Quantification of murine endothelial cell adhesion molecules in solid tumors

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LEUKOCYTE TRAFFICKING in inflamed tissues is mediated by adhesive interactions between surface receptors on leukocytes and their ligands on microvascular endothelial cells (26, 33, 36). Enhanced surface expression of these endothelial cell adhesion molecules (CAMs) occurs after endothelial cell activation with cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1, and interferon-γ, thereby facilitating the adhesion and extravasation of leukocytes (5). The expression of selectins (P-selectin, E-selectin) allows leukocytes to roll along the surface of endothelial cells (6). This low-affinity cell-cell interaction is often accompanied by an increased activation/surface expression of integrins on leukocytes, which can establish bonds with a different family (immunoglobulin supergene) of counter-receptors (e.g., intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1)) on endothelial cells that mediate stable adhesion and emigration of leukocytes (34). Given the importance of endothelial CAMs in the initiation and perpetuation of inflammatory responses and in other cell recruitment processes, such as tumor metastasis, much attention has been devoted to defining the changes in endothelial CAM expression that occur in diseased tissues.

Studies of leukocyte-endothelial cell adhesion in tumor microvessels have revealed diminished adhesive interactions under both basal (7, 11, 37) and cytokine-stimulated (11, 37) conditions. This observation is consistent with immunohistochemical (20) and cytofluorimetric (14, 29) studies that predicted a reduced endothelial CAM expression in tumor microvessels. It has been suggested that the proposed downregulation of endothelial CAMs facilitates tumor progression by allowing tumor cells to avoid immunosurveillance by circulating lymphocytes. There are, however, several other immunohistochemical studies of tumor vasculature that invoke an enhanced expression of endothelial CAMs, resembling an inflammatory phenotype, in non-small cell lung carcinoma (35), colorectal cancer (39), hemangiomas (19), and breast cancer (10). The complexity of endothelial CAM expression in tumor microvessels is also exemplified by recent reports that implicate angiogenic agents in the modulation of these adhesion glycoproteins (13, 22). A limitation of the aforementioned studies, however, is the absence of quantitative data that allow for an objective and systematic comparison of basal and stimulated endothelial CAM expression in tumors and normal vascular beds. Such information may provide mechanistic insights concerning the altered leukocyte trafficking that occurs in solid and metastatic tumors, and it could lead to the development of therapeutic interventions that selectively enhance the immunogenicity of tumor vessels.

Recently, a technique employing radiolabeled monoclonal antibodies (MAbs) against endothelial CAMs has been used to obtain quantitative estimates of endothelial CAM expression in different vascular beds (28). This technique has been used to measure P- and E-selectin (9) as well as ICAM-1 and VCAM-1 expression (16) in normal and acutely inflamed vascular beds.
In the present study, the dual-radiolabeled MAb technique was used to quantify constitutive and TNF-α-induced expression of ICAM-1, ICAM-2, VCAM-1, P-selectin, E-selectin, and platelet-endothelial cell adhesion molecule 1 (PECAM-1) in different vascular beds of C57Bl/6 mice that developed tumors after subcutaneous inoculation with RM-1 prostate carcinoma cells. Immunohistochemistry was also employed to localize endothelial CAMs within RM-1 tumors.

**MATERIALS AND METHODS**

Animals and tumor cell line. Male C57Bl/6J mice (n = 129) weighing 24.0 ± 3.9 g (mean ± SD) were obtained from Harlan Laboratories (Frederick, MD) and maintained according to guidelines set forth by the Division of Animal Resources, Louisiana State University Medical Center. The tumor cell line RM-1 was kindly provided by Dr. Steven Jennings (LSU Medical Center, Shreveport, LA). This cell line is a ras + myc-transformed mouse prostate carcinoma cell line that was chosen for its high tumorigenicity (3). RM-1 tumor cells were cultured in DMEM (GIBCO Laboratories, Grand Island, NY) containing 5% FCS and antibiotics. Cultures were maintained in a humidified 5% CO2 atmosphere at 37°C. Tumor cells used for the experiments were selected at passages 10–15. RM-1 cells were harvested during log-phase growth, washed with DMEM, and counted using hemacytometry. Viability was assessed using the trypan blue exclusion technique.

Mice in the tumor-bearing group had their backs shaved and were cleaned with betadyne before being injected with tumor cells. Tumors were initiated by subcutaneous (dorsum) inoculation of 1.0 × 106 viable cells in 0.1 ml of Hanks’ balanced salt solution (HBSS). Constitutive and TNF-α (R&D Systems, Minneapolis, MN)-induced expression of endothelial CAMs was determined 10–15 days postinoculation, when tumors reached a weight of ~1 g (average 0.7 g). These animals were compared with an age- and weight-matched control group of non-tumor-bearing mice that received an injection of 0.1 ml of HBSS in the dorsum 10–15 days before experimental analysis. P-selectin- and ICAM-1-deficient mice, provided by Pharmacia-Upjohn (Kalamazoo, MI), were also injected with RM-1 cells to produce tumors.

