Brief Definitive Report

Endothelial Vascular Cell Adhesion Molecule 1 Expression Is Suppressed by Melanoma and Carcinoma

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Summary

Vascular cell adhesion molecule 1 (VCAM-1) mediates extravasation of circulating leukocytes into inflamed tissues, and presumably, plays a role in the immigration of cytotoxic effector lymphocytes into tumor metastases. Since metastases are rarely cleared by blood-borne cells from the immune system, we asked whether the tumor may escape host defense by interfering with the mechanism of effector cell extravasation. Here we show that in mice and humans, VCAM-1 expression is repressed on tumor-infiltrating vascular endothelial cells in the lungs. On lung blood vessels distant from the tumor, VCAM-1 is constitutively expressed. When melanoma and endothelioma cells were cultured on either side of a Nucleopore membrane, the expression of VCAM-1 on the endothelioma cells was inhibited and VCAM-1 gene transcription was suppressed. We propose that the downregulation of VCAM-1 is a mechanism by which vascularized melanoma and carcinoma avoid invasion by cytotoxic cells of the immune system.

Over the last decade, adoptive immunotherapy protocols for the treatment of metastatic malignancies have been developed (1). Lymphocytes were isolated, expanded in vitro, and administered back intravenously to the tumor-bearing host. The tumor-reactive lymphocytes then accumulated at sites of the metastases (2) and eradicated the malignancies by as yet unknown mechanisms. The selective accumulation of tumor-reactive lymphocytes requires adhesion molecules on vascular endothelium that mediate the arrest and extravasation. Since cellular cytotoxicity, either developed in situ or mediated by adoptive immunotherapy protocols, represents a fundamental threat to the tumor, escape mechanisms avoiding arrest and extravasation could have evolved. Indeed, these studies show that the most successful accumulation and responses to tumor immunotherapy were obtained when pretreating the tumor-bearing host with cyclophosphamide (1, 2) a cytostatic drug well-known to disrupt the endothelial cell layer integrity (3). The endothelium may be an effective barrier disabling cytotoxic cells to reach and kill the tumor.

Vascular cell adhesion molecule 1 (VCAM-1) is a cytokine-inducible endothelial adhesion molecule that plays a major role during inflammation. Lungs of mice constitutively expressed VCAM-1 on endothelial cells lining large vessels. Since VCAM-1 mediates lymphocyte infiltration (4, 5), one would expect that colonization of lung tumor metastases would also be enabled by VCAM-1. We studied the expression of VCAM-1 on LPS- and TNF-α-activated endothelial cells under the influence of melanoma cells. The results reported here suggest that B16 melanoma cells inhibit VCAM-1 expression on activated endothelia in vivo and in vitro. The phenomenon was also observed in human tumors, indicating that this mechanism may be a general one.

Materials and Methods

Animals, Cell Lines, and Human Tissue. Female C57BL/6 mice were 10–14 wk old. The endothelioma cell line End. 3 was a generous gift of Dr. W. Risau (Max Planck Institute, Bad Nauheim, Germany). The murine melanoma cell line B16/129-10, was kindly provided by Dr. W. Birchmeier (Max Delbrück-Centrum, Berlin-Buch, Germany), and the 3T3 cells were from the American Type Culture Collection (ATCC), Rockville, MD. Cells were grown in DME (Gibco, Paisley, Scotland) supplemented with 10% FCS (Boehringer Mannheim, Germany) (complete medium). Human tissue was taken from biopsies after surgery at the Justus Liebig University. Collected specimens included one primary small cell lung carcinoma (male, 68-yr-old), three primary mamma carcinoma (female, 50–61-yr-old), and three primary skin melanomas.

Antibodies and Other Reagents. Purified rat IgG was from Jackson ImmunoResearch (West Grove, PA). Supernatants of anti-VCAM-1 antibodies M/K-2.7 (ATCC) and anti-MECA 32 (a kind gift of Dr. E. Butcher, Stanford University School of Medicine, Stanford, CA) were used. Mouse anti-human VCAM-1, BBA5, was from R&D Systems Europe Ltd. (Abingdon, UK). Anti-factor VIII antiserum was purchased from Behringwerke AG (Marburg, Germany). The visualizing reagent for FACS® analysis (Becton Dickinson & Co., Mountain View, CA) was FITC-conjugated goat anti-rat IgG (Jackson ImmunoResearch). Human and murine recombinant TNF-α were kindly provided by Dr. W. Leslauer (F. Hoffmann-La Roche Ltd., Basel, Switzerland). Human recombinant IL-1α was from Dr. P. Lomedico (F. J. Exp. Med. © The Rockefeller University Press • 0022-1007/95/02/0811/06 $2.00 Volume 181 February 1995 811–816
bEnd.3 cells were detached. For time-kinetic studies, bEnd.3 cells were incubated for a total of 72 h. The capacity of the endothelioma cell line to respond to TNF-α was significantly diminished. All experiments described here were done with bEnd.3 cells with comparable low passage numbers. The co-culture was maintained for 24, 16, 4 and 0 h, resulting in a total incubation time for bEnd.3 cells of 72 h in all experimental groups. Where the bEnd.3 cells were cultured with tumor or control cells in the presence of melanoma TNF-α, the endothelioma cells were inhibited in their VCAM-1 expression, but larger vessels were positive for VCAM-1, indicating that in ~80% of the cases, VCAM-1 expression was indeed amplified.

