a role for VEGF as an inhibitor of leukocyte-vessel wall interactions (32), the results of other studies seem to be in contrast with our findings (7, 33). These latter studies report an increase in adhesion molecule expression on HUVEC after VEGF stimulation. However, in these studies VEGF stimulation was always ≥24 h. In a pilot study, we also observed an initial up-regulation of ICAM-1 expression after VEGF stimulation of HUVECs; in contrast, prolonged stimulation (which is also the case in the presence of a tumor) results in a down-regulation of ICAM-1 expression and leukocyte adhesion (data not shown). In one other study, performed by Detmar et al. (34), leukocyte-vessel wall interactions were found to be increased in skin vessels of VEGF-transgenic mice. However, this experimental approach in which the effects of a continuous overexpression of VEGF were investigated is completely different from the situation within tumors where VEGF [and also other (growth) factors] is produced locally in increasing quantities.

The present study is the first to show a systemic effect of a large tumor: leukocyte-vessel wall interactions appeared to be down-regulated in ear microvessels of mice bearing a large tumor on their flank. This systemic effect is less pronounced as compared with the situation within a tumor; leukocyte adhesion was only partially down-regulated, whereas inside a tumor the level of adhesion was reduced to zero. This may be because the circulating concentration of angiogenic factors in mice with a large flank tumor is lower than the local concentration inside a small ear tumor. The fact that the systemic leukocyte count was higher in TNF-α-treated mice with a large tumor on their flank than in TNF-α-treated control mice also suggests a

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</table>

Data are presented as median values and interquartile ranges in parentheses.

Fig. 6. ICAM-1 is down-regulated on mouse ECs isolated from a tumor as compared with ECs isolated from normal tissue. Normal and tumor tissues from Swiss/nude (A) and C57Bl/6 mice (B) were mechanically and enzymatically digested. The obtained single-cell suspensions were cultured for 3 days. Subsequently, the cells were harvested, and the expression of ICAM-1 on ECs was determined by FACS analysis. Data were divided by control values and presented as means of fluorescence intensity and corresponding SE values. C, normal C57Bl/6 ECs were cultured with various concentrations of bFGF. Subsequently, the cells were harvested, and the expression of ICAM-1 on ECs was determined by FACS analysis. D, b.END5 cells were cultured for 3 days with 50 ng/ml bFGF or 50 ng/ml VEGF. TNF-α was added 6 h before harvesting. Afterward, the cells were harvested and the expression of ICAM-1 and VCAM-1 on ECs was determined by FACS analysis. *, P < 0.05; **, P < 0.01 as compared with the control group; †, P < 0.05; ††, P ≤ 0.07 as compared with TNF-α group; bars, ±SE.
systemic inhibitory effect of the flank tumor on leukocyte-vessel wall interactions. Because it is known for that some tumors produce soluble TNF-α-receptors (35, 36), this might also be an explanation for the observed systemic effect of a large tumor on leukocyte-vessel wall interactions. However, we also show data (Fig. 6D) on the direct effect of angiogenic factors on ECs, which exclude tumor cell interference in this process. Therefore, we believe that the observed effects are explained by an EC-mediated mechanism rather than a mechanism controlled by tumor-derived signals.

The current results indicate that in the nude mouse model, adhesion but not rolling is modulated by tumor derived factors. This suggests that ICAM-1 but not the selectins are sensitive to regulation by these factors, which would be a discrepancy with the human situation, where E- and P-selectin were found to be suppressed in tumor ECs (11, 37). It is more likely that the absence of effects on leukocyte rolling is because of selection of the nude mouse model, because we did observe a significant effect on leukocyte rolling inside the tumor in C57Bl/6 mice. We hypothesize that either tumor regulation of leukocyte rolling is confined to a functional T-cell population or that this effect is dependent on specific (combinations of) cytokines that are expressed by an intact immune system.

During its development, a growing tumor needs to undergo the so-called angiogenic switch, which is the starting point of the attraction of new blood vessels to grow beyond the size of 1–2 mm in diameter. It is possible that the small ear tumors studied in our experiments did not display a full angiogenic profile. Although this certainly would be an issue for spontaneous tumor models, in our model the injected cells already expressed VEGF and/or bFGF during culture. Evidence for the fact that we injected the cells in an advanced angiogenic state is provided by the fact that we observed the formation of a hematoma-like spot within 24 h after injection, which might be because of a permeability increase induced by VEGF and the presence of new vasculature already after 2–3 days (Fig. 1, A and B). Moreover, it was established that the small ear tumors are able to grow to the size of more advanced tumors (diameter >3 mm), indicating that angiogenic factors are being excreted by the tumor cells. Also, we obtained using real-time quantitative RT-PCR show that LS174T cells contain mRNA for VEGF in culture already. Therefore, we favor the view that our ear tumor model is relevant for the study of the early outgrowth of metastases rather than the outgrowth of a primary tumor.

In conclusion, the currently described tumor models introduce a noninvasive method for studying leukocyte-vessel wall interactions in vivo. We have demonstrated that leukocyte-vessel wall interactions are suppressed within a tumor but increase as a function of distance from the tumor. Moreover, in case of a large tumor burden there appears to be a systemic down-regulation of leukocyte interactions with the vessel wall. This effect is mediated by down-regulation of endothelial adhesion molecules, probably by angiogenic factors, leading to the escape of tumors from the immune system. Future studies will address the issue of the reversal of the observed phenomena by treatment with angiogenesis inhibitors and the improvement of (adoptive) immunotherapy by inhibitors of angiogenesis as a novel treat-ment strategy for cancer.

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