Monoclonal antibodies. The MAbs used for in vivo quantification of ICAM-1 and VCAM-1 expression were YN-1, a rat IgG1 directed against murine ICAM-1, and MK1.9.1, a rat IgG1 targeted against mouse VCAM-1 (both provided by Dr. Mary Gerritsen, Genentech, San Francisco, CA). The MAbs used for in vivo assessment of P- and E-selectin were RB40.34 (Pharminigen), a rat IgG1 against mouse P-selectin and 10E6, a rat IgG2 against mouse E-selectin (24), respectively. For ICAM-2 determination, 3C4 (Pharminigen), a rat IgG2a that reacts with mouse ICAM-2 was used. MEC13.3 (Pharminigen), a rat IgG2a directed against murine PECAM-1, and P-23, a nonbinding murine IgG1 directed against human P-selectin, were also used in the experimental protocols.

Radioiodination of MAbs. Binding MAbs YN-1, MK1.9.1, RB40.34, 10E6, 3C4, and MEC13.3 were labeled with 125I and the nonbinding MAb P-23 was labeled with 131I (NEN, Boston, MA) using the iodogen method. Briefly, 250 μg of protein were incubated with 250 μCi of sodium 125I (or sodium 131I) and 125 μg of iodogen at 4°C for 12 min. PBS was then added to bring the total volume to 2.5 ml. The radiolabeled MAb was then separated from free 125I or 131I by gel filtration on a Sephadex PD-10 column (Pharmacia, Uppsala, Sweden). The column was equilibrated and then eluted with PBS containing 1% BSA. Two 2.5-ml fractions were collected, the second of which contained the radiolabeled MAb. Previous studies have demonstrated the ability of MAbs to retain their functional activity after radioiodination as evidenced by a similar effectiveness of labeled and unlabeled MAbs in blocking leukocyte adherence in rat mesenteric venules (28). Labeled MAbs were stored in 500-μl aliquots at 4°C.

Surgical procedure. Mice were anesthetized subcutaneously with a cocktail of ketamine and xylazine at doses of 150 and 7.5 mg/kg, respectively. The left jugular vein and right
vasculature of wild-type (C57Bl/6) and ICAM-1 constitutive value. BRM-1 tumor vessels. Values are means ± SE. *P < 0.05 vs. constitutive value. B: constitutive ICAM-1 expression in tumor vasculature of wild-type (C57Bl/6) and ICAM-1 −/− mice. Values are means ± SE. *P < 0.005 vs. C57Bl/6 mice.

Fig. 2. A: constitutive and TNF-α-stimulated ICAM-1 expression in RM-1 tumor vessels. Values are means ± SE. *P < 0.05 vs. constitutive value. B: constitutive ICAM-1 expression in tumor vasculature of wild-type (C57Bl/6) and ICAM-1 −/− mice. Values are means ± SE. *P < 0.005 vs. C57Bl/6 mice.

carotid artery were cannulated with polyethylene tubing (PE-10). In the ICAM-1 experiments, a mixture of 125I-labeled anti-ICAM-1 MAb (125I-YN-1; 10 µg), unlabeled anti-ICAM-1 MAb (40 µg), and an amount of 131I-labeled P-23 (131I-P-23) necessary to ensure a total 131I injected activity of 400,000–600,000 counts/min (cpm) was administered through the jugular vein cannula. Pilot studies utilizing a 10-µg dose of 125I-YN-1 in conjunction with 0–60 µg of cold YN-1 demonstrated that the combination of 10 µg of 125I-YN-1 and 40 µg of cold YN-1 provides optimum activity for accurately assessing ICAM-1 expression and ensuring receptor saturation under constitutive and cytokine-challenged conditions. In the VCAM-1 experiments, 10 µg of 125I-labeled anti-VCAM-1 MAb (125I-MK1.9.1) in combination with 20 µg of cold MK1.9.1 and an appropriate amount of 131I-P-23 (400,000–600,000 cpm) assured receptor saturation. For ICAM-2, complete receptor occupation was achieved with 10 µg of 125I-labeled 3C4 (125I-3C4) and 60 µg of cold 3C4, which was administered with 131I-P-23. Determination of PECAM-1 (CD31/endoCAM) necessitated 10 µg of 125I-labeled anti-PECAM-1 (125I-MEC13.3) with 30 µg of cold anti-PECAM in combination with P-23. For the selectin studies, a mixture of 10 µg of either 125I-labeled P-selectin MAb (125I-RB40.34) or E-selectin MAb (125I-10E6) and a dose of P-23 capable of providing 400,000–600,000 cpm 131I were used. These doses were selected on the basis of pilot studies demonstrating optimum activity and receptor saturation in the tissues examined under constitutive and stimulated conditions.

