VCAM-1–, ELAM-1–, and ICAM-1–Independent Adhesion of Melanoma Cells to Cultured Human Dermal Microvascular Endothelial Cells

Kwang H. Lee, Thomas J. Lawley, Yuelin Xu, and Robert A. Swerlick
Department of Dermatology, Emory University School of Medicine, Atlanta, Georgia, U.S.A.

We have examined the mechanisms by which tumor cells bind to endothelial cells utilizing cultured melanoma cells and microvascular endothelial cells derived from human dermis (HDMEC). The ability of biologic response modifiers (BRM) to modulate the adhesion of melanoma cells to HDMEC was defined and those results were compared with results from human umbilical vein endothelial cells (HUVEC). SK-MEL-2, WM 266-4, and Hs 294T melanoma cells all bound to HDMEC and HUVEC monolayers and adherence of melanoma cells was enhanced in a dose- and time-dependent manner by the treatment of HDMEC with interleukin 1 (IL-1) alpha or tumor necrosis factor (TNF) alpha. Similar increases in binding to HDMEC or HUVEC were induced after BRM stimulation, although baseline melanoma cell binding to HUVEC tended to be slightly higher than to HDMEC. In contrast, whereas phorbol 12-myristate 13-acetate (PMA) augmented melanoma cell adherence to HDMEC, PMA failed to increase adherence to HUVEC. The alterations in melanoma cell binding were induced only after pretreatment of endothelial and not melanoma cells with PMA. Studies of the expression of cell adhesion molecules (CAM) on HDMEC and HUVEC using enzyme-linked immunosorbent assay showed that vascular cell adhesion molecule 1 (VCAM-1) is not induced by PMA on HDMEC and intercellular adhesion molecule 1 (ICAM-1) is downregulated on HDMEC by PMA treatment. Endothelial leukocyte adhesion molecule 1 (ELAM-1) is induced by PMA, IL-1alpha, or TNFalpha, but its expression does not correlate with increased melanoma cell binding MoAb recognizing VCAM-1 – inhibited TNFalpha-induced increases in melanoma cell binding to HUVEC. However, anti–VCAM-1 antibody failed to clock melanoma cell binding to PMA or IL-1alpha – stimulated HDMEC and only partially inhibited melanoma cell binding to TNFalpha-stimulated HDMEC. This study demonstrates that PMA and IL-1alpha – induced increases in melanoma cell adherence to HDMEC are not mediated via known CAM, including ICAM-1, VCAM-1, or ELAM-1, and may be affected through microvessel-specific novel proteins not previously described on endothelial cells. *J Invest Dermatol 98:79–85, 1992*

The development of tumor metastasis is dependent upon the ability of tumor cells to migrate from the primary tumor, enter the blood or lymph, and then exit at a distant site. Evidence also supports the view that hematogenous metastasis requires direct adhesive interactions between tumor cells and vascular endothelial cells [1]. Various tumor cells have been shown to adhere directly to endothelial monolayers in vitro [2–7], and in some experimental models, the ability of cultured tumor cell lines to form metastases correlates with their ability to bind to vascular endothelial cells in vitro [2,4]. The localization of tumors to different anatomic sites may be governed by unique characteristics of endothelial cells in different vascular beds.

The development of a variety of monoclonal antibodies directed against cell adhesion molecules (CAM) and the definition of their ligands have resulted in a rapid expansion in our understanding of cell-cell and cell-matrix interactions [8–10]. Recently, three cytokine-inducible endothelial cell adhesion molecules, intercellular adhesion molecule 1 (ICAM-1), endothelial leukocyte adhesion molecule 1 (ELAM-1), and vascular cell adhesion molecule 1 (VCAM-1), have been identified from large-vessel endothelial cells, cloned, and characterized [11–16]. It is known that these molecules

Manuscript received July 9, 1991; accepted for publication August 29, 1991.

This work was supported by NIH grant R01 AR 36632 and by the Dermatology Foundation and was presented in part at the Society of Investigative Dermatology national meeting, 1991, and published in abstract form (J Invest Dermatol 96:548, 1991).

Reprint requests to: Dr. Kwang Hoon Lee, Department of Dermatology, 201 Woodruff Memorial Building, Emory University School of Medicine, Atlanta, GA 30322.

Abbreviations:
BRM: biologic response modifiers
CAM: cell adhesion molecule
ELAM-1: endothelial leukocyte adhesion molecule 1
HDMEC: human dermal microvascular endothelial cell
HUVEC: human umbilical vein endothelial cell
ICAM-1: intercellular adhesion molecule 1
IL-1: interleukin 1
INCAM-110: inducible cell adhesion molecule 110
PDBu: phorbol 12,13 dibutyrate
PMA: phorbol 12-myristate 13-acetate
TMB: tetramethylbenzidene
TNF: tumor necrosis factor
VCAM-1: vascular cell adhesion molecule 1

0022-202X/92/$03.50

play an important role in leukocyte-vascular endothelial cell adhesion [12,14,17]. Recent studies have also demonstrated that activation of large-vessel endothelial cells by cytokines can alter the adhesion of human melanoma cells and carcinoma cells in vitro [5–7] and the expression of ELAM-1 and a protein closely related to VCAM-1 called inducible cell adhesion molecule 110 (INCAM-110) on large-vessel endothelium appears to correlate with the ability of specific tumor cells to adhere to human umbilical vein endothelial cells (HUVEC) [7]. However, the regulation of tumor cell adherence to human microvascular endothelium by proinflammatory cytokines has not been examined. We therefore examined whether the biologic response modifiers interleukin-1 (IL-1) alpha, tumor necrosis factor (TNF) alpha, and phorbol 12-myristate 13-acetate (PMA) alter the binding of cultured melanoma cells to human dermal microvascular endothelial cells (HDMEC), and compared the adherence of melanoma cells to HDMEC to adherence of melanoma cells to HUVEC.

