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Research Article

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Leukocyte Adhesion in Angiogenic Blood Vessels

Role of E-selectin, P-selectin, and β2 Integrin in Lymphotoxin-mediated Leukocyte Recruitment in Tumor Microvessels

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Abstract

Interaction of circulating leukocytes with tumor microvasculature is a critical event in the recruitment of effector cells into the tumor stroma. We have examined the ability of lymphotoxin (TNF-β), to stimulate rolling, adhesion, and transmigration of leukocytes in angiogenic blood vessels induced by tumor spheroids of Lewis lung carcinoma (LLC) implanted in dorsal skinfold chambers of nude mice. In the absence of cytokine stimulation, circulating leukocytes failed to appreciably interact with tumor microvessels (TMV), although significant rolling and adhesion was observed in normal vessels. However, stimulation with lymphotoxin (LT) resulted in a rapid increase in the number of fast and slow rolling leukocytes in TMV. Treatment with anti–P-selectin mAb 5H1 resulted in inhibition of fast rollers alone, while combination treatment with anti–P-selectin and anti–E-selectin (9A9) mAbs effectively blocked slow rolling of leukocytes. Superfusion of the lymphotoxin-stimulated neovasculature with leukotriene B4 (LTB4) resulted in stable cell adhesion followed by emigration of leukocytes into the tumor stroma. LTB4-mediated adhesion and transmigration was significantly inhibited by treatment with anti-β2 mAb 2E6. These studies delineate a multistep cascade of leukocyte adhesion in TMV and demonstrate that stimulation of the neovasculature with cytokines and chemoattractants can result in P- and E-selectin–dependent rolling and β2-dependent stable adhesion followed by transmigration into the tumor stroma. (*J. Clin. Invest.* 1997. 99:2246–2253.) Key words: selectins • tumors • cytokines • angiogenesis • leukocyte recruitment

Introduction

Angiogenesis and the establishment of tumor microvessels (TMV)[1] is an important regulatory feature for the growth and development of tumors (1, 2). The ability of immune effector cells to infiltrate tumors during immune surveillance is dependent on their ability to interact with endothelial cells lining the angiogenic blood vessels and extravasate into the tumor stroma. It is well established that the recruitment of circulating leukocytes into the interstitial tissue is dependent on a multistep cascade of events involving sequential rolling, firm adhesion, and transmigration that are mediated by distinct adhesion and activation pathways (3, 4). Selectins (3, 4) and α4-integrins (5, 6) mediate rolling of leukocytes in postcapillary venules of the systemic circulation, while firm adhesion of rolling leukocytes is dependent on α4/vascular cell adhesion molecule (VCAM)-1 (6) or β2/intercellular adhesion molecule (ICAM)-1 interactions (7). In contrast, relatively little is known about factors that regulate various steps of leukocyte adhesion and their transmigration across TMV in vivo. In a rat model of tumor microcirculation, leukocyte rolling was observed to be significantly lower in TMV compared with normal vessels, both under control conditions and after stimulation with fMLP, LPS, or TNF-α (8). Likewise in a murine model of tumor angiogenesis, leukocytes failed to roll in significant numbers in TMV of nude mice, while activation with TNF-α resulted in an increase in leukocyte rolling in these vessels (9). The absence of significant rolling or adhesion of leukocytes in TMV could be related to the downregulation or lack of expression of vascular adhesion molecules in tumor neovasculature. Recent studies of the tumor vasculature have revealed that expression of vascular adhesion molecules might be restricted to the normal tissue surrounding the tumors (10), and cellular infiltrates associated with tumors are primarily confined to the periphery of tumors (11), suggesting entry of infiltrating cells into the tumor stroma from the surrounding normal tissue. While many investigators have observed increased expression of P-selectin, E-selectin, ICAM-1, ICAM-2, and VCAM-1 in primary tumor sites of human melanomas, renal and breast carcinomas (12–16), others have failed to observe expression of VCAM-1 or E-selectin in tumor vessels (17, 18).

In the present report we investigated the ability of LT (lymphotoxin or TNF-β), a proinflammatory cytokine to induce rolling, adhesion, and transmigration of leukocytes in Lewis lung carcinoma (LLC)-induced TMV using a dorsal skinfold chamber model of tumor angiogenesis in nude mice (19, 20). LT has been shown to be cytotoxic and possess growth inhibitory or antitumor activity on some malignant tumors (21–24), despite being less toxic than TNF-α in mice (23). LT has also been observed to possess stimulatory effects on B cells (25–27). Moreover, LT induces leukocyte adhesion to cultured endothelial cells in vitro (28) and upregulates VCAM-1 and ICAM-1 in transgenic mice (29). The ability of circulating leukocytes to interact with neovasculature is important for their extravasation into the tumor stroma. Using intravital microscopy, we have therefore examined the efficacy of LT in combination with LTB4, an inflammatory mediator and a po-

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1. *Abbreviations used in this paper:* FR, fast roller; ICAM, intercellular adhesion molecule; LLC, Lewis lung carcinoma; LT, lymphotoxin; SR, slow roller; TMV, tumor microvessels; VCAM, vascular cell adhesion molecule.

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tent chemotactic substance that causes emigration from post capillary venules (30), to mediate early events of leukocyte adhesion and their transmigration from TMV into stroma of microtumors.

Methods

Preparation of multicellular tumor spheroids

LLC tumor cells were cultured in DME (Gibco Laboratories, Grand Island, NY) containing 10% FCS (Gemini Bioproducts Inc., Calabasas, CA). Cultures were maintained in a humidified 5% CO_2 atmosphere at 37°C. To prepare multicellular aggregates or tumor spheroids, cells were first washed with fresh complete medium and then resuspended in a volume of 5 ml complete medium (5×10^6 cells/ml) and placed into a 50-ml flask and rocked on a gyratory shaker in a humidified gas mixture of 5% CO_2 and 95% room air at 37°C. Solid tumor spheroids were formed after 24–48 h of shaking. Tumor spheroids with similar diameters (600–1,000 μm) were selected and then washed three times with PBS, pH 7.4. Spheroids were individually picked with sterile glass pipette and used for implantation in the dorsal skinfold chambers. This procedure insures single spheroids of similar size for implantation in the chamber.