A blood sample was obtained from the carotid cannula 5 min after the MAb mixture was injected. The animals were then heparinized (40 U heparin sodium) and rapidly exsanguinated by perfusion of bicarbonate-buffered saline through the jugular vein cannula with simultaneous blood withdrawal through the carotid cannula. The vascular system was flushed with 15 ml of bicarbonate-buffered saline through the carotid cannula after the inferior vena cava was transected at the thoracic level. Entire organs were harvested and weighed.

Calculation of endothelial CAM expression. The method for calculating the expression of endothelial CAMs has been described previously (16, 28). In brief, the 125I (binding MAb) and 131I (nonbinding MAb) activities in different tissues and in 50-µl samples of cell-free plasma were counted in a 14800 Wizard 3 gamma counter (Wallac, Turku, Finland) with automatic correction for background activity and spillover. The total injected activity in each experiment was calculated by counting a 2-µl sample of the mixture containing the radiolabeled MAbs. The radioactivities remaining in the tube used to mix the MAbs and the syringe used to inject the mixture were subtracted from the total injected activity. The accumulated activity of each MAb in an organ was expressed as the percentage of the injected dose (%ID) per gram of tissue. Endothelial CAM expression was calculated by subtracting the accumulated activity per gram of tissue of the nonbinding MAb (131I-P-23) from the activity of the binding MAb (125I-YN-1, 125I-MK1.9.1, 125I-10E6, 125I-RB40.34, 125I-ICAM-2, or 125I-MEC13.3) and expressed as micrograms of MAb per gram of tissue.

CAM expression (µg MAb/g) = \[ \frac{[\%ID(125I)_{\text{plasma}} - \%ID(131I)_{\text{tissue}}] \times \%D(125I)_{\text{plasma}}}{\%D(131I)_{\text{plasma}}} \times \frac{\text{Total binding MAb (µg)}}{100} \]

Assessment of tissue vascular permeability. To compare the amount of protein extravasation from the tumor microcirculation with that occurring in other vascular beds, we estimated the percentage of circulating nonbinding MAb (P-23) that gained access to the extravascular space in tumors and normal tissues using the following formula

Protein extravasation = \[ \frac{131I-P-23 \text{ tissue (cpm/g)}}{131I-P-23 \text{ plasma (cpm/g)}} \times 100 \]

A similar approach was previously employed to estimate permeability of the vasculature in solid rat tumors to IgG (25).

CAM binding assay. To determine whether tumor cells expressed receptors for the different endothelial CAMs analyzed in this study, RM-1 tumor cells were grown to confluence in 48-well plates, and 6 wells each were incubated with 1 µg of radiolabeled RB40.34 or 10E6 or 4 µg of radiolabeled YN-1, MK1.9.1, or MEC13.3. After 30 min of incubation, wells were washed four times with HBSS and trypsinized, and 125I activity in the cell lysate was analyzed on the gamma counter.

Immunohistochemistry. Mice were injected subcutaneously with 1.0 × 106 RM-1 cells and, after 10–15 days, anesthetized with a ketamine-xylazine cocktail. Tumors weighing ~1.0 g were removed and placed in Zamboni’s fixative and refrigerated at 4°C overnight. Cryostat sections were cut at 10 µm and taken up on poly-L-lysine-coated slides. Slides were dried
over P2O5 in a vacuum desiccator for 30 min. The slides were then blocked with 10% normal donkey serum (Sigma, St. Louis, MO) and immunostained with the same MAbs used for the in vivo studies: E-selectin (10E6), 20 µg/ml; P-selectin (RB40.34), 20 µg/ml; ICAM-1 (YN-1), 30 µg/ml; VCAM-1 (MK1.9.1), 70 µg/ml; and PECAM-1 (MEC13.3), 20 µg/ml. After overnight incubation with the primary MAbs, the slides were rinsed in PBS and incubated for 1 h with AffiniPure donkey anti-rat IgG FITC (Jackson ImmunoResearch Laboratories, West Grove, PA). The slides were then rinsed with PBS and mounted in Mowiol-Glycerol + Vectashield mount medium to preserve fluorescence. Tissue staining was visualized using a krypton/argon laser on a Bio-Rad MRC 1024 scanning laser confocal microscope (Bio-Rad Laboratories, Hercules, CA), and the distribution of staining was assessed by comparing selectin, ICAM-1, and VCAM-1 with vessels staining positive to PECAM-1.