DNA isolation, restriction enzyme digestion, and agarose gel electrophoresis were performed according to standard methods.

Results

Malignant Melanoma Cells Repress VCAM-1 Expression on Venular Endothelial Cells. Mice were injected with highly metastasizing B16/129-10 melanoma cells, a subclone of the original B16 F1 melanoma. Mice were injected with LPS or control PBS 12 d before they were killed. The lungs of these animals were analyzed by immunohistochemistry. Endothelial cells were identified by labeling lung sections with MECA 32 mAb (shown for LPS challenged mice, Fig. 1 a). The expression of MECA 32 on vascular endothelium within metastases appears not to be influenced by the tumor environment (Fig. 1 b). On vascular endothelium, VCAM-1 was constitutively expressed in large vessels, such as arterioles and venules (Fig. 1 c), indicating the occurrence of chronic inflammatory reactions in mice held under conventional conditions. Considerably less VCAM-1 was expressed on vessels of similar size infiltrating melanoma metastases (Fig. 1 d). It seems, that the presence of melanoma metastases results in the repression of VCAM-1 on adjacent vascular endothelia. We observed that veins measuring less than ~80 μm in diameter were inhibited in their VCAM-1 expression, but larger vessels were not affected (Fig. 1 d).

The frequency at which such downregulatory events occur, was measured (Table 1). In normal and LPS-stimulated mice 95 ± 2% of the venules outside metastatic lesions are positive for VCAM-1 in the lung. Within B16 metastases, however, only 18 ± 2% of the draining vessels were found to be positive for VCAM-1, indicating that in ~80% of the cases, VCAM-1 expression was repressed by the tumor.

Malignant Melanoma Cells Repress VCAM-1 on TNF-α-Stimulated Endothelial Cells. To mimic in vitro the conditions encountered on vascular endothelia within tumors, a co-culture system was established using B16 melanoma and bEnd.3 endothelioma cells. Intimate contact of tumor and endothelial cells was obtained by incubating both cell types on either side of a 0.4-μm Nucleopore filter membrane. Transmigration of either exclude dead cells and debris. Analysis was performed with the LYSIS data handling system.

Reverse-transcribed PCR. Total RNA from cultured bEnd.3 cells was isolated and cDNA synthesized as described elsewhere (8). The primers used for PCR amplification of VCAM-1 cDNA were 5’CTCCAGGGAAACACGATC3’ and 5’AGTGGACAGTGACGGTCTC3’. For the amplification of hypoxanthine phosphoribosyl transferase (HPRT) cDNA, a housekeeping gene, the primers used were 5’GCTGGTGAAAGGACCTCTY and 5’CACAGGACTGAAACACCTGC3’. The PCR reaction was run over 30 cycles after 5 s at 96°C, 15 s at 50°C, and 60 s at 72°C on a thermal cycler (AMS Biotechnology, Lugano, Switzerland). For some reactions, 2.5 ng of mouse genomic DNA was included as an additional control. To confirm that the amplified band represents murine VCAM-1 cDNA, single digests with HpaII and SacI were performed. When digested with HpaII, the resulting bands were, as anticipated, 317 and 292 bp; when digested with SacI, bands of 450 and 159 bp appeared, showing that VCAM-1 cDNA was indeed amplified.

DNA isolation, restriction enzyme digestion, and agarose gel electrophoresis were performed according to standard methods.