Animal model and surgical techniques

Dorsal skinfold chambers in mice were prepared as previously described (19, 20, 31, 32). Briefly, 8–10-week-old nude mice (25–35 grams body weight) were anesthetized with a subcutaneous injection of a saline solution containing a cocktail of ketamine hydrochloride and Xylazine (7.5 and 2.5 mg, respectively, per 100 mg body weight) and placed on a heating pad. One pair of identical titanium frames were implanted into a dorsal skinfold parallel to the dorsum so as to sandwich the stretched double layer of skin. One layer of the dorsal skin was removed in a circular area of 15 mm diameter using an operation microscope to facilitate implantation of the titanium chambers. The underlying thin layer of striated skin muscle (M. cutaneous max.), subcutaneous tissue, and epidermis was covered with a cover slip enclosed in one of the frames and animals allowed to recover from anesthesia. After a recovery period of 2–4 d, the coverslip of the chamber was removed and tumor spheroids were carefully placed over the upper tissue layer of the chamber and the chamber closed again with the coverslip. The animals were housed individually in a room maintained at 21–22°C with free access to water and standard laboratory chow.

Intravital microscopy

Unanesthetized mice were placed in a plexiglass tube with the chamber extending from a longitudinal slit and immobilized on a platform. Microscopy was performed using a Leitz Biomed intravital microscope. Observations were made 1 wk after implantation of spheroids to determine the extent of angiogenesis using Nikon Inc. (Melville, NY) 4X (numerical aperture [NA] = 0.10), Nikon 10X (NA = 0.30), and Leitz 25X (NA = 0.60) objectives. Once extensive angiogenesis was established in the skin chamber (usually 10–14 d), the platform was placed on the microscope stage for observation of the tumor microcirculation. Passage of mouse leukocytes in TMV was made visible either by transillumination or by stroboscopic epiillumination of acridine orange–labeled leukocytes using a video triggered Xenon arc and a Leitz Ploemopak using appropriate filter blocks. All images were recorded through a silicone intensified tube camera (SIT68; DAGE MTI, Michigan City, IN) attached to the microscope and connected to a monitor (Panasonic) and an S-VHS video recorder (HC-6600; JVC, Tokyo, Japan) using a Nikon 10X or a Leitz 25X water immersion objective. Leukocytes visibly interacting with the microvascular endothelium and passing at a slower rate than the main blood stream were considered as rolling cells, while those remaining stationary for $>$ 1 min were considered as adherent cells.

Antibodies

Function blocking monoclonal antibodies raised against murine: P-selectin (mAb 5H1; rat IgG1) and E-selectin (mAb 9A9; rat IgG2b) (32–35), anti-α4 (mAb PS/2; rat IgG2b; generously provided by Dr. Eugene C. Butcher, Stanford University, Stanford, CA) (36) and anti-β2 antibodies (mAb 2E6; hamster IgG) (37) were used. Appropriate isotype-matched control antibodies were used as controls. Final concentration of endotoxin levels determined by Limulus amebocyte lysate test (BioWhittaker, Inc., Walkersville, MD) were $<$ 0.6 EU/ml.

Experimental procedure

Effect of LT on leukocyte rolling in TMV. Nude mice with fully developed neovasculature (10–14 d after tumor spheroid implantation) were examined for blood flow in TMV. Mice with poorly vascularized blood vessels ($\sim$ 20–30%) were excluded from the study. Mice with good blood flow in TMV were injected (i.v.) with LT (generous gift from Dr. Gordon Vehar, Genentech Inc., San Francisco, CA) at a concentration of 0.28 mg/kg body weight. The animals were then monitored at regular time intervals (0, 1, 2, 4, 6, 8 and 24 h) for maximal leukocyte rolling. Subsequently, animals were given either (2 mg/kg body weight) function blocking anti–P-selectin (mAb 5H1), anti–E-selectin (mAb 9A9), anti-α4 (mAb PS/2) or anti-β2 (mAb 2E6) antibodies sequentially or alone to determine their effect on leukocyte rolling and adhesion. Appropriate isotype matched normal antibodies were used as controls. Peripheral blood was drawn from nude mice before and after treatment with LT and the total systemic leukocytes/milliliter of blood determined.

Effect of LTB4 on firm adhesion and transmigration. The effect of LTB4 superfusion on stable arrest (firm adhesion) and emigration of rolling leukocyte into the tumor stroma was also investigated. Briefly, mice were stimulated with LT for 6–8 h and then administered (1 ml/kg body weight, i.v.) acridine orange (2.0 mg/ml PBS). The coverslip from the skinfold chamber was carefully removed and the tissue within the chamber was superfused (100–200 μl) with different concentration of LTB4 (10^{-7} to 10^{-9} M) for a period of 2 min. The chamber was closed ensuring that no air bubbles were trapped and the effect of LTB4 superfusion on leukocyte adhesion and transmigration was determined. In addition, the effect of β2 integrin blockade on leukocyte adhesion and emigration was examined. mAb 2E6 (2 mg/kg body weight) or isotype matched control mAbs were administered i.v. along with acridine orange before LTB4 superfusion.