Experimental protocols. ICAM-1 and VCAM-1 expression were determined under constitutive conditions and at 5 h after TNF-α challenge (25 µg/kg) in both normal and tumor-bearing mice. A recent study revealed that TNF-α elicits a dose-dependent increase in ICAM-1 and VCAM-1 and that peak expression of these endothelial CAMs occurs 5 h after intraperitoneal injection of 25 µg/kg TNF-α (16). This dose of TNF-α was utilized for analysis of the remaining endothelial CAMs, although the time course after stimulation with this cytokine was varied to correlate with documented values of maximal expression of the various endothelial CAMs. To measure the response to TNF-α stimulation, we analyzed P-selectin, E-selectin, and PECAM-1 at 4 h, 3 h, and 5 h after intraperitoneal injection, respectively. ICAM-2 expression was measured only in RM-1 tumor-bearing mice. The effect of TNF-α administration on ICAM-2 expression was assessed 5 h after cytokine administration. A separate group of age (and weight)-matched tumor- and non-tumor-bearing mice was used for constitutive measurements of endothelial CAM expression. To assess the specificity of the 125I-labeled MAbs to bind their corresponding ligands, the accumulation of 125I-YN-1 and 125I-RB40.34 was measured in ICAM-1-deficient mice (n = 8) and P-selectin knockout mice (n = 8), respectively.

Statistical analysis. All data are expressed as means ± SE for each treatment group, except for data in Tables 1 and 2, which are expressed as means ± SD. The data were analyzed by ANOVA with Scheffe’s (post hoc) test. An unpaired Student’s t-test was used when only two groups were being compared. Statistical significance was set at a value of P < 0.05. For normalization of the various endothelial CAMs (ECAM) to vascular surface area (PECAM-1 expression), it was assumed that the errors of endothelial CAM and PECAM-1 expression are independent and uncorrelated. Therefore, the ratio of endothelial CAM expression to PECAM-1 expression was calculated as the ratio of the mean values of these variables, where the SD of the ratio of endothelial CAM expression to PECAM-1 expression is calculated as

\[ SD_{\text{ratio}} = \frac{Z}{\sqrt{\frac{S_{\text{ECAM}}^2}{\bar{Y}_{\text{ECAM}}^2} + \frac{S_{\text{PECAM-1}}^2}{\bar{X}_{\text{PECAM-1}}^2}}} \]

where X, Y, and Z are the mean values for PECAM-1, ECAM, and the ratio of ECAM to PECAM-1, respectively, and SD_{ECAM} Fig. 3. Constitutive and TNF-α-stimulated vascular cell adhesion molecule 1 (VCAM-1) expression in tumor-bearing and normal mice in lung (A), heart (B), and small intestine (C). Values are means ± SE for TBM constitutive (n = 7), TBM TNF-α (n = 5), NM constitutive (n = 4), and NM TNF-α (n = 4) groups. *P < 0.05 vs. corresponding constitutive value.

Fig. 4. Constitutive and TNF-α-stimulated VCAM-1 expression in RM-1 tumor vessels. Values are means ± SE. *P < 0.05 vs. constitutive value.
and SDPECAM-1 refer to the SD of ECAM and PECAM-1 values, respectively.

RESULTS

Constitutive and TNF-α-challenged ICAM-1 expression. The basal accumulation of radiolabeled ICAM-1 MAb (125I-YN-1) in different organs revealed significant constitutive expression of this endothelial CAM. There were, however, significant differences in the level of constitutive expression between tissues, with the lung (Fig. 1) exhibiting the highest expression and the tumor (Fig. 2) showing the lowest. Intraperitoneal injection of TNF-α elicited a significant increase in ICAM-1 expression in all organs examined, including the tumor. Both constitutive and TNF-α-induced ICAM-1 expression in the various tissues did not differ between tumor-bearing and normal (non-tumor-bearing) mice (Fig. 1). Constitutive measurements of ICAM-1 expression in different tissues (including the tumor) of ICAM-1-deficient mice revealed negligible levels compared with estimates derived from wild-type (C57Bl/6J) mice (Figs. 1 and 2B). Tumor-bearing ICAM-1-deficient mice stimulated with TNF-α (n = 4) also exhibited negligible ICAM-1 expression in the tumor microvasculature, even when compared with constitutive ICAM-1 values in tumors of wild-type mice (data not shown).

Constitutive and TNF-α-challenged VCAM-1 expression. VCAM-1 was also found to be constitutively expressed in all vascular beds studied, including the tumor (Figs. 3 and 4), although the level of expression in each tissue was lower than that noted for ICAM-1. Constitutive measurements of VCAM-1 expression in different tissues (including the tumor) of ICAM-1-deficient mice revealed negligible levels compared with estimates derived from wild-type (C57Bl/6J) mice (Fig. 5). Tumor-bearing ICAM-1-deficient mice stimulated with TNF-α (n = 4) also exhibited negligible ICAM-1 expression in the tumor microvasculature, even when compared with constitutive ICAM-1 values in tumors of wild-type mice (data not shown).