Flow Cytometric Analysis. Endothelioma cells were detached from the Nucleopore membrane by incubation with 0.5 ml trypsin/EDTA (Gibco) for 5 min at 37°C. After blocking the trypsin activity and washing with complete medium, the cells were processed for FACScan® analysis as described (7). Flow cytometric analysis was performed with a FACScan® (Becton Dickinson) using logarithmic amplification. The cells were electronically gated to
Figure 1. Histochemical staining of endothelial cells in normal and malignant murine lung after LPS-challenge. The animals were treated as described in Materials and Methods. Frozen sections of the lung (6 μm) were labeled with specific antibodies and developed with alkaline phosphatase-coupled secondary antibodies. (a) Labeling of normal lung by the endothelial cell-specific mAb MECA 32. (b) MECA 32 labeling of a venule within a B16 melanoma metastasis. (c) VCAM-1 staining by mAb M/K 2.7 showing the same venule as in (a) on a serial section. (d) VCAM-1 staining by mAb M/K 2.7 showing the same venule as in (b) within a B16 metastasis. (Arrows) Venules in normal tissue and metastatic lesions within the lung of one representative mouse (Experiment #1, n = 2). (Arrowheads) A large vein adjacent to a metastasis. Original magnification: ×250, bar, 34 μm.

Table 1. Repression of VCAM-1 Expression on Tumor-draining Endothelia in B16 Melanoma Metastases

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Normal lung</th>
<th>Metastasis</th>
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<tr>
<td></td>
<td>1</td>
<td>2</td>
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<tr>
<td>VCAM-1</td>
<td>269/285</td>
<td>183/189</td>
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<tr>
<td></td>
<td>94%</td>
<td>97%</td>
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<tr>
<td>MECA 32</td>
<td>283/283</td>
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Lungs of B16 tumor-bearing mice were prepared as described in Materials and Methods. In Experiment 1, the animals were pretreated with 15 μg of LPS i.v., mice in experiments 2 and 3 were not pretreated. Immunohistochemical analysis of blood vessels draining metastases and normal lung tissue was performed on frozen sections after labeling for VCAM-1 or the endothelial marker MECA 32, respectively. Blood vessels measuring <80 μm in diameter, but not capillaries, were analyzed. The total tumor areas of 39, 22, or 44 sections were analyzed in experiments 1, 2, or 3, respectively, for VCAM-1 and MECA 32. Since normal tissue is more abundant than tumorous tissue, 2, 4, or 4 sections were analyzed outside metastases. Sections stained for VCAM-1 were serial sections of tissues analyzed for MECA 32 expression outside the tumor. The data are expressed as numbers of VCAM-1 or MECA 32 positive vessels/total numbers of blood vessels. Percentages of positive vessels are indicated underneath. The data represent values of 2, 8, and 4 independently treated mice in experiments 1, 2, and 3, respectively.
cell line from one side of the porous filter to the other was never observed, however direct cell–cell contact by filopodia could not be excluded (not shown). First we determined whether VCAM-1 expression can be upregulated on the endothelial cell line upon treatment with either LPS, IL-1α, human and murine TNF-α (not shown). This effect was observed and was also attained when the endothelial cells were grown on Nucleopore filters (shown for TNF-α treatment, Fig. 2, C and D). After co-culture of the endothelial cells with the melanoma cell line in the presence of TNF-α, VCAM-1 expression was lost in a time-dependent fashion. Maximal repression of VCAM-1 expression was obtained after 24 h (Fig. 2 E and not shown). However, when endothelial cells were co-cultured with the fibroblast cell line 3T3, TNF-α–induced VCAM-1 expression persisted (Fig. 2 F), indicating that downregulation by melanoma cells is not a mere co-culture effect.

**Malignant Melanoma Cells Reduce VCAM-1 mRNA Abundance.** Metastasizing tumor cells are known to secrete proteolytic enzymes (9), which may cleave VCAM-1 from endothelial cell surfaces or, VCAM-1 expression could be regulated at the level of transcription. To analyze the mechanism, mRNA expression in endothelial cells was assessed by RT-PCR. VCAM-1 RNA was readily detected in endothelial cells activated by TNF-α. VCAM-1 RNA abundance was reduced by coinoculation of activated endothelia with melanoma cells (Fig. 3). This effect was maximal after 4 h and persisted for up to 48 h (not shown). Thus, the inhibition of VCAM-1 expression on endothelia regulated by the tumor is a consequence of diminished VCAM-1 RNA abundance. This might occur because of attenuated VCAM-1 transcription or reduced VCAM-1 mRNA stability.

**VCAM-1 Downmodulation Occurs also in Human Malignancies.** To test whether this downregulatory effect of VCAM-1 expression on endothelia occurs more generally in tumors, human melanoma and small cell carcinoma metastases in the lung were analyzed. For the identification of endothelial cells, the tissues were stained with anti-factor VIII (Fig. 4). Normal vessels within the lung expressed VCAM-1 (Fig. 4 c). Again, VCAM-1 expression was lost on blood vessels within the metastatic lesion (Fig. 4 d). Suppression of VCAM-1 expression was also observed on blood vessels within three primary melanomas and three primary mamma carcinoma (not shown).