Image analysis

Rolling of leukocytes in the skin chamber vessels was assessed from video recordings and analyzed as previously described (32). Rolling was analyzed by manually counting the total number of rolling cells passing through a reference point in a vessel segment and expressed as rollers/minute. Adherent leukocytes were defined as cells that remained stationary in the tumor vessels for at least 1 min and expressed as cells/100 μm length of tumor vessels. Leukocyte extravasation into tumor stroma was quantified by enumerating the number of cells that had transmigrated out (50–200 μm away from the vessel wall) in parallel with a 200-μm tumor vessel segment. In addition, rolling velocity of individual cells in TMV was determined by frame-by-frame analysis of recorded video images and expressed as micrometer/second. Leukocytes rolling at velocities $<$ 3 μm/s were considered as slow rollers, while those leukocytes rolling at $>$ 5 μm/s were considered as fast rollers. In addition the wall shear rate was calculated for each vessel as previously described (32, 38): $Vc/1.6 \times 1/D \times 8$ (s^{-1}) where D and Vc represent centerline blood flow velocity and vessel diameter, respectively. The corresponding wall shear stress (dyn/cm^{-2}) within TMV was determined assuming a blood viscosity of 0.025 poise. Statistical analyses were made using analysis of variance and multiple comparisons tests. For all tests, P values $<$ 5% were considered significant. Data are presented as mean±SEM (unless specified as mean±SD). Statistical calculations were computed with a statistical software package (SigmaStat; Jandel Scientific, San Rafael, CA).

Results

Tumor spheroids of LLC induce neovascularization in dorsal skinfold chambers of nude mice. Tumor spheroids (600–1,000 μm) prepared from LLC were implanted in the dorsal skinfold chambers of nude mice (Fig. 1 *a*). After 10–14 d after implantation, tumor spheroids had induced a well defined vascular network in the tumor area (Fig. 1 *b*). Evaluation of the microcirculatory parameters such as leukocyte–endothelial interactions in these mice revealed appreciable rolling of leukocytes in normal venules (23±12 rollers/min) (diameter 41.6±4.4 μm; $n = 20$ vessels in nine mice; i.e., $n = 20/9$) (Fig. 2 *a*) , but not in TMV (1.04±0.2 rollers/min) (diameter 38.1±2.0 μm; $n = 31/7$) (Fig. 1 *c* and Fig. 2 *a*). As previously described (32, 39), the baseline rolling of leukocytes observed in normal vessels of the skin chamber was due to a continuous and sustained expression of P-selectin, and mAbs against murine P-selectin (mAb 5H1) but not E-selectin (mAb 9A9) inhibited this rolling (data not shown).

Stimulation with LT induces leukocyte rolling in TMV. Since significant rolling of leukocytes was not observed in TMV during control conditions, mice were administered with the proinflammatory cytokine LT, and its effect on inducing rolling in TMV as well as in normal vessels was determined over a 24-h period. LT induced a significant increase in the flux of interacting cells in tumor (Fig. 1 *d*, 4 h) as well as in normal vessels in a time-dependent manner. In comparison to a base line rolling of 23±12 rollers/min in normal vessels ($n = 6/3$), stimulation with LT resulted in a two- to threefold increase in rolling, and a peak response was observed between 6 to 8 h after LT administration (47±17.1 rollers/min at 6 h after LT, $P = 0.02$). Stimulation of TMV with LT in contrast resulted in a dramatic increase in the flux of rolling leukocytes, from a base line of 1.04±0.2 rollers/min to 21.6±3.6 rollers/min at 6 h ($n = 31/7$; $P = 0.0013$) (Fig. 2 *b*). Maximal rolling in TMV was also observed between 6 to 8 h after LT administration (Fig. 1 *d* at 4 h and Fig. 2 *b*). The effect of LT stimulation lasted up to 24 h, after which the flux of rolling leukocytes was reduced to the prestimulation levels in both normal vessels and TMV. Although, LT stimulation resulted in an increase in the rolling in TMV, the systemic counts before and after LT stimulation were not significantly altered (2.8 ± 10^6/ml vs 3.1 ± 10^6/ml).

In 6 out of 12 animals stimulated with LT, a fraction of the leukocyte population was observed to be apparently adherent to TMV (14/27 vessels examined). A careful examination of the recorded video images revealed that a significant proportion of these apparently adherent cells were actually rolling cells moving at extremely low velocities. To ascertain that the slow rolling cells were not adherent cells, the animals were pretreated with a neutralizing anti–murine β2 mAb 2E6 immediately after LT administration. In all the animals tested ($n = 14/6$), the anti-β2 mAb failed to reduce the number of slow rolling cells (24.4±5.2 rollers/min vs 24.2±11.2 rollers/min during control period, $P = 0.88$). Overall three types of interacting cells were observed in TMV in response to stimulation with LT: fast rollers (FR), slow rollers (SR), and adherent cells. The mean rolling velocity of SR in TMV was 0.6±0.7 μm/s (mean±SD; range 0.04 to 3 μm/s; $n = 19/8$) (Fig. 2 *c*) while the velocity of FR was 54.9±36.9 μm/s (mean±SD; range 5 to 200 μm/s; $n = 23/8$) (Fig. 2 *d*). Thus, leukocytes rolling at velocities < 3.0 μm/s were considered as SR while those with rolling velocities > 5 μm/s were considered as FR. The 10-fold difference in velocities between FR and SR was not due to alterations in the wall shear stress in TMV. The wall shear stress in vessels with FR alone was 1.7±0.2 dynes/cm^2 (range: 0.9 to 3.4 dynes/cm^2) and 1.4±0.1 dynes/cm^2 (range: 0.9 to 2.3 dynes/