Table 1. Constitutive PECAM-1 estimates and PECAM-1-normalized values of ICAM-1 and VCAM-1 expression

<table>
<thead>
<tr>
<th></th>
<th>PECAM-1, µg MAb/µg tissue</th>
<th>ICAM-1, µg MAb/µg tissue</th>
<th>ICAM/PECAM</th>
<th>VCAM-1, µg MAb/µg tissue</th>
<th>VCAM/PECAM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung</td>
<td>15.116 ± 3.464</td>
<td>13.477 ± 2.430</td>
<td>0.892 ± 0.260</td>
<td>0.787 ± 0.426</td>
<td>0.052 ± 0.031</td>
</tr>
<tr>
<td>Heart</td>
<td>3.509 ± 1.73</td>
<td>0.901 ± 0.142</td>
<td>0.257 ± 0.095</td>
<td>0.239 ± 0.118</td>
<td>0.068 ± 0.041</td>
</tr>
<tr>
<td>Small intestine</td>
<td>2.952 ± 0.700</td>
<td>1.905 ± 0.777</td>
<td>0.645 ± 0.304</td>
<td>0.223 ± 0.079</td>
<td>0.075 ± 0.052</td>
</tr>
<tr>
<td>Skin</td>
<td>0.576 ± 0.236</td>
<td>0.443 ± 0.269</td>
<td>0.769 ± 0.563</td>
<td>0.145 ± 0.045</td>
<td>0.252 ± 0.129</td>
</tr>
<tr>
<td>Tumor</td>
<td>0.475 ± 0.146</td>
<td>0.152 ± 0.049</td>
<td>3.20 ± 0.142</td>
<td>0.062 ± 0.040</td>
<td>0.131 ± 0.093</td>
</tr>
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</table>

Values are means ± SD. PECAM-1, platelet-endothelial cell adhesion molecule 1; ICAM-1, intercellular adhesion molecule 1; VCAM-1, vascular cell adhesion molecule 1; MAb, monoclonal antibody.

Fig. 5. Constitutive and TNF-α-stimulated P-selectin expression in tumor-bearing, normal, and P-selectin-deficient (P-sel −/−) tumor-bearing mice in lung (A), heart (B), and small intestine (C). Values are means ± SE for TBM constitutive (n = 8), TBM TNF-α (n = 8), NM constitutive (n = 4), NM TNF-α (n = 5), and P-sel −/− TBM (n = 4) groups. *P < 0.05 vs. corresponding constitutive value. **P < 0.0001 vs. TBM constitutive value.

Fig. 6. Constitutive and TNF-α-stimulated P-selectin expression in tumor vasculature of C57Bl/6 mice compared with constitutive expression in P-sel −/− tumor-bearing mice. Values are means ± SE. *P < 0.05, **P < 0.001 vs. C57Bl/6 constitutive value.
Constitutive PECAM-1 expression. Previous studies indicate that PECAM-1 expression provides an index of microvascular density in different tissues (8). Consequently, we measured PECAM-1 expression in the different vascular beds in an effort to normalize the expression values for the other endothelial CAMs to vascular surface area. Table 1 summarizes the values for constitutive PECAM-1 expression in the different tissues studied and presents the ICAM-1 and VCAM-1 data normalized to PECAM-1 expression. The basal PECAM-1 values reveal that the lung exhibits the highest expression of this endothelial CAM, whereas the tumor expressed the lowest amount of PECAM-1.

Constitutive and TNF-α-challenged P-selectin expression. Normal and tumor-bearing mice expressed similar levels of P-selectin in different vascular beds under both baseline (constitutive) and TNF-α-stimulated conditions. All organs, including RM-1 tumors, displayed a robust response to TNF-α administration (Figs. 5 and 6), with the small intestine showing the most intense response, i.e., a >10-fold increase in P-selectin expression at 4 h after TNF-α stimulation. Tumor-bearing P-selectin knockout mice were utilized to assess the specificity of 125I-RB40.34 MAb in binding to its ligand. The accumulation of 125I-RB40.34 in these mutants was zero for all organs examined, including the tumor. Moreover, constitutive levels of P-selectin in tumors of wild-type mice were fully 10-fold higher than tumor values obtained in TNF-α-stimulated P-selectin knockout mice (n = 4; data not shown).

Constitutive and TNF-α-induced E-selectin expression. The constitutive expression of E-selectin in different vascular beds was lower than that measured for the other endothelial CAMs (Fig. 7). The constitutive expression of E-selectin in RM-1 tumors was higher than determined in heart and small intestine but not in lung. E-selectin expression increased in all tissues of tumor-bearing mice after TNF-α, except the tumor (Fig. 8). The accumulation of MAb 10E6 remained essentially unchanged after TNF-α stimulation in RM-1 tumors. Figure 9 demonstrates a tumor blood vessel staining positive for E-selectin.