MATERIALS AND METHODS

Endothelial Cell Culture HDMEC were isolated from human neonatal foreskins as described previously [18]. Briefly, foreskins were cut into small pieces, treated with 0.3% trypsin (Sigma Chemical Co., St. Louis, MO), and 1% EDTA (Sigma) and individual segments were compressed with a scalpel blade to express microvascular fragments. The microvascular segments were layered onto a 35% Percoll (Pharmacia AB, Sweden) gradient in Hanks' balanced salt solution (HBSS) and spun at 400 × *g* for 15 min at room temperature. The fraction with a density less than 1.048 g per ml was applied to gelatin (Sigma)-precoated tissue-culture dishes and cultured in endothelial basal media (Clonetics Corp., San Diego, CA) with epidermal growth factor 5 ng/ml (Clonetics), hydrocortisone acetate 1 μg/ml (Sigma), dibutyryl cyclic AMP 5×10^{-5} M (Sigma), penicillin 100 U/ml, streptomycin 100 μg/ml, ciprofloxacillin 10 μg/ml (Miles Laboratories, Westhaven, CN), amphotericin B 250 μg/ml (Sigma), and 30% human serum (Irvine Scientific, Santa Ana, CA). The resulting cell cultures were consistently 100% pure, as assessed by morphologic and immunochemical criteria. Experiments were conducted with endothelial cells at passages 2–8.

HUVEC were isolated from fresh umbilical cords by collagenase (Worthington Biochemical Co., Freehold, NJ) treatment and maintained in tissue culture as described previously [19]. Isolated HUVEC were cultured in growth media consisting of medium 199 (Gibco Laboratories, Grand Island, NY), supplemented with endothelial cell growth supplement 50 μg/ml (Biomedical Technologies, Stoughton, MA), glutamine 2×10^{-9} M (Sigma), penicillin 100 U/ml, streptomycin 100 μg/ml, and amphotericin B 250 μg/ml (Sigma), and 20% fetal bovine serum (FBS) (Gibco). HUVEC were used between passages 2–8. In order to normalize for differences in culture conditions, HUVEC were routinely cultured in HDMEC media for 3–5 d prior to use in experiments.

Melanoma Cell Lines Human melanoma cell lines SK-MEL-2, Hs 294T, and WM 266-4 were obtained from the American Type Culture Collection (ATCC, Rockville, MD). All cell lines were cultured on tissue culture dishes in Dulbecco's modified Eagle's medium (DMEM) (Gibco) and supplemented with glutamine 2×10^{-9} M (Sigma), and 10% FBS (Gibco). Cells were passaged with trypsin-versene (Irvine Scientific).

Antibodies Hybridoma 84H10 producing MoAb to ICAM-1 (mouse IgG_1) is grown in our laboratory and anti-ICAM-1 antibody was used as diluted ascites or as purified antibody after octanoic acid precipitation. MoAb 4B9 (mouse IgG_1) recognizing VCAM-1 was a gift of Dr. J. Harlan (University of Washington, Seattle, WA). MoAb 7A9 (mouse IgG_1) recognizing ELAM-1 was the generous gift of Dr. Walter Newman (Otsuka America Pharmaceuticals, Rockville, MD). MoAb HP2/1 recognizing the $alpha_4$ integrin chain (CDw49d) was purchased from AMAC (Westbrooke, ME).

Melanoma Cell–Endothelial Cell Adherence Assay HDMEC and HUVEC were plated in gelatin-coated 96-well flat-bottomed culture plates. They were preincubated with either cell culture media alone or with different BRM at appropriate concentrations and times: IL-1alpha (gift of Dr. I. Green, NIH, Bethesda, MD), TNFalpha (Genetech Corporation, San Francisco, CA), PMA (Sigma), and phorbol 12,13 dibutyrate (PDBu, Sigma). Tumor cell lines were labeled with ^{51}Cr (ICN Biomedicals, Costa Mesa, CA) by incubating 300 μCi per 8×10^6 cells for 18 h at 37°C. They were then removed from tissue culture plates with 5 mM EDTA (Sigma) in PBS with 1% bovine serum albumin (BSA) (Sigma), washed, resuspended to 8×10^5 ml in RPMI with 10% FBS and 100 μl of cell suspension was added to each well containing HDMEC or HUVEC and incubated for 30 min. After incubation at 37°C, the plates were washed and filled with HBSS with 0.5% BSA. The plates were then covered with thick filter paper and lids, sealed with parafilm, inverted, and centrifuged (600 × *g*, 10 min) to remove nonadherent cells. Remaining adherent cells were then lysed with 1% triton-X (Sigma) and harvested supernatants were read in a gamma counter. The percentage of bound melanoma cells was calculated as follows:

$$\%\ \text{melanoma cell binding} = \frac{\text{adherent counts-background counts}}{\text{counts added per well-background counts}} \times 100.$$

In some experiments, HDMEC monolayers, melanoma cells, or both were preincubated with 100 μl of MoAb for 45 min. One hundred microliters of 10–100 μg/ml of purified antibody, or 1:100 diluted ascites, were used. After preincubation with antibodies, the adherence assay was performed as described above in the continuous presence of antibodies. Statistical analyses were performed using an independent Student t test.

ELISA for the Study of Expression of CAM on Endothelial Cells Endothelial cells were plated into 96-well flat-bottomed microtiter plates at a concentration of 4×10^4 cells per well and were preincubated with either cell culture media alone or with IL-1alpha, TNFalpha, or PMA for 1–72 h at 37°C. A total of 100 μl of either MoAb 84H10 (ICAM-1), 4B9 (VCAM-1), or 7A9 (ELAM-1) at concentrations ranging from 1–10 μg/ml was added to each well and the plates were incubated at 37°C for 1 h. After washing, 100 μl of peroxidase-conjugated goat anti-mouse IgG (Biorad, Richmond, CA), diluted 1:500, was added to each well and plates were incubated for 1 h. The plates were again washed and the binding of antibody was quantitated colorimetrically by the addition of tetramethylbenzidene (TMB, 1 mg/ml, Sigma). One ml of a 100-mg/ml stock solution of TMB in acetone was added to 100 ml in distilled water. Ten microliters of 30% H_2O_2 was added immediately prior to use. The chromogenic reaction was stopped with 25 μl 8N H_2SO_4 and the plates were read spectrophotometrically at 450 nm on a Titertek ELISA reader. Statistical analyses were performed using an independent Student t test.