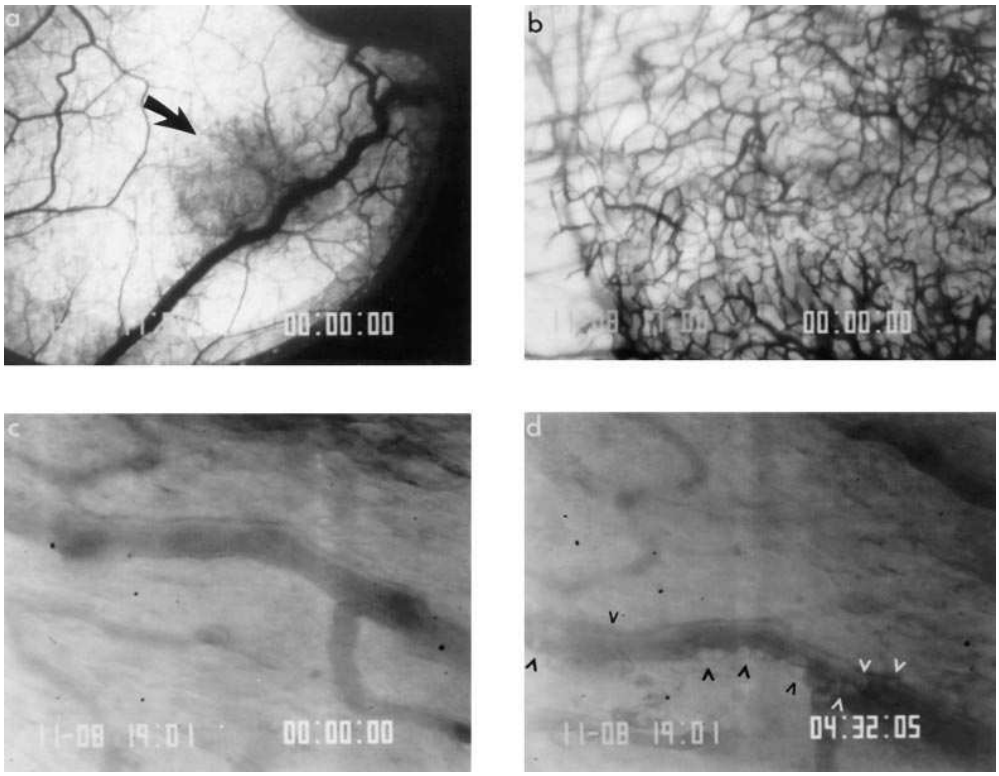

Figure 1. (*a*) Photomicrograph showing an overview of an implanted Lewis lung carcinoma (LLC) spheroid (*arrow*) 10 d after implantation in a dorsal chamber of a nude mouse using trans-illumination (4× objective). (*b*) Photomicrograph of the angiogenic response induced by LLC spheroid 10 d after implantation (10× objective). A well established vascular network with extremely high density is normally evident within 10–14 d of spheroid implantation. (*c*) Blood flow in TMV (average diameter: 38±2 μm) before LT stimulation. Blood flow is from left to right. No rolling was observed in TMV in absence of cytokine stimulation. (*d*) Stimulation with LT (4 h) results in induction of leukocyte rolling in TMV.

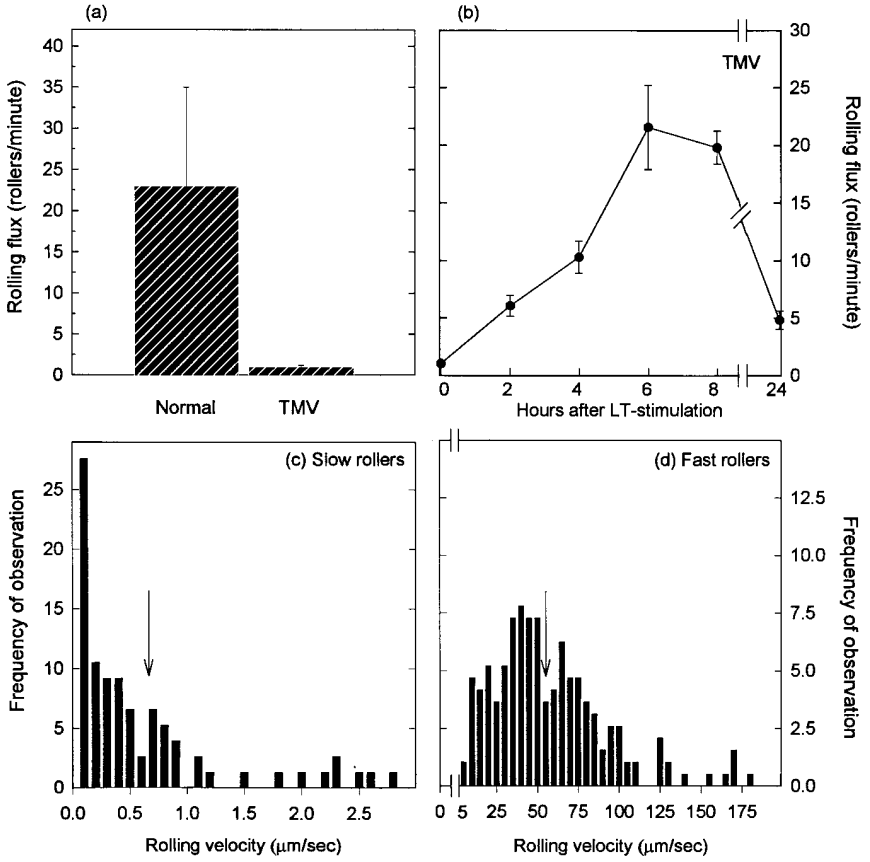

Figure 2. (*a*) Spontaneous rolling of leukocytes is observed in normal vessels but not TMV. Tumor spheroids of LLC were implanted in the dorsal skinfold chambers for a period of 10–14 d. In absence of cytokine stimulation, no significant rolling was observed on the endothelial cells lining the walls of TMV (diameter: 38.1±2.0 μm; n = 31 vessels in seven mice), while considerable rolling was evident in normal blood vessels (diameter: 41.6±4.4 μm; n = 20 vessels in nine mice) outside the tumor vasculature. (*b*) LT stimulation induces leukocyte rolling in TMV. Administration of LT (0.28 mg/kg body weight, i.v.) resulted in an increase in the flux of rolling leukocytes in TMV. A maximum response was observed 6–8 h after LT stimulation. Rolling flux was quantitated as described in the Methods section. (*c* and *d*) Frequency distribution profiles of slow rollers (*SR*) and fast rollers (*FR*) after stimulation with LT. The flux of FR and SR was determined in TMV 6 h after LT stimulation. Subsequently the velocity of individual FR and SR was manually determined by off-line analysis of recorded video images and expressed as micrometers per second. The histograms reveal frequency observations of 70–200 interacting cells from 17–24 vessels obtained from eight mice. Arrows indicate the mean rolling velocity of slow and fast rollers.

cm^2) in vessels with FR + SR (P = 0.102). Moreover, there was no significant difference in shear rates between control and LT-treated tumor vessels (data not shown).