Table 2. Constitutive P- and E-selectin expression normalized to constitutive PECAM-1 values

<table>
<thead>
<tr>
<th></th>
<th>PECAM-1, µg MAb/g tissue</th>
<th>P-selectin, µg MAb/g tissue</th>
<th>P-selectin/PECAM</th>
<th>E-selectin, µg MAb/g tissue</th>
<th>E-selectin/PECAM</th>
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<td>0.003 ± 0.003</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
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<td>3.509 ± 1.173</td>
<td>0.008 ± 0.002</td>
<td>0.002 ± 0.001</td>
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<td>0.086 ± 0.036</td>
<td>0.021 ± 0.010</td>
<td>0.036 ± 0.023</td>
</tr>
<tr>
<td>Tumor</td>
<td>0.475 ± 0.146</td>
<td>0.032 ± 0.011</td>
<td>0.067 ± 0.031</td>
<td>0.013 ± 0.008</td>
<td>0.027 ± 0.019</td>
</tr>
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</table>

Values are means ± SD. 0 indicates a value not significantly different from zero.
P- and E-selectin expression normalized to PECAM-1 expression. Table 2 summarizes the data for constitutive expression of P- and E-selectin when normalized for constitutive expression of PECAM-1. When the selectin data are normalized to PECAM-1 values (corrected for vascular density), the values for constitutive selectin expression in the RM-1 tumors appear to be higher than the values predicted for lung, heart, and small intestine.

Constitutive and TNF-α-challenged PECAM-1 expression. Figure 10 shows that both constitutive and TNF-α-induced expression of PECAM-1 did not differ between normal and tumor-bearing mice. As previously reported (8), cytokine challenge does not alter PECAM-1 expression in lung, heart, and small intestine. However, in RM-1 tumors (Fig. 11), PECAM-1 expression was significantly reduced (~70%) 5 h after intraperitoneal injection of TNF-α (P < 0.05). The highest level of PECAM-1 expression was noted in the lung, with the lowest level in the tumor (Figs. 10 and 11).

ICAM-2 expression in RM-1 tumor-bearing mice. ICAM-2 expression in RM-1 tumor-bearing mice (Fig. 12) resembles that of PECAM-1 in both its high density of expression on vascular endothelial cells and its behavior in response to TNF-α administration. Consistent with in vitro reports (38), ICAM-2 expression in normal vascular beds is refractory to stimulation with TNF-α. However, ICAM-2 expression in tumor vessels (Fig. 13) is significantly attenuated 5 h after cytokine stimulation.

Vascular permeability. Table 3 compares the 5-min extravasation values of 125I-P-23 MAb in different vascular beds of tumor-bearing mice. The data indicate that immunoglobulin extravasation is most pronounced in the liver, intermediate in the lung and RM-1 tumor, and least pronounced in the small intestine. Stimulation with TNF-α did not alter the rate of P-23 accumulation in any of the tissues studied.

Cell adhesion assay. Confluent monolayers of RM-1 cells failed to bind any of the radiolabeled MAbs used in this study, which indicates that the tumor cells per se do not express the endothelial CAMs measured in vivo (data not shown).

Immunohistochemistry. Although no attempt was made to quantify endothelial CAMs using immunohistochemistry, confocal images demonstrated the vascular localization of ICAM-1, VCAM-1, E-selectin, P-selectin, and PECAM-1 in RM-1 tumors (data not shown). Unlike findings in previous reports (20) that have documented a reduction in endothelial CAM labeling on vessels in the interior of vascular tumors, RM-1 tumors consistently demonstrated a diffuse pattern of endothelial CAM staining that was not confined to peripheral vessels. Immunohistochemistry was not performed on normal tissues.

**DISCUSSION**

Leukocyte-endothelial interactions within tumor microvasculature are critical for mounting a host immune response against tumor tissue, as well as successful treatment of cancer patients with adoptive cell transfer therapy. Until now, investigations into this area have been largely confined to immunohistochemical analysis and intravital observations. Reports based on these techniques have shown some heterogeneity of endothelial CAM expression between different types of tumors, with some tumors constitutively expressing various
CAMs on their endothelial cells (10, 19, 35, 39) and other tumors appearing deficient in leukocyte adhesion receptors (7, 11, 14, 20, 29, 37). Whereas these studies have proven useful in detecting the presence or absence of endothelial CAMs, they are unable to provide quantitative measurements of endothelial CAM expression in tumor microvessels. Such information could prove beneficial in evaluating strategies to make tumors more immunoresponsive. Using a solid tumor model, we provide the first quantitative measurements of endothelial CAM expression in nonstimulated and TNF-α-challenged tumor microvessels. In addition, our study allows for a comparison of the level of expression of endothelial CAMs in tumors with simultaneously obtained values from normal tissue.