RESULTS

Effect of BRM on Melanoma Cell–Endothelial Cell Adherence Stimulation of HDMEC with IL-1alpha (80 U/ml) or TNFalpha (200 U/ml) for 24 h resulted in significant increases in Hs 294T and SK-MEL-2 binding. Binding of Hs 294T melanoma cells increased from baseline 15.9% to 34.3% after IL-1alpha treatment and 34.6% after TNFalpha treatment (Fig 1). Similarly, cytokine stimulation of SK-MEL-2 cells increased binding from 15.7% to 35.7% and 33.2% after stimulation with IL-1alpha or TNFalpha. Cytokine stimulation of endothelial cells also resulted in similar increases in binding of WM 266-4 melanoma cells to HDMEC monolayers.

The increases in melanoma cell binding to HDMEC induced by IL-1alpha and TNFalpha were time and dose dependent. Increased binding was clearly induced as early as after 2 h of stimulation with IL-1alpha or TNFalpha. Maximal increases were seen after 24 h of IL-1alpha stimulation, and these increases were maintained when

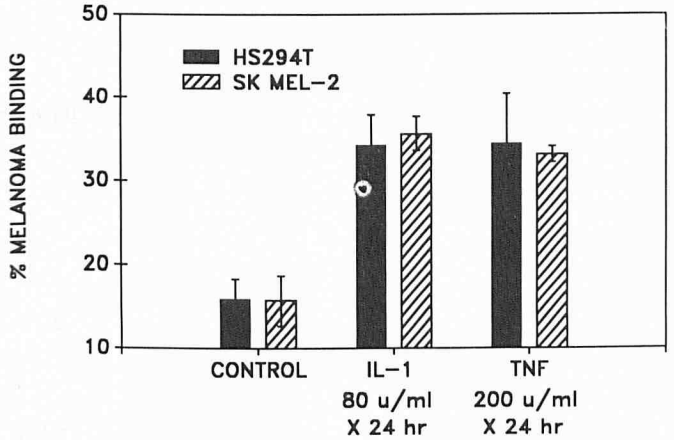

Figure 1. Cytokines induce increase in melanoma cell binding to HDMEC. HDMEC were incubated with IL-1alpha (80 μ/ml) or TNFalpha (200 μ/ml) for 24 h and then co-incubated with radiolabeled Hs 294T or SK-MEL-2 melanoma cells as described in *Materials and Methods*. Both IL-1alpha and TNFalpha induced significant increases in melanoma cell binding ($p < 0.01$).

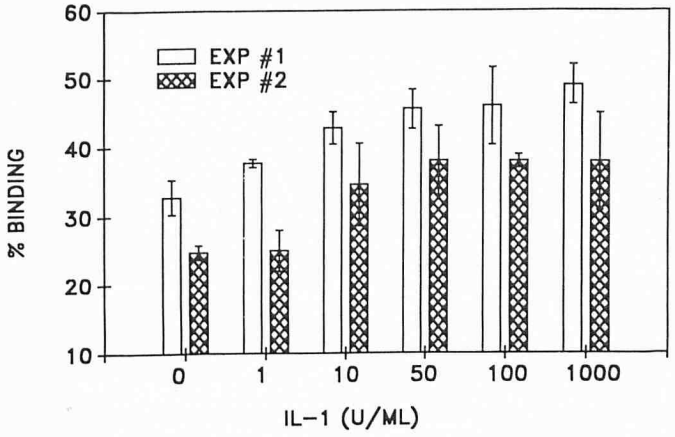

Figure 3. Dose response of IL-1alpha–induced increases in melanoma cell binding. HDMEC were incubated with doses of IL-1alpha from 1–1000 μ/ml for 24 h. Significant increases in SK-MEL-2 binding were observed after stimulation with 10 μ/ml of IL-1alpha ($p < 0.05$). Maximal increases in melanoma cell binding were induced at doses of 50–100 μ/ml ($p < 0.01$).

examined at 48–72 h (Fig 2). In contrast, TNF stimulation resulted in maximal adherence after 4 h, which then fell minimally to modestly by 24 h, and returned to baseline after 48 h.

Doses of IL-1alpha and TNFalpha as low as 10 μ/ml induced increases in melanoma cell binding when examined after 24 h of stimulation. For example, stimulation of HDMEC with 10 μ/ml, 100 μ/ml, and 1000 μ/ml of TNFalpha resulted in increased SK-MEL-2 binding from baseline 24% to 33%, 44%, and 49%, respectively. IL-1alpha demonstrated a similar dose response (Fig 3). Pretreatment of melanoma cells with cytokines for time periods ranging from 4–24 h did not affect melanoma cell-HDMEC binding.

Stimulation of HUVEC with cytokines also resulted in increases in binding of either WM 266-4, Hs 294T, and SK-MEL-2 melanoma cells to HUVEC similar to those noted in experiments using HDMEC. HUVEC tended to demonstrate slightly higher adherence of melanoma cells to unstimulated cells.

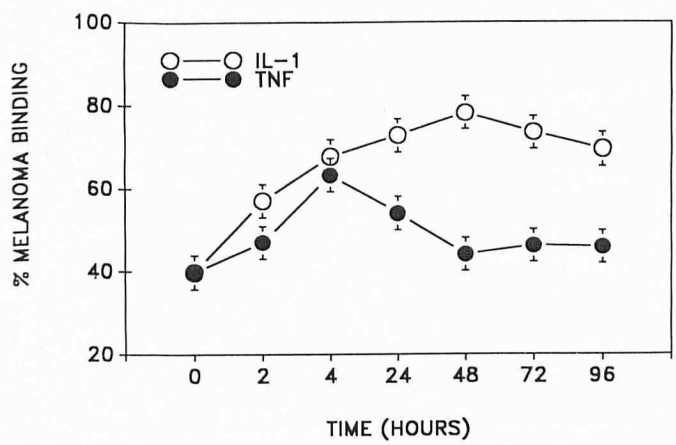

Figure 2. Time course of IL-1alpha– and TNFalpha-induced increases in melanoma cell binding. HDMEC were incubated with IL-1alpha (40 μ/ml) or TNFalpha (40 μ/ml) for periods of time ranging from 1–96 h. The binding of WM 266-4 melanoma cells was then assessed. IL-1–induced significant increases after 2 h ($p < 0.05$), which persisted up to 96 h ($p < 0.01$), whereas TNFalpha-induced increases were present at 4 and 24 h ($p < 0.01$), but binding returned to baseline by 48 h.