P-selectin independently supports fast rolling of leukocytes (FR), while E- and P-selectin in combination mediate slow rolling (SR) in TMV. To define the roles of individual adhesion receptors in LT-mediated rolling and adhesion of leukocytes in TMV, the effects of function blocking antibodies raised against E- and P-selectins, α4, β2-integrins, and isotype-matched control antibodies were determined (Figs. 3 and 4). Mice were initially stimulated with LT and selected animals were simultaneously administered anti-β2 mAb 2E6. 6 h after LT stimulation, mice were treated with the different mAbs (either alone or in combination but administered sequentially) and the effects on the flux of FR, SR or as combined flux was determined. Treatment with anti–P-selectin mAb 5H1 resulted in a 89.4±3.6% inhibition of FR (mice with FR alone: n = 9/5; P = 0.006 vs control) (Fig. 3 *a*), while in mice with FR and SR, anti–P-selectin mAb 5H1 resulted in 44.3±7.8% inhibition of rolling (n = 12/5; P = 0.02 vs control) (Fig. 3 *a*). The remaining

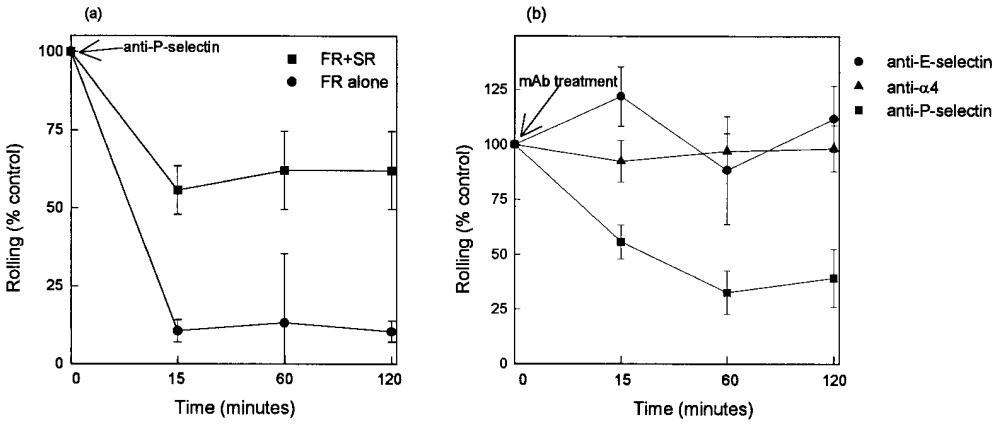

Figure 3. (*a*) P-selectin independently supports fast rolling of leukocytes (*FR*). The effect of anti–P-selectin mAb 5H1 (2 mg/kg body weight) on the flux of rolling leukocytes in mice with FR alone and mice with both FR and SR was determined. Anti–P-selectin mAb completely inhibited leukocyte rolling in mice with FR alone (n = 5 mice), while only partially inhibition of rolling was observed in mice with FR + SR (n = 5 mice). (*b*) The effect of anti-α4, anti–E and P-selectin mAbs on flux of rolling leukocytes in TMV. LT-stimulated mice were treated with different antiadhesion molecule mAbs and their effects in mice with FR or FR + SR in TMV was determined (n = 3–5 mice).

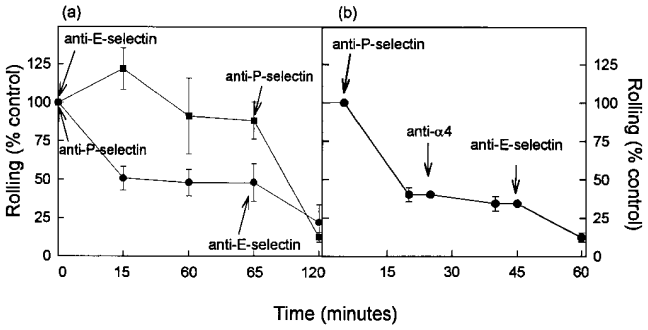

Figure 4. (*a*) Blocking of both E- and P-selectin inhibits fast and slow rolling leukocytes. Treatment of LT stimulated mice with anti–E- and anti–P-selectin mAbs in combination but not alone resulted in complete inhibition of FR + SR in TMV (n = 13 vessels in five mice). Arrows indicate the time when the antibodies were administered. (*b*) Combination treatment with anti–P-selectin followed by anti-α4 (PS/2) mAbs had no significant effect on the flux of leukocyte rolling, while subsequent treatment with anti–E-selectin mAbs in both cases resulted in complete inhibition of rolling (FR + SR). Arrows indicate the time when the antibodies were administered.

rolling cells observed in TMV were predominantly SR in nature. Treatment of nude mice with anti–E-selectin mAb 9A9 alone did not alter the flux of FR (n = 6/3) or FR + SR (n = 4/3) (Fig. 3 *b*). Likewise, treatment of LT-stimulated mice with anti-α4 mAb PS/2 at the same concentration had no significant effect on the flux of the FR or SR in TMV (Fig. 3 *b*). Treatment of mice with isotype-matched control antibodies (rat IgG2b, or IgG1) failed to inhibit leukocyte rolling in TMV (data not shown).