The goals of the present study were achieved using the dual-radiolabeled MAb technique, which enables the user to quantify the surface expression of endothelial CAMs in different regional vascular beds. This technique has been previously employed to measure E-selectin expression in porcine skin after intradermal injection of IL-1 (17), to assess the kinetics of ICAM-1 and VCAM-1 expression in different vascular beds of the mouse (16) and rat (28) after endotoxin or TNF-α administration, and to evaluate the changes in murine P- and E-selectin expression after challenge with either histamine or endotoxin (9). These studies provided evidence supporting the feasibility, reproducibility, and accuracy of the technique as well as evidence indicating significant heterogeneity of the kinetics and magnitude of endothelial CAM expression (constitutive and induced) between vascular beds (9, 16, 18, 28).

For Fig. 10. Constitutive and TNF-α-stimulated PECAM-1 expression in tumor-bearing and normal mice in lung (A), heart (B), and small intestine (C). Values are means ± SE for TBM constitutive (n = 5), TBM TNF-α (n = 4), NM constitutive (n = 5), and NM TNF-α (n = 5) groups.

Fig. 11. Constitutive and TNF-α-stimulated PECAM-1 expression in RM-1 tumor vessels. Values are means ± SE. *P < 0.05 vs. constitutive value.

Fig. 12. Constitutive and TNF-α-stimulated ICAM-2 expression in RM-1 tumor-bearing mice in lung (A), heart (B), and small intestine (C). Values are means ± SE for constitutive (n = 7) and TNF-α (n = 9) groups.
example, we have previously shown that MAb directed against specific endothelial CAMs do not accumulate to a significant extent in tissues of mice that are genetically deficient in the corresponding adhesion molecule (9, 16). The present study extends this observation to tumor microvessels, inasmuch as ICAM-1- and P-selectin-specific MAbs failed to accumulate in RM-1 tumors of the corresponding endothelial CAM-deficient mice (Figs. 2 and 6).

The quantitative data generated by the dual-radiolabeled MAb technique in the present study reveals several interesting and novel features of endothelial CAM expression in the vasculature of primary tumors. These features include 1) a low binding of the PECAM-1-specific MAb in tumors relative to other vascular beds, suggesting a comparably lower vascular density; 2) constitutive levels of ICAM-1, VCAM-1, and P-selectin in tumor microvessels that fall within the ranges measured in other vascular beds (when data are normalized for PECAM-1 expression); 3) constitutive levels of E-selectin that are higher than those in all vascular beds, except in the skin overlying the tumor; 4) no difference in constitutive or TNF-α-induced expression of the different endothelial CAMs in normal tissues between tumor-bearing and control (no tumor) mice, suggesting that plasma cytokine levels are not greatly elevated in RM-1 tumors; 5) intensity of the upregulation of VCAM-1, ICAM-1, and P-selectin induced by TNF-α in RM-1 tumors that is comparable to that observed in other vascular beds; 6) E-selectin expression in the vasculature of RM-1 tumors that is unresponsive to TNF-α stimulation; and 7) PECAM-1 and ICAM-2 expression in RM-1 tumors that is dramatically reduced by TNF-α administration. These observations may have important implications in understanding the trafficking of inflammatory cells within tumor microvasculature.

Recently, we have shown that PECAM-1 expression in different vascular beds can be used as an index of endothelial cell surface area or vascular density (8). PECAM-1 is expressed at endothelial intercellular junctions, where it functions in homotypic endothelial cell adhesion (1). It is not surprising, therefore, that the pulmonary vasculature, with its massive vascular density, expresses the highest level of PECAM-1 of all organs studied. The observation that RM-1 tumors show relatively little accumulation of the PECAM-1 MAb suggests that this tumor has a relatively low vascular density and likely reflects the slow process of angiogenesis (i.e., endothelial sprout formation, budding, canalization, etc.).

Normalization of the constitutive values of ICAM-1 and VCAM-1 relative to PECAM-1 expression suggests that the density of expression of VCAM-1 and ICAM-1 in tumors is comparable to that observed in some other tissues. Furthermore, these endothelial CAMs responded to TNF-α with the same intensity in tumors and normal tissues, suggesting that endothelial cells in RM-1 tumors possess the membrane receptors and signal transduction cascades needed for this cytokine to elicit ICAM-1 and VCAM-1 upregulation. P-selectin expression in the nonstimulated tumor was found to be similar (when normalized to PECAM-1) to that observed in the small intestine, where the rolling receptor is believed to contribute to the maintenance of a resident population of interstitial leukocytes (2). This observation was somewhat unexpected because of reports describing a smaller flux of rolling leukocytes in venules of tumors compared with fluxes detected in normal tissues (11, 37). However, a recent study determined that P-selectin is equally important as a rolling receptor in tumor vessels and in vessels of inflamed organs because leukocyte rolling in tumor vessels is significantly reduced after administration of anti-P-selectin MAbs (7). The findings of the present study reveal that P-selectin upregulation in response to cytokine challenge is also comparable between otherwise normal tissues and RM-1 tumors.