Taken together, these data show that downregulation of the endothelial cell adhesion molecule VCAM-1 might represent a common mechanism occurring in melanoma and carcinoma metastases in humans.

**Discussion**

Our studies show that metastases of melanoma and carcinoma cells downregulate cytokine-induced VCAM-1 expression on vascular endothelial cells. We were able to demonstrate a downregulation of the VCAM-1 message within 4 h, followed by a decrease of VCAM-1 protein expression on the endothelial cell surface after 24 h in an in vitro assay. This is a novel demonstration of a tumor-derived activity that in-

![Figure 2. Surface expression of VCAM-1 on endothelial cells co-cultured with melanoma cells, after TNF-α treatment: evaluation by cytofluorimetry. Co-cultured cells were treated as described in Materials and Methods. (A and B) Background labeling as assessed with control rat IgG, followed by fluorescein-conjugated secondary antibody; (C and D) bEnd.3 cells labeled by anti-VCAM-1 mAb M/K 2.7; (E) bEnd.3 cells co-cultured with B16 melanoma cells on the other side of the Nucleopore membrane for 24 h and stained by anti VCAM-1; and (F) bEnd.3 cells co-cultured with 3T3 murine fibroblasts for 24 h and stained by anti-VCAM-1. One representative out of three independent experiments is shown.]
Figure 3. Analysis of VCAM-1 mRNA expression in endothelial cells co-cultured with melanoma cells after TNF-α treatment: evaluation by RT-PCR. BEnd.3 endothelial cells were cultured on Nucleopore filters, either alone (−), or with B16 melanoma cells (+) on the other side of the filter. After 4 h of incubation, endothelial cells were collected, and RNA was isolated and reversibly transcribed into cDNA. The VCAM-1 and HPRT-specific cDNAs were amplified by PCR. (M): molecular size marker, (Ø): no DNA control.

Table 1. Time course of VCAM-1 mRNA expression in endothelial cells co-cultured with melanoma cells after TNF-α treatment

<table>
<thead>
<tr>
<th>Time (hrs)</th>
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Figure 4. Expression of VCAM-1 in normal and malignant human lung endothelia. A small-cell bronchial carcinoma of a 68-yr-old, male patient was analyzed. Frozen sections (5 μm) were labeled by specific antibodies and developed with alkaline phosphatase-coupled secondary antibodies. (a) Staining of endothelial cells in a tumor-free area with anti-factor VIII. (b) Factor VIII staining of venules within the carcinoma lesion. (c) VCAM-1 staining of vessels in the tumor-free area using the mAb BBA5. (d) VCAM-1 staining of vessels within a carcinoma metastasis. (Arrow) Arterioles and venules in normal and metastatic tissue. The result represents one representative of a total of seven individual tumors analyzed. One to three sections per tumor were inspected. Original magnification: ×160, bar, 22 μm.

of E-selectin expression is mediated by the tumor-derived cytokine TGFβ-1 (13). Although B16 melanoma cells release TGFβ-1, this cytokine had no effect on VCAM-1 expression when added to stimulated endothelioma cells (our unpublished results) and this agrees with data reported by others (14).

It seems that the ability of tumor cells to downregulate lymphocytic adhesiveness in tumor-draining vessels is not a particular property of the tumors analyzed here. Using intravital microscopy, lymphocyte adherence to blood vessels in mammary adenocarcinomas in rats was found to be significantly diminished (15). When fluorescently labeled lymphocytes were transferred together with proinflammatory cytokines, the lymphocytes showed the characteristic rolling along and adhesion to vascular endothelia, which, however, was not the case in blood vessels within tumor metastases. Although the role of cell adhesion molecules has not been investigated in these studies, tumor-dependent downregulation is certainly a possibility to be taken into consideration. Such a mechanism may in fact represent a common strategy of vascularized melanoma and carcinoma metastases to escape attack by cytotoxic effector cells.

Clearly, these findings are of relevance with regard to the design of cell transfer immunotherapy in cancer patients. One would expect successful therapeutic effects only with tumors that do not interfere with cell adherence receptors on vascular endothelia. On the other hand, cytotoxic effector cells which use adhesion receptor–ligand combinations not controlled by tumor cells may be considered for therapy. Experiments examining the relationship between tumor-seeking effector cells and cell adhesion molecules available on tumor-associated vascular endothelia are currently in progress (7).

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