Since anti–P-selectin mAb 5H1 resulted in only 44.3±7.8% decrease in rolling (predominantly inhibition of FR in mice with FR + SR), we determined if other adhesion molecules in combination with P-selectin, were involved in SR. While an initial treatment of anti–P-selectin mAb 5H1 resulted in a 49.2±7.7% inhibition of the total flux (n = 13/5), subsequent treatment with anti–E-selectin mAb 9A9 resulted in a 78±11.6% inhibition of the total flux (P = 0.007 vs control; P = 0.04 vs mAb 5H1 treatment) (Fig. 4 *a*). In two out of five animals tested (n = 5 vessels) sequential treatment with the two mAb resulted in complete inhibition of rolling ($P < 0.001$), while the remaining animals demonstrated residual slow rollers in the TMV (n = 8 vessels). In an analogous manner, treatment with anti–E-selectin mAb (which had no effect on the rolling flux of SR or FR), followed by treatment with anti–P-selectin mAb 5H1, resulted in complete or significant inhibition of SR + FR (87.6±3.1%; $P < 0.001$ vs untreated mice) (Fig. 4 *a*). Combination treatment with anti–P-selectin mAb followed by anti-α4 mAbs did not result in further inhibition of rolling (i.e., remaining SR) (Fig. 4 *b*). However, subsequent administration of anti–E-selectin mAb resulted in complete inhibition of SR (Fig. 4 *b*) suggesting the involvement of P-selectin along with E-selectin in mediating slow rolling in tumor vessels.

LT induces leukocyte adhesion in TMV of mice with slow rolling but not fast rolling leukocytes. In addition to mediating slow rolling of leukocytes, stimulation of mice with LT resulted in adhesion of leukocytes. However, adhesion was evident only in vessels with SR + FR but not in tumor vessels with FR alone. These leukocytes were found to be adherent (10.6±1.7 cells/100 μm; n = 34/13) to the vessel walls for at least 1 min and were distinct from slow rollers that were in constant but slow motion. Treatment of mice (FR + SR) with anti-β2 mAb 2E6 failed to reduce the number of adherent leukocytes in these vessels (11.3±2.3 cells/100 μm; n = 17/8 (Fig. 5). However treatment of mice with anti–E- and anti–P-selectin mAbs in combination resulted in 54.5±23% inhibition (4.8±2.4 cells/100 μm; n = 15/6; P = 0.03 vs control) in the number of adherent cells (Fig. 5). When administered individually, anti–P-selectin mAb 5H1 (25.3±18.2% inhibition; 7.9±1.9 cells/100 μm; n = 13/5; P = 0.17 vs control) or anti–E-selectin mAb 9A9 (9.4±1.8 cells/100 μm) had no significant effect on the number of adherent cells (Fig. 5). Leukocyte adhesion was absent not only in vessels with FR alone but also in the control group administered vehicle alone. These results suggest that slow rolling of leukocytes is a prerequisite for LT-mediated adhesion in the tumor neovasculature.

LTB4-mediated firm adhesion and transmigration of leukocytes in LT-stimulated TMV is β2 integrin dependent. Although LT induced adhesion of leukocytes in vessels with SR + FR, none of these adherent cells were observed to emigrate into the tumor stroma. However, local superfusion of LT-stimulated tumor neovasculature with increasing concentrations of LTB4 (10^{-9} to 10^{-7} M) resulted in a dose-dependent increase in stable adhesion (in vessels with FR and FR + SR) followed by emigration of leukocytes into the extravascular space. Superfusion of vessels (FR alone) with LTB4 at a concentration of 10^{-9} M failed to induce significant adhesion of rolling leukocytes (1.2±0.9 adherent cells/100 μm; n = 24/9), while at concentrations of 10^{-8} M (8.3±1.7 adherent cells/100 μm) and 10^{-7} M (9.3±1.2 adherent cells/100 μm), a dramatic increase in

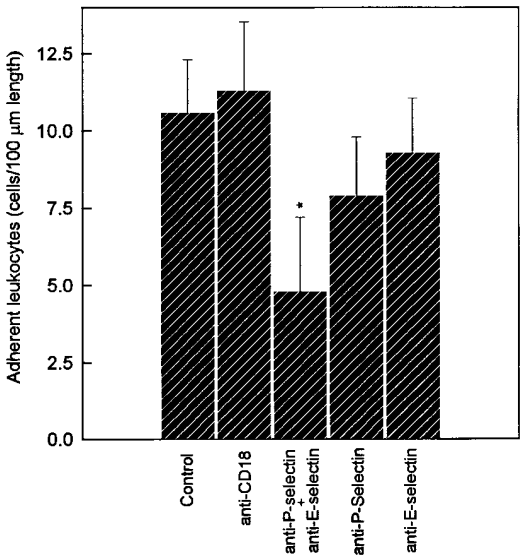

Figure 5. Anti–E- and P-selectin mAbs in combination block LT-mediated leukocyte adhesion in TMV. Treatment of mice stimulated with LT for 6 h with anti-β2 mAb 2E6 or anti–E- or anti–P-selectin mAbs individually failed to inhibit the adhesion observed in TMV, while combination treatment with anti–E- and P-selectin mAbs resulted in partial inhibition of leukocyte adhesion. (n = 13–34 vessels in 5–13 mice) (*$P < 0.05$).

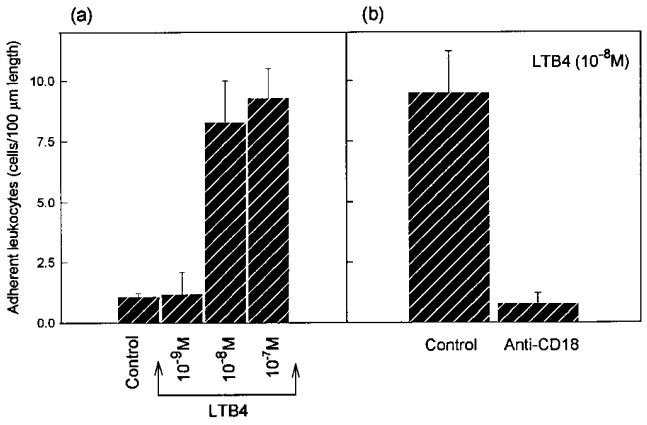

Figure 6. (*a*) LTB4 induces stable arrest of rolling leukocytes in LT-stimulated TMV. LT-stimulated mice were first administered with acridine orange followed by superfusion with LTB4. Dose-dependent increase in the adhesion of FR in TMV was observed within minutes of LTB4 superfusion (n = 24 vessels in nine mice). (*b*) LTB4-mediated stable arrest of rolling leukocytes (FR and FR + SR) in TMV is β2 integrin dependent. The number of adherent cells/100 μm length of tumor vessel in absence or presence of anti-β2 mAb was determined by off-line analysis of recorded video images. The data represents mean (±SEM) of adherent leukocytes/100 μm length of tumor vessels with FR and FR + SR.