E-selectin, another leukocyte rolling receptor that is exclusively found on endothelial cells, has been demonstrated with immunohistochemical staining in several types of tumors (10, 19, 23, 35, 39). Our measurements of E-selectin expression in nonstimulated RM-1 tumors indicate that the surface density of this rolling receptor on tumor endothelium is higher than that in all other tissues studied, except in skin directly over the tumor. Previous studies have demonstrated that E-selectin functions as a ligand on skin microvasculature for the cutaneous lymphocyte antigen, a lymphocyte homing receptor (4, 30). It is interesting to note that although normal tissues and RM-1 tumors.

Table 3. Nonbinding 125I-labeled MAb accumulation in tissues

<table>
<thead>
<tr>
<th></th>
<th>Liver</th>
<th>Lung</th>
<th>Tumor</th>
<th>Heart</th>
<th>Small Bowel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constitutive</td>
<td>12.85 ± 0.68</td>
<td>2.55 ± 0.68</td>
<td>2.46 ± 0.16</td>
<td>0.93 ± 0.18</td>
<td>0.57 ± 0.11</td>
</tr>
<tr>
<td>TNF-α</td>
<td>13.45 ± 3.08</td>
<td>2.14 ± 0.19</td>
<td>2.08 ± 0.37</td>
<td>0.81 ± 0.18</td>
<td>0.50 ± 0.11</td>
</tr>
</tbody>
</table>

Values are means ± SD of percentages of injected dose of monoclonal antibody (MAb) in tissues. TNF-α, tumor necrosis factor-α.
tumor microvessels normally express large amounts of E-selectin, TNF-α stimulation does not elicit an increased surface expression of this endothelial CAM. An explanation for this abnormal response of E-selectin in RM-1 tumors to cytokine challenge is not readily available. However, the apparently normal responses of VCAM-1, ICAM-1, and P-selectin to TNF-α challenge in tumor microvessels suggest that the abnormality likely occurs at the post-TNF-α-receptor level. Transforming growth factor (TGF)-β, an angiogenic factor that is produced in large quantities by RM-1 tumors (3), may contribute to the abnormal E-selectin expression response. It has been demonstrated that TGF-β inhibits (>50%) both basal and stimulated E-selectin expression on cultured human umbilical vein endothelial cells (12). This effect is selective for E-selectin, because TGF-β has no effect on ICAM-1 and VCAM-1 expression in the same model. This intriguing possibility warrants further investigation.

An interesting observation in this study was the unique action of TNF-α on PECAM-1 and ICAM-2 expression in tumors. Whereas PECAM-1 expression remained unchanged after TNF-α administration in all normal tissues, RM-1 tumors exhibited approximately a 70% reduction. A similar response was observed for ICAM-2, an endothelial CAM that, in vitro, has been reported to be refractory to cytokine stimulation (38). Such a reduction in PECAM-1 and ICAM-2 expression may reflect either shedding or internalization of these adhesion glycoproteins and/or endothelial cell injury. Endothelial injury after TNF-α administration has been described in both animal tumor models (15, 27, 31) and patients undergoing isolated limb perfusion therapy with TNF-α (32). In patients with irresectable sarcomas and melanomas undergoing isolated limb perfusion, Renard et al. (32) described a sequence of events beginning with swollen endothelial cells, which was followed by upregulation of E-selectin and VCAM-1 and by neutrophil recruitment. Our findings showing that the VCAM-1, P-selectin, and ICAM-1 expression responses are as robust in RM-1 tumors as in normal tissues, whereas the tumors show a profound decline in PECAM-1 expression, are difficult to reconcile simply on the basis of endothelial cell injury. Hence, additional work is needed (possibly using cultured endothelial cells derived from RM-1 tumors) to determine the cause of the TNF-α-induced downregulation of PECAM-1 and ICAM-2 in this tumor.

It must be emphasized that the present study utilizes a basic tumor model that may not be representative of all tumor types. Nonetheless, the approach used to quantify endothelial CAM expression in our report should prove useful for delineating any differences that may exist between tumor types and for evaluating strategies aimed at facilitating immune recognition of tumor tissue, such as neutralization of growth factors that have been shown to inhibit leukocyte-endothelial interactions (13, 22). This methodology should also prove useful for investigations directed toward elucidating the temporal relationship between endothelial CAM expression and the accumulation of metastatic tumor cells in different regional vascular beds, and for determining whether the upregulation of endothelial CAMs associated with metastasis to specific organs is a cause or a consequence of the metastatic process.

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