CELL REGULATION, Vol. 2, 347–355, May 1991

Tumor cell surface $\alpha^4\beta_1$ integrin mediates adhesion to vascular endothelium: demonstration of an interaction with the *N*-terminal domains of INCAM-110/VCAM-1

Darren B. Taichman,* Myron I. Cybulsky,*
Isabelle Djaffar,*† B. Michael Longenecker,‡
Joaquin Teixidó,§ G. Edgar Rice,*
Alejandro Aruffo,‖,¶ and Michael P. Bevilacqua*
*Vascular Research Division
Department of Pathology
Brigham and Women's Hospital
¶Department of Molecular Biology
Massachusetts General Hospital
*¶Harvard Medical School
§Division of Tumor Virology
Dana Farber Cancer Institute
Boston, Massachusetts 02115
‡Department of Immunology
University of Alberta
Edmonton, Alberta, T6G 2H7 Canada

Hematogenous metastasis involves adhesive interactions between blood-borne tumor cells and the vessel wall. By the use of *in vitro* assays, the adhesion of human melanoma, osteosarcoma, and kidney carcinoma (but not colon carcinoma) cell lines was shown to involve the cytokine-inducible endothelial cell surface protein inducible cell adhesion molecule 110 (INCAM-110) and the $\alpha^4\beta_1$ integrin, molecules normally involved in endothelial-leukocyte interactions. Tumor adhesion to human endothelial cell monolayers was increased 1.9- to 8.2-fold by endothelial activation with the cytokine tumor necrosis factor (TNF) and inhibited by the anti-INCAM-110 monoclonal antibody (mAb) E1/6. Each of these tumor cells expressed members of the β_1 integrin family of adhesion molecules, and antibodies to the α^4 and β_1 integrin subunits inhibited tumor-endothelial adhesion (48–87% inhibition). A cDNA encompassing the three *N*-terminal Ig-like domains of vascular cell adhesion molecule 1 (VCAM-1) encoded a protein recognized by the anti-INCAM-110 mAb E1/6 and, when captured onto plastic, supported melanoma cell adhesion by an α^4 integrin-dependent mechanism. In contrast to mAb E1/6, a second anti-INCAM-110 mAb Hu8/4 neither inhibited adhesion to activated endothelium nor bound the first three Ig-like domains of INCAM-110/VCAM-1. These data indicate that the adherence of several human tumors to activated endothelium is mediated by an interaction of $\alpha^4\beta_1$ integrin and the *N*-terminal Ig-like domains of endothelial INCAM-110/VCAM-1. Tumor acquisition of the α^4 integrin subunit and endothelial expression of INCAM-110 may affect the frequency and distribution of metastasis.

† Present address: U150 Inserm, Hôpital Lariboisiere, Paris, France.
‖ Present address: Oncogen, Inc., Seattle, WA 98121.

Introduction

The interaction of blood-borne tumor cells with the vascular endothelium is a key component of hematogenous metastasis. Increasing evidence suggests that tumor cell adhesion to the endothelial lining is influenced by endothelial activation (Dejana *et al.*, 1988; Rice *et al.*, 1988) or tissue-specific differences in endothelium (Alby and Auerbach, 1984; Pauli and Lee, 1988) and depends on the expression of specific cell surface molecules (Rice and Bevilacqua, 1989). Inducible cell adhesion molecule 110 (INCAM-110),[1] a 110-kDa glycoprotein found on the surface of cytokine-activated endothelial cells, was originally defined by its capacity to mediate the adhesion of metastatic melanoma cell lines (Rice and Bevilacqua, 1989). More recently, INCAM-110 has also been shown to serve as a receptor for blood lymphocytes and monocytes (Rice *et al.*, 1990) and to be expressed on activated microvascular endothelium during inflammatory and immunological processes (Rice *et al.*, 1990, 1991). Another cytokine-inducible cell surface protein, endothelial-leukocyte adhesion molecule 1 (ELAM-1; Bevilacqua *et al.*,

[1] Abbreviations: ELAM-1, endothelial leukocyte adhesion molecule 1; HEC, human (umbilical vein) endothelial cells; ICAM-1, intercellular adhesion molecule 1; INCAM-110, inducible cell adhesion molecule 110; mAb, monoclonal antibody; PCR, polymerase chain reaction; TNF, tumor necrosis factor; Tris-HCl, tris(hydroxymethyl)aminomethane-HCl; VCAM-1, vascular cell adhesion molecule 1.