the number of firmly arrested leukocytes in TMV was observed ($P < 0.0001$ vs control: 1.1±0.5 adherent cells/100 μm) (Fig. 6 *a*). Likewise LTB4 (10^{-8} M) treatment of vessels with SR + FR resulted in three- to fivefold increase in leukocyte adhesion (data not shown). Furthermore, superfusion of LT-stimulated tumor vessels with LTB4 (10^{-8} M) resulted in a significant decrease in the number of rolling cells. Compared with 17.9±2.6 rollers/min during the control period, only 1.9±1 rollers/min ($P = 0.0004$) were observed in the LTB4-superfused tumor vessels (average flux in vessels with FR or FR/SR). Pretreatment of mice with anti-β2 mAb 2E6 before LTB4 administration resulted in very significant inhibition of adhesion (0.8±0.4 cells/100 μm vs 9.5±1.9 cells/100 μm in untreated animals; $P < 0.0001$) (Fig. 6 *b*). In addition to mediating firm adhesion, LTB4 induced emigration of adherent leukocytes from the microvessels (3–12 transmigrated cells/across 200 μm vessel segment) into the tumor stroma (Fig. 7 *a*). Likewise, pretreatment of LT-stimulated mice with anti-β2 mAb 2E6 before LTB4 administration resulted in significant inhibition of adhesion and complete inhibition of transmigration across TMV (Fig. 7 *b*). Moreover, the mAb treatment also resulted in an increase in the fraction of rolling leukocytes (24.4±4.9 rollers/min compared with 1.9±1.0 rollers/min in LTB4-treated control group; $P < 0.0001$). In contrast, normal IgG control had no effect on LTB4-mediated firm adhesion and transmigration (data not shown). Furthermore, in absence of LT-stimulation, LTB4 failed to induce significant rolling (2.3±0.21 rollers/min; FR only) or adhesion (1.95±0.23 adherent cells/100 μm length). Similarly, LTB4 failed to induce any transmigration of leukocytes into the tumor stroma in absence of LT stimulation. These studies demonstrate that cytokine dependent firm adhesion is a prerequisite for chemoattractant-mediated transmigration in TMV of nude mice.

Discussion

The regulated expression of vascular adhesion molecules during immune surveillance of tumors and their involvement in the recruitment of leukocytes into tumor stroma is a poorly understood process. Adhesion molecules orchestrate a sequential interaction of marginating leukocytes with vascular endothelium through specific adhesion and activation-dependent pathways (4, 5). In the present study, we have examined the function of various leukocyte and vascular adhesion molecules in the recruitment of circulating leukocytes through angiogenic blood vessels to sites of active growth of tumors. Using intravital microscopy and a dorsal skinfold model of tumor angiogenesis (19, 20), several important features of TMV have been identified: (*a*) leukocytes fail to roll efficaciously or interact with unstimulated tumor neovasculature, but demonstrate extensive rolling in normal venules of the skin of nude mice during control conditions. It is conceivable that the lack of leukocyte rolling might be related to the inability of TMV to express

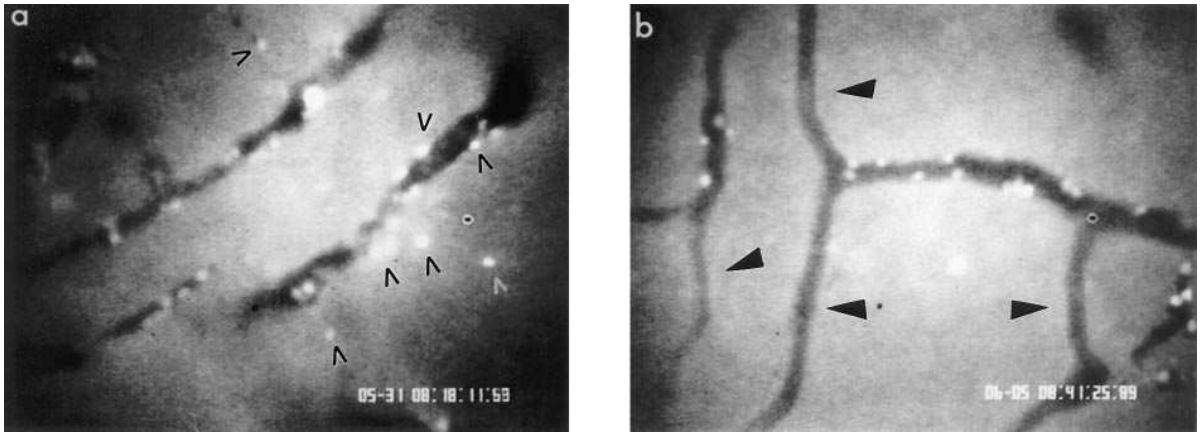

Figure 7. LTB4 mediates β2-integrin–dependent transmigration of adherent leukocytes. LT-stimulated mice were first injected with acridine orange. The animals were then treated with either (*a*) control IgG or (*b*) anti-β2 mAb 2E6 (2 mg/kg body weight). Subsequently, the LT-stimulated TMV were superfused with 10^{-8} M LTB4 and its effect of leukocyte transmigration determined. Open arrows represent acridine orange–labeled leukocytes that have emigrated into the tumor stroma in response to superfusion with LTB4 in control mice (*a*), while closed arrows represent blood vessels devoid of leukocyte adhesion in mice treated with anti-β2 mAb (*b*).

significant levels of vascular adhesion molecules in absence of specific cytokine stimulation. (*b*) Tumor-induced neovascular endothelial cells have the ability to express vascular E- and P-selectins (as determined by function blocking antibodies) that are upregulated in response to activation with cytokines such as LT and subserve an important function of supporting leukocyte rolling (FR and SR) in TMV. The presence of either FR or FR + SR in different tumor vessels was not due to differences in hemodynamic parameters such as wall shear stress and vessel diameters. (*c*) The rolling induced by LT-stimulation is not sufficient to establish stable arrest and spontaneous emigration of leukocytes from TMV. This activation-dependent process (4, 5) can however be simulated by local superfusion of the angiogenic blood vessels with LTB4, a metabolite of arachidonic acid and a potent inflammatory and chemotactic mediator (30).