Phorbol Ester Effect on Melanoma Cell-Endothelial Cell Adherence PMA or PDBu stimulation of HDMEC or HUVEC monolayers led to discordant results. PMA (20 ng/ml × 24 h) or PDBu (100 ng/ml × 24 h) stimulation of HDMEC monolayers resulted in increased binding of all melanoma cell lines. SK-MEL-2 binding increased from 28.6 ± 0.9% to 45.8 ± 3.7% and 58.4 ± 4.6% after 24 h of stimulation with PMA and PDBu, respectively (Fig 4). Similar increases were observed with WM 266-4 (Fig 4) and Hs 294T melanoma cells (data not shown). This enhanced melanoma cell adhesion to HDMEC was detectable after 4 h incubation with PMA, peaked at 24 h, maintained through 72 h and was maximal at 20 ng/ml (Fig 5). In contrast, PMA or PDBu stimulation of HUVEC failed to induce increased melanoma cell binding and generally resulted in a moderate decrease in binding. PMA stimulation

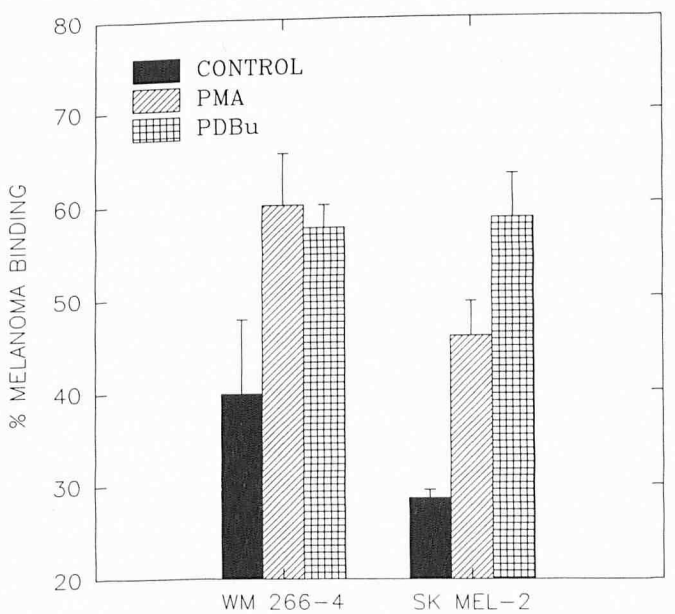

Figure 4. Effect of PMA and PDBu on melanoma cell binding to HDMEC. HDMEC stimulated with PMA (20 ng/ml) or PDBu (100 ng/ml) for 24 h bind significantly increased numbers of both WM 266-4 and SK-MEL-2 melanoma cells ($p < 0.05$).

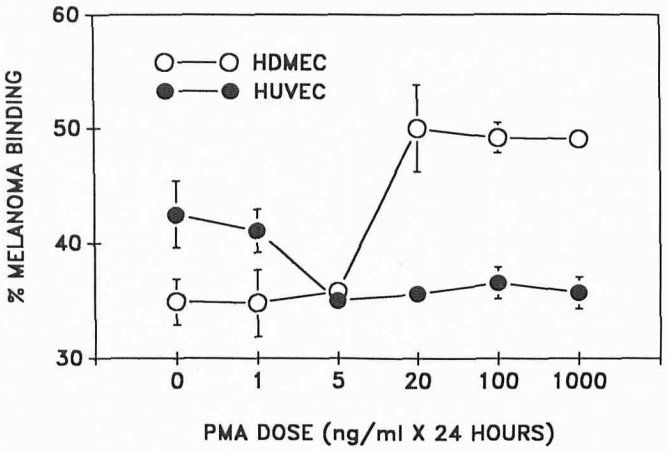

Figure 5. Differential effects of PMA stimulation on melanoma cell binding to HDMEC and HUVEC. HDMEC *(open circles)* and HUVEC *(closed circles)* were stimulated with increasing doses of PMA for 24 h. Stimulation of HDMEC resulted in a dose-dependent increase in WM 266-4 melanoma cell binding at doses equal to or greater than 20 ng/ml ($p < 0.01$), whereas PMA stimulation of HUVEC resulted in a moderate dose-dependent decrease in binding, at doses equal to or greater than 5 ng/ml ($p < 0.05$).

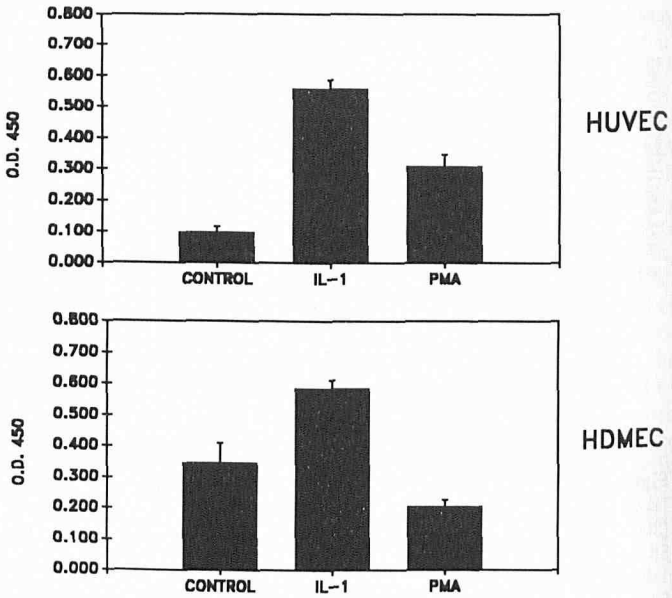

Figure 6. Differential effects of PMA on HDMEC and HUVEC ICAM-1 expression. HDMEC and HUVEC were stimulated with IL-1alpha (80 μ/ml) or PMA (100 ng/ml) for 24 h and then assayed for cell-surface ICAM-1 expression by ELISA. Both HDMEC and HUVEC stimulated with IL-1 significantly increased expression of ICAM-1 ($p < 0.01$). Stimulation with PMA resulted in increased ICAM-1 expression on HUVEC significantly ($p < 0.01$), but decreased expression of ICAM-1 on HDMEC significantly ($p < 0.05$).

of HUVEC decreased binding of SK-MEL-2 cells to HUVEC from 40.4% to 33.8% (Fig 5). PMA or PDBu stimulation of HUVEC also did not result in increased binding of WM 266-4 and Hs 294T melanoma cells (data not shown).