© 1991 by The American Society for Cell Biology

1987, 1989), functions as a receptor for neutrophils but can also mediate the adhesion of a metastatic colon carcinoma (Rice and Bevilacqua, 1989). These findings suggest that adhesion molecules on activated endothelium not only regulate leukocyte movement throughout the body but also are utilized by circulating tumor cells in binding to the vessel wall.

In the present investigation, we examined tumor cells for surface molecules involved in adhesive interactions with vascular endothelium. Specifically, we assessed the role of several members of the β_1 integrin group of cell-matrix and cell-cell adhesion proteins (Hynes, 1987; Ruoslahti and Pierschbacher, 1987). Recently, lymphocyte cell surface $\alpha^4\beta_1$ was shown to function as a receptor for vascular cell adhesion molecule 1 (VCAM-1; Elices *et al.*, 1990), a molecule identified by RNA subtraction cloning (Osborn *et al.*, 1989). Here we establish the immunological identity of INCAM-110 and VCAM-1. We demonstrate that certain human melanomas and osteosarcomas, but not colon carcinomas, express the $\alpha^4\beta_1$ integrin, which supports their adhesion to activated endothelial cells. Further, a recombinant fusion protein containing the three *N*-terminal Ig-like domains of endothelial INCAM-110/VCAM-1 is recognized by an antibody that specifically blocks tumor adhesion to activated endothelium. Finally, this fusion protein supports tumor cell adhesion by an α^4 integrin-dependent mechanism.

Results

Tumor cells adhere to TNF-activated endothelium by distinct mechanisms

Activation of human endothelial cell (HEC) monolayers with tumor necrosis factor (TNF) increased the adhesion of several types of tumor cells. The adhesive behavior of human melanomas representing different stages of melanocytic differentiation was compared with normal melanocytes. Melanoma SK-MEL-131 clone 1.36–1.5 demonstrates early (poorly differentiated) markers, whereas SK-MEL-23 clones 22.a and 1–15 express intermediate and late phenotypic markers, respectively (Houghton *et al.*, 1987). Each of the melanoma cell lines adhered to TNF-activated HEC by an INCAM-110-dependent mechanism, as demonstrated by inhibition with anti-INCAM-110 monoclonal antibody (mAb) E1/6 (Table 1). These observations extend previous findings with the metastatic melanoma cell lines SK-MEL-24 and Hs 294T (Rice and Bevilacqua, 1989). In contrast to these malignant cells, the adhesion of isolated human melanocytes was not increased by TNF treatment of the endothelial cells (2 experiments, not shown).

Table 1. Effects of endothelial activation and anti-INCAM-110 mAb E1/6 on tumor cell adhesion

Tumor cell line	Fold increase in adhesion	% Inhibition by anti-INCAM-110
Melanoma SK-MEL-24	4.7 ± 0.6 (13)	50 ± 8 (3)
Melanoma Hs 294T	2.0 ± 0.2 (13)	51 ± 9 (5)
Melanoma SK-MEL-131 (clone 1.36-1.5)	8.2 ± 1.0 (3)	74 ± 9 (3)
Melanoma SK-MEL-23 (clone 22.a)	5.6 ± 1.6 (4)	54 ± 9 (4)
Melanoma SK-MEL-23 (clone 1-15)	1.9 ± 0.1 (3)	36 ± 7 (3)
Osteosarcoma MG-63	1.9 ± 0.2 (14)	45 ± 7 (10)
Kidney carcinoma CAKI-1	2.1 ± 0.0 (2)	29 ± 5 (3)
Colon carcinoma HT-29	1.9 ± 0.2 (16)	NS (6)*