Growth of metastatic tumor cells is dependent not only upon the ability to induce an active angiogenic response (1, 2) but also the capacity to evade the host immune response. Although remarkable progress has been made in defining effector cells involved in the immune surveillance of tumors, overall, the treatment of metastatic microtumor colonies has remained elusive. In addition to other factors, the lack of efficient recruitment of tumor killing leukocytes into the core of the tumor stroma could be a contributing feature. The relative abundance of immune effector cells on the periphery but not in the core of a tumor (11) imply that TMV may not be particularly effective targets for immune effector cell adhesion/recruitment. This is also evident from our observations as well as those reported by others (8, 9) demonstrating a significantly diminished rolling of leukocytes in TMV compared with normal vessels, and could be due to markedly reduced expression of adhesion molecules in the TMV. Although increased levels of E-selectin, P-selectin, VCAM-1, and ICAM-1,2,3 have been associated with tumors (12–16), the expression of VCAM-1 was found to be suppressed in the small tumor vessels (< 80 μm) of the lung (17). Similarly, the expression of E-selectin was not evident in tumor vessels of melanomas (18) while the expression of PNAd and MAdCAM-1 was absent in the endothelium of noninfiltrated tumors (40). The downregulation of E-selectin and VCAM-1 expression in TMV has been suggested to be mediated by soluble factors released by tumors (17) including TGF-β1 (40, 41). Moreover, it is conceivable that other soluble factors such as VEGF and bFGF, not only induce tumor angiogenesis (20, 42, 43), but also downregulate expression of adhesion molecules by the neovasculature. Thus tumor-dependent downregulation of vascular adhesion molecules in TMV and the inability of circulating leukocytes to interact with tumor vasculature may be a critical component in the evasion of host immune surveillance.

Stimulation of tumor-induced vasculature with specific cytokines is likely to induce an inflammatory response and promote leukocyte margination and rolling. Consistent with this hypothesis are recent observations demonstrating an increase in leukocyte rolling after stimulation of tumor vessels of nude mice with TNF-α (9), although the adhesion molecules involved in rolling were not identified. Our results demonstrate that LT can significantly induce a selective E- and P-selectin–dependent rolling in TMV. The inability of anti-α4 mAbs to inhibit leukocyte rolling in TMV in contrast might be due to the absence of VCAM-1 expression by tumor vessels (17). However, the relative importance of L-selectin or α4 integrin in mediating leukocyte rolling in TMV under different inflamed settings such as those induced by other cytokines or in other strains of mice is not known. In nude mice, stimulation of TMV with LT results in a P-selectin–dependent (PSGL-mediated) initial rolling of leukocytes (FR), while those microvessels expressing significant levels of E-selectin and/or those leukocyte expressing counter receptors for E-selectin participate in strengthening of the initial adhesive interaction to mediate rolling at reduced velocities (SR). It is conceivable that the transition from FR to SR might be a rate limiting step in the effective recruitment of leukocytes into the tumor stroma. Though it is not clear, what factors precisely control the transition of fast to slow rollers in TMV, in absence of chemokine activation, slow rolling of leukocytes appears to be a prerequisite for their spontaneous adhesion in LT-stimulated tumor vessels. Furthermore, the systemic leukocyte counts of nude mice with the implanted LLC tumor spheroids was not effected by treatment with LT and is suggestive of the lack of nonspecific inflammation.

Although ICAM-1 expression appears to be induced in the neovasculature, in the absence of further activation, either stable arrest (of rolling or weakly adherent leukocytes) or transmigration of leukocytes is not an abundant feature of LT-stimulated tumor vessels. This latter effect is easily compensated by superfusion of the neovasculature with LTB4 which results in firm adhesion followed by transmigration of adherent leukocytes. Treatment of mice with anti-β2 mAb 2E6 resulted in significant inhibition of both these steps, suggesting a β2-activation–dependent mechanism of leukocyte stable arrest and emigration in TMV. Our studies provide evidence that appropriate stimulation of TMV with specific cytokines and chemotactic mediators such as LT and LTB4 (30, 38) can result in efficient recruitment of leukocytes into the tumor stroma. Since extravasation of leukocytes is crucial to their subsequent ability to induce tumor killing, further studies will determine if treatment of LT-stimulated mice with chemokines, especially those that lack the ELR motif (44) can result in an effective recruitment of leukocytes as well as inhibition of growth of microtumors and angiostatic response in vivo. It is possible that such a combination treatment could augment the inhibition of tumor growth mediated by LT (21). Gene transfer of plasmacytomas using human LT expressing plasmids in nude mice resulted in inhibition of tumor growth that was associated with infiltration of macrophages and granulocytes.

In summary we demonstrate that tumor-induced angiogenic blood vessels in nude mice inherently fail to support leukocyte rolling or adhesion even after significant blood flow has been established. However, this evasive mechanism of tumor surveillance can be countered by targeting the neovasculature with LT and LTB4 which results in a P- and E-selectin–dependent rolling and β2-mediated leukocyte extravasation into the tumor stroma. These results suggest an attractive approach which, when coupled to gene or immunotherapy with cytokines, may potentiate significant mobilization of effector cells such as cytotoxic T-lymphocytes and eosinophils and inhibition of tumor growth.

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