In order to determine whether phorbol ester stimulation affected binding by acting upon endothelial cells, melanoma cells, or both, HDMEC or SK-MEL-2 cells were preincubated with PMA 20 ng/ml for 24 h at 37°C, washed, and then assessed for binding. When HDMEC were pretreated, the adherence of SK-MEL-2 cells to HDMEC increased from 27.4 ± 1.3% to 41.3 ± 3.4%. When melanoma cells were pretreated with PMA, the adherence was not affected (control 26.9 ± 2.2, PMA 23.3 ± 1.4%). Furthermore, we examined the effect of short-term incubation of melanoma cells with PMA (20 ng/ml for 30 min) and found that it also did not increase melanoma cell adherence to HDMEC monolayers (data not shown).

Expression of ICAM-1, VCAM-1, and ELAM-1 on Endothelial Cells Because cytokine inducible CAM on endothelial cells have been implicated as adherence proteins mediating melanoma cell binding, we measured the cell-surface expression of ICAM-1, VCAM-1, or ELAM-1 on HDMEC and HUVEC using ELISA. Our results demonstrated that the expression of ICAM-1 on both HDMEC and HUVEC was increased after treatment with IL-1alpha or TNFalpha, as has been previously reported. ICAM-1 was also increased on HUVEC by stimulation with PMA (Fig 6) but, in contrast, PMA decreased the expression of ICAM-1 on HDMEC by 30–70% after 24 h (Fig 6).

Unstimulated HUVEC and HDMEC did not express ELAM-1, but ELAM-1 expression could be induced by treatment with IL-1alpha or TNFalpha (Fig 7). Maximal expression was observed after 4 h of stimulation and expression returned approximately to baseline after 24–38 h. PMA also induced minimal expression of ELAM-1 on HUVEC or HDMEC after 4 h (Fig 7), but expression was not present after 24 h.

VCAM-1 was also not expressed on unstimulated HDMEC or HUVEC. Both IL-1alpha and TNFalpha induced expression of VCAM-1 on HUVEC, but only TNFalpha induced expression of VCAM-1 on HDMEC (Fig 8). PMA (100 ng/ml × 24 h) stimulation of HDMEC or HUVEC did not result in expression of cell surface VCAM-1 (Fig 8).

Blockade of Melanoma Cell-HDMEC Adhesion with MoAb Against CAM In order to elucidate which adhesion pathways are operative in melanoma cell-HDMEC adhesion, we examined the effect of MoAb directed against known adherence proteins on HDMEC-melanoma cell adherence. As has been previously reported, increased binding of Hs 294T melanoma cells to TNFalpha-stimulated HUVEC was inhibited by MoAb 4B9 recognizing VCAM-1 (Fig 9*A*). MoAb 4B9 did not block TNFalpha-induced increases of melanoma cell binding to HDMEC, although a slight inhibition of binding was observed in some experiments (Fig 9*B*). Increased melanoma cell binding to IL-1alpha-stimulated HDMEC was not affected by MoAb 4B9 (Fig 9*C*), and additionally, 4B9 also failed to block melanoma cell binding to PMA-stimulated HDMEC (Fig 9*D*). Furthermore, anti-ICAM-1 antibodies and antibodies recognizing the alpha$_4$ integrin chain had no effect on melanoma cell adhesion to stimulated or unstimulated HDMEC (Table I).

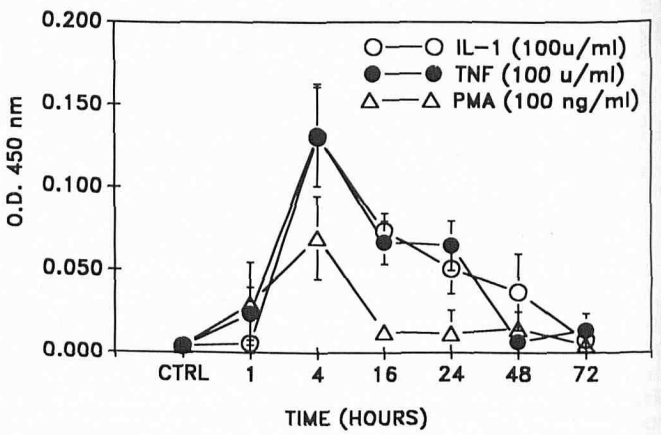

Figure 7. Expression of ELAM-1 on HDMEC. HDMEC were stimulated with IL-1alpha (100 μ/ml), TNFalpha (100 μ/ml), or PMA (100 ng/ml) and assayed for expression of ELAM-1 expression after 1, 4, 16, 24, 48, and 72 h of stimulation. ELAM-1 expression was maximal after 4 h of stimulation and returned to baseline after 16–72 h.

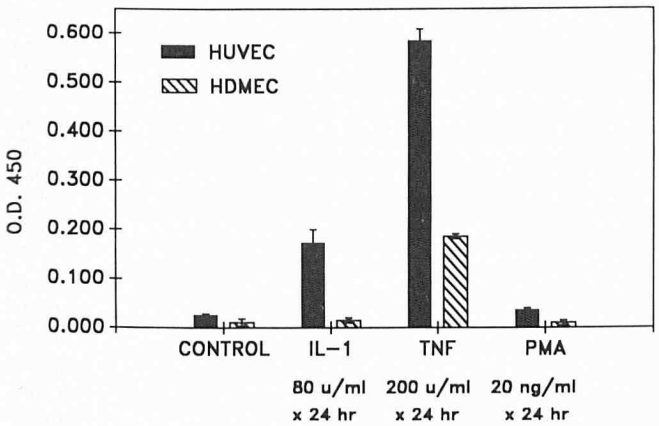

Figure 8. Induction of VCAM-1 on HDMEC and HUVEC by IL-1alpha, TNFalpha, and PMA. HDMEC and HUVEC were stimulated with IL-1alpha (80 μ/ml), TNFalpha (200 μ/ml), or PMA (20 ng/ml) for 24 h and assayed for expression of VCAM-1 by ELISA. Stimulation of HUVEC with IL-1alpha or TNFalpha resulted in a significant expression of cell surface VCAM-1 ($p < 0.01$), whereas only TNFalpha induced significant expression on HDMEC ($p < 0.01$).

DISCUSSION

The sequence of events leading to the development of metastasis is complex and includes local movement and invasion of tumor cells to the lymphatics or the vasculature, adherence to the endothelium or subendothelial basement membrane at a secondary site, and, finally, growth at this distant site [1]. Of these sequential processes, adherence of tumor cells to vascular endothelium is a critical step in blood-borne metastasis. The localization of metastasis to a given site may be dependent upon the ability of tumor cells to specifically bind to endothelium and migrate into extravascular spaces. Several reports have demonstrated a correlation between increased tumor cell adherence in vitro and increased metastatic capability in vivo [2,4,20].

The role of endothelial cells in the localization of tumor metastasis has also been examined. Since Greene and Harvey [21] proposed that the localization of a metastatic colony to a particular organ might depend on the formation of an initial bond between the tumor cells and the adhesive molecules on the luminal side of the vascular endothelium, many reports suggest that endothelial cell surface-associated specificities may play a significant role in determining the pattern of metastasis [2,4,20]. This is manifested clinically by the predilection of certain tumors to metastasize to specific vascular beds. Furthermore, inflammation and injury also appear to increase the likelihood that tumor cells may localize to a given site [22,23]. Recent evidence suggests that cytokine-induced effects on the vasculature may explain these phenomena in that in vitro stimulation of cultured HUVEC increases the adhesion of tumor cells [5–7].

The binding of tumor cells to endothelium is governed by the expression of specific adherence proteins on the surface of both tumor cells and endothelium. These interactions have been initially defined in humans using umbilical vein endothelial cells as a model.

Table I. Effect of Anti-ICAM-1, Anti-VCAM-1, and Anti-VLA-4 MoAb on Adherence of WM 266-4 Melanoma Cells to HDMEC[a]

MoAb	Percent Adherence	
	Untreated HDMEC	IL-1-Treated HDMEC
None	20.8 ± 1.6	30.0 ± 2.9
84H10	21.8 ± 4.7	31.4 ± 1.3
4B9	18.0 ± 2.0	27.7 ± 5.1
HP2/1	20.2 ± 1.8	30.4 ± 3.1
4B9 + HP2/1	18.7 ± 0.8	31.8 ± 2.7

[a] Wm 266-4 melanoma cells were pretreated with MoAb HP2/1 (CDw49d) 20 μg/ml at 37°C for 45 min. Untreated or IL-1-treated (100 μ/ml for 24 h) HDMEC were pretreated with MoAb 84H10(20 μg/ml) or 4B9(20 μg/ml) for 45 min at 37°C. Assays were done in the continuous presence of antibody. Percent adherence was determined after 30 min incubation at 37°C. Data represent the mean percent ± SD of quadruplicate microtiter wells in a single experiment.

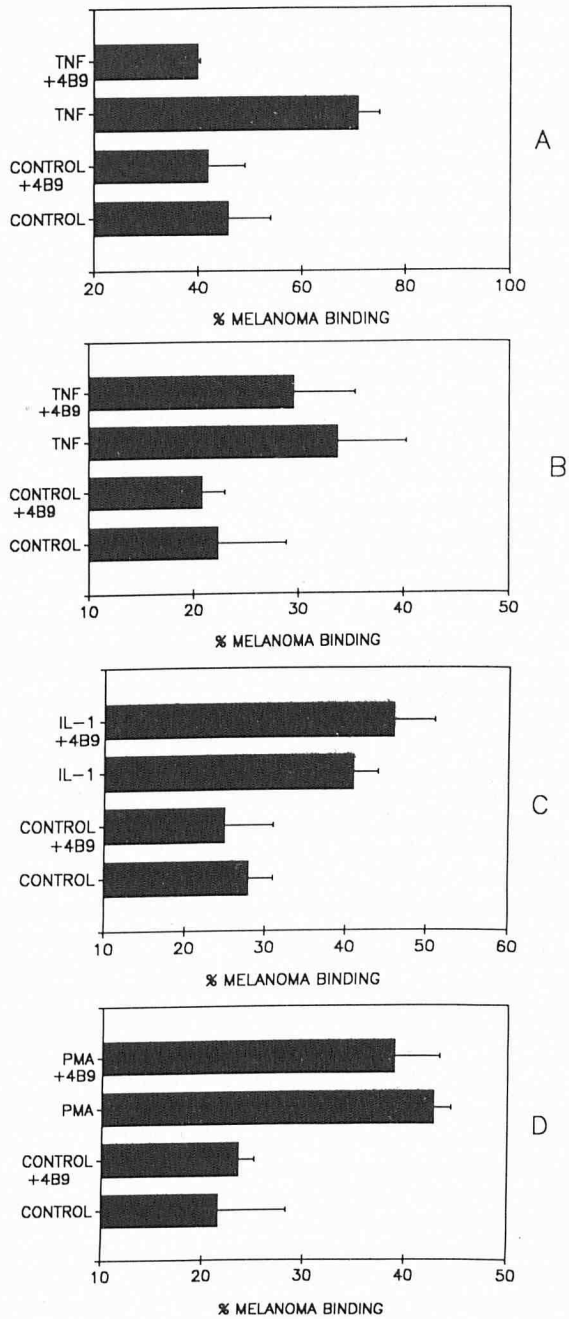

Figure 9. Effect of MoAb to known CAM on melanoma cell-endothelial cell adherence. MoAb 4B9 recognizing VCAM-1 significantly inhibited TNFalpha- (200 μ/ml × 24 h) induced increased binding of Hs 294T melanoma cells to HUVEC ($p < 0.01$) (*A*), but did not significantly inhibit TNFalpha- (200 μ/ml × 24 h) induced increases of binding of Hs 294T melanoma cells to HDMEC (*B*). MoAb 4B9 also did not significantly inhibit increased binding of WM 266-4 melanoma cells to IL-1alpha- (40 μ/ml × 24 h) treated HDMEC (*C*), or SK-MEL-2 binding to PMA- (20 ng/ml × 24 h) treated HDMEC (*D*). HDMEC monolayers were pretreated for 30 min with MoAb 4B9 (10–20 μg/ml) and melanoma adherence assays were conducted in the continuous presence of antibody.

The binding of both melanoma cells and colon carcinoma cells to HUVEC is mediated by the binding of the tumor cells to specific, cytokine-inducible ligands on HUVEC. Interestingly, these tumors recognize distinct inducible endothelial cell ligands [7].

The ability to study microvascular endothelial cells derived from skin offers additional tools to build upon the insights into tumor binding developed using large-vessel HUVEC. Previous studies using animal models demonstrate that specific tumors clearly localize preferentially to the microvasculature [3,4] and this specificity may be related to unique surface characteristics and phenotypes of microvascular endothelial cells. We have previously demonstrated that HDMEC possess a phenotype distinct from HUVEC, particularly in respect to their expression of cell adhesion molecules [24,25] and therefore examined tumor-cell adherence to microvascular endothelium using melanoma cells as a model.

The binding of tumor cells to unstimulated HDMEC was roughly comparable to the binding to HUVEC, although it tended to be slightly lower. Treatment of HDMEC or HUVEC with either IL-1alpha or TNFalpha resulted in increases in the binding of melanoma cells. Although both IL-1alpha and TNFalpha induced rapid increases in melanoma cell binding to HDMEC, increases associated with IL-1alpha treatment persisted up to 72 h, whereas TNFalpha treatment resulted in binding that fell back to baseline by 48–72 h. This suggests that these cytokines may induce changes in adherence via different mechanisms.

PMA treatment of large- and small-vessel endothelial cells demonstrated marked differences in responses. PMA stimulation of HDMEC resulted in a time- and dose-dependent increase in the binding of melanoma cells, with maximal increases seen after 24 h of stimulation. In contrast, PMA stimulation of HUVEC resulted in no increases or moderate decreases in the binding of melanoma cells. This effect was observed when HUVEC were cultured in either HUVEC or HDMEC media. The increased melanoma cell binding to PMA-treated HDMEC was clearly due to an effect on HDMEC and not melanoma cells, because pretreatment of melanoma cells with PMA resulted in no increases in binding. This is in contrast to observations regarding tumor cell binding to extracellular matrix where PMA stimulation of tumor cells resulted in increased binding to matrix [26].

Previous studies have also demonstrated that cytokine treatment of endothelial cells results in increases in tumor cell binding via induction of specific adherence proteins on the surface of endothelial cells that bind to the tumor cells [5–7]. These proteins include ELAM-1 and INCAM-110, which has subsequently been shown to be an alternatively spliced variant of the T-cell binding protein VCAM-1 [27]. Additionally, histopathologic studies of melanoma have also suggested that the invasiveness of this tumor correlates with the expression of the adhesion molecule ICAM-1 [28].

It is most likely that PMA treatment of HDMEC results in the expression or upregulation of endothelial cell adhesion molecules that mediate increased tumor cell binding. However, our data suggest that it is a molecule distinct from either ICAM-1, ELAM-1, or VCAM-1. Under our experimental conditions, all three melanoma cell lines bound to some degree to unstimulated endothelial cell monolayers that do not express VCAM-1 or ELAM-1. It is possible that this binding may be mediated via interactions with HDMEC ICAM-1, but our data also suggest that this is unlikely. Unstimulated HDMEC express 2–3 times greater cell-surface ICAM-1 than HUVEC [24,25], but tended to demonstrate slightly less melanoma cell binding. PMA treatment of HDMEC also results in a marked decrease in ICAM-1 expression, which is coincident with a marked increase in their melanoma-cell binding. In contrast, HUVEC expression of ICAM-1 is markedly increased by PMA treatment, but PMA treatment induced no increase in melanoma cell binding. Furthermore, MoAb 84H10, which blocks T-cell adherence to HDMEC monolayers, has no effect on melanoma cell binding to HDMEC [17].

It is also unlikely that the binding of melanoma cells to HDMEC can be fully explained on the basis of ELAM-1 or VCAM-1 (or INCAM-110)–dependent adherence pathways. Although IL-1alpha, TNFalpha, and PMA induce ELAM-1 on HDMEC, the kinetics of ELAM-1 induction are distinct from the time course of cytokine-induced increases in melanoma cell binding. ELAM-1 is rapidly induced and maximal expression is observed after 4–8 h of IL-1alpha or TNFalpha stimulation, but falls precipitously after 24–48 h. Melanoma cell adherence was not maximal until after 24 h of IL-1 or PMA stimulation, and remained elevated for at least 48–96 h.

PMA or IL-1alpha treatment of HDMEC also did not result in any significant HDMEC cell-surface expression of VCAM-1. Despite this, both were also extremely active in inducing persistent increases in melanoma cell binding to microvascular endothelial cells. These increases were not affected by MoAb 4B9. In contrast, TNF-stimulated HUVEC, which express VCAM-1, bind increased numbers of melanoma cells, and this increased binding was effectively blocked by MoAb 4B9. Increased melanoma cell binding to IL-1alpha- or PMA-treated HDMEC, which do not express VCAM-1, was not inhibited by MoAb 4B9, although MoAb 4B9 does inhibit the binding of VLA-4–expressing Ramos cells to VCAM-1–expressing HDMEC.* Additionally, the inability of anti–VLA-4 antibody to block melanoma binding to stimulated HDMEC provides even further evidence that VLA-4–VCAM-1 (or INCAM-110) interactions do not play an important role in melanoma cell–HDMEC binding.

Our data demonstrate that cytokine or protein kinase C agonist stimulation of HDMEC results in increases in melanoma cell binding in vitro, that some of these effects appear to be specific for microvascular endothelial cells, and that it is extremely unlikely that these increases in binding can be completely explained by previously described pathways involving ICAM-1, VCAM-1, or ELAM-1. These data suggest that melanoma cell adherence to HDMEC may be mediated via novel, as-of-yet undescribed microvascular endothelial-specific adherence molecules. Further studies using MoAb against specific PMA-inducible epitopes that may play a role in HDMEC–melanoma cell adherence will permit further characterization of this pathway.

REFERENCES

1. Crissman JD, Hatfield J, Schaldenbrand M, Sloane BF, Honn KV: Arrest and extravasation of B16 amelanotic melanoma in murine lungs: a light and electron microscopic study. Lab Invest 53:470–478, 1985
2. Korach S, Poupon MF, Du Villard J, Becker M: Differential adhesiveness of rhabdomyosarcoma-derived cloned metastatic cell lines to vascular endothelial monolayers. Cancer Res 46:3624–3629, 1986
3. Pauli BU, Lee CL: Organ preference of metastasis: the role of organ-specifically modulated endothelial cells. Lab Invest 58:379–387, 1988
4. Auerbach R, Lu WC, Pardon E, Gumkowski F, Kaminska G, Kaminski M: Specificity of adhesion between murine tumor cells and capillary endothelium: an *in vitro* correlate of preferential metastasis *in vivo*. Cancer Res 47:1492–1496, 1987
5. Dejana E, Bertocchi F, Bortolami MC, Regonesi A, Tonta A, Breviario F, Giavazzi R: Interleukin 1 promotes tumor cell adhesion to cultured human endothelial cells. J Clin Invest 82:1466–1470, 1988
6. Rice GE, Gimbrone MA Jr, Bevilacqua MP: Tumor cell-endothelial interactions. Increased adhesion of human melanoma cells to activated vascular endothelium. Am J Pathol 133:204–210, 1988
7. Rice GE, Bevilacqua MP: An inducible endothelial cell surface glycoprotein mediates melanoma adhesion. Science 246:1303–1306, 1989
8. Juliano RL: Membrane receptors for extracellular matrix macromolecules: relationship to cell adhesion and tumor metastasis. Biochim Biophys Acta 907:261–278, 1987
9. Carlos TM, Harlan JM: Membrane proteins involved in phagocyte adherence to endothelium. Immunol Rev 114:5–28, 1990
10. Albelda SM, Buck CA: Integrins and other cell adhesion molecules: FASEB J 4:2868–2880, 1990

* Swerlick RA: Unpublished observation.

11. Bevilacqua MP, Pober JS, Mendrick DL, Cotran RS, Gimbrone MA Jr: Identification of an inducible endothelial-leukocyte adhesion molecule. Proc Natl Acad Sci USA 84:9238–9242, 1987
12. Bevilacqua MP, Stengelin S, Gimbrone MA, Jr, Seed B: Endothelial leukocyte adhesion molecule 1: an inducible receptor for neutrophils related to complement regulatory proteins and lectins. Science 243:1160–1165, 1989
13. Rothlein R, Dustin ML, Marlin SD, Springer TA: A human intercellular adhesion molecule (ICAM-1) distinct from LFA-1. J Immunol 137:1270–1274, 1986
14. Osborn L, Hession C, Tizard R, Vassallo C, Luhowskyj S, Chi-Rosso G, Lobb R: Direct expression cloning of vascular cell adhesion molecule 1, a cytokine-induced endothelial protein that binds to lymphocytes. Cell 59:1203–1211, 1989
15. Simmons D, Makgoba MW, Seed B: ICAM, an adhesion ligand of LFA-1, is homologous to the neural cell adhesion molecule NCAM. Nature 331:624–627, 1988
16. Staunton DE, Marlin SD, Stratowa C, Dustin ML, Springer TA: Primary structure of ICAM-1 demonstrates interaction between members of the immunoglobulin and integrin supergene families. Cell 52:925–933, 1988
17. Dustin ML, Springer TA: Lymphocyte function-associated antigen-1 (LFA-1) interaction with intercellular adhesion molecule-1 (ICAM-1) is one of at least three mechanism for lymphocyte adhesion to culture endothelial cells. J Cell Biol 107:321–331, 1988
18. Kubota Y, Kleinman HK, Martin GR, Lawley TJ: Role of laminin and basement membrane in the morphological differentiation of human endothelial cells into capillary-like structures. J Cell Biol 107:1589–1598, 1988
19. Jaffe EA, Nachman RL, Becker CG, Minick CR: Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. J Clin Invest 52:2745–2756, 1973
20. Varani J, Lovett EJ, Elgebaly S, Lundy J, Ward PA: *In vitro* and *in vivo* adherence of tumor cell variants correlated with tumor formation. Am J Pathol 101:345–352, 1980
21. Greene HSN, Harvey EK: The relationship between the dissemination of tumor cells and the distribution of metastases. Cancer Res 24:799–811, 1964
22. Marley NF, Marley WM: Skin metastasis in an area of radiation dermatitis. Arch Dermatol 118:129–131, 1982
23. Shine T, Wallack MK: Inflammatory oncotaxis after testing the skin of the cancer patient. Cancer 47:1325–1328, 1981
24. Swerlick RA, Kubota Y, Garcia-Gonzalez E, Lawley TJ: Immunophenotypic analysis of human dermal microvascular endothelial cells (HDMEC). J Invest Dermatol 92:525, 1989
25. Swerlick RA, Garcia-Gonzalez E, Kubota Y, Xu Y, Lawley TJ: Studies of the modulation of MHC antigen and cell adhesion molecule expression on human dermal microvascular endothelial cells. J Invest Dermatol 97:190–196, 1991
26. Varani J, Fantone JC: Phorbol myristate acetate-induced adherence of Walker 256 carcinosarcoma cells. Cancer Res 42:190–197, 1982
27. Cybulsky MI, Fries JWU, Williams AJ, et al: Alternative splicing of human VCAM-1 in activated vascular endothelium. Am J Pathol 138:815–820, 1991
28. Johnson JP, Stade BG, Holzmann B, Schwable W, Riethmuller G: De novo expression of intercellular adhesion molecule 1 in melanoma correlates with increased risk of metastasis. Proc Natl Acad Sci USA 86:641–644, 1989