HEC monolayers were treated 4–6 h with TNF and incubated with mAb E1/6 (anti-INCAM-110). Tumor cells were applied for a 30-min adhesion assay. Response to TNF treatment is expressed as fold increase over basal adhesion. Effect of anti-INCAM-110 mAb E1/6 is expressed as percent inhibition compared with adhesion to TNF-stimulated HEC in the presence of control mAb H4/45 (anti-P96) or without mAb treatment. mAbs were utilized as neat hybridoma supernatants. Data are given as mean ± SE of indicated number of experiments (n). None of the tumor cell lines was affected by control mAb. All values are statistically significant ($p < 0.05$, Student's t test) except as noted (NS). The following levels of basal adhesion to unstimulated HEC monolayers were observed: ~10% of cells applied (SK-MEL-131 [clone 1.36-1.5] and SK-MEL-23 [clones 22.a and 15-1]); ~20% (Hs 294T); 30–40% (SK-MEL-24, CAKI-1, and HT-29).

* In parallel experiments, colon carcinoma HT-29 adhesion was inhibited 58 ± 8% (n = 4) by anti-ELAM-1 mAb H18/7.

Increased adhesion to activated endothelium was also observed with nonmelanoma tumor cell lines. Binding of the human osteosarcoma MG-63, kidney carcinoma CAKI-1, and colon carcinoma HT-29 cell lines was increased 1.9- to 2.1-fold by TNF treatment of HEC monolayers. As with the melanomas, the adhesion of osteosarcoma and kidney carcinoma cells was inhibited by anti-INCAM-110 mAb E1/6 (Table 1). In contrast, colon carcinoma cell adhesion was not affected by mAb E1/6 but was inhibited by anti-ELAM-1 mAb H18/7 (Rice and Bevilacqua, 1989). Thus, melanoma, osteosarcoma, and kidney carcinoma cell adhesion was sup-

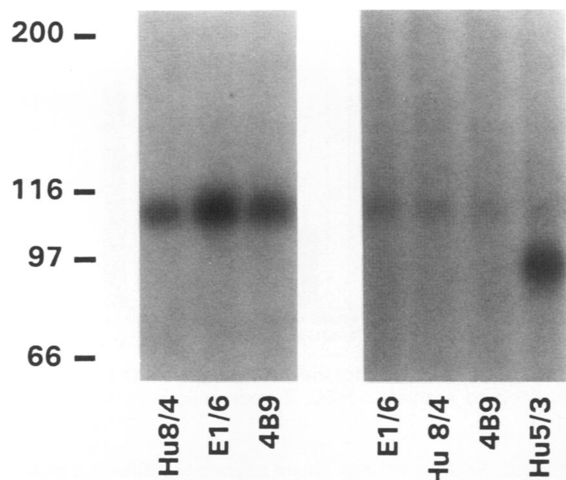

Figure 1. **Autoradiogram of gel (SDS-PAGE) comparing specificities of mAbs to lysates of ^{125}I-labeled TNF-activated HEC (left).** mAb E1/6 has been previously described to recognize INCAM-110 (Rice and Bevilacqua, 1989) and mAb 4B9 to recognize VCAM-1 (Carlos *et al.,* 1990). Shown on right is crossover immunoprecipitation with preclearing by (anti-INCAM-110) mAb E1/6. mAb Hu 5/3 recognizes human ICAM-1 (Luscinskas *et al.,* 1991).

ported by endothelial INCAM-110, whereas binding of colon carcinoma cells was ELAM-1 dependent.

Immunologic identity of endothelial INCAM-110 and VCAM-1

Recently, a cDNA encoding VCAM-1 was isolated from cytokine-activated endothelial cells by RNA subtraction cloning (Osborn *et al.,* 1989). This cDNA encodes a protein with six Ig-like domains that can support the adhesion of lymphoid cell lines after expression in COS cells. In a separate study, a mAb (4B9) that recognized cells transfected with this cDNA inhibited the adhesion of lymphocytes to vascular endothelial cells (Carlos *et al.,* 1990). Adhesion of lymphocytes to VCAM-1 is similar to that observed for INCAM-110 (Rice and Bevilacqua, 1989; Rice *et al.,* 1990). Immunoprecipitation with anti-INCAM-110 mAb E1/6 or anti-VCAM-1 mAb 4B9 yielded a 110-kDa polypeptide(s) from surface-iodinated, TNF-activated HEC lysates (Figure 1). Depletion of lysates with mAb E1/6 (anti-INCAM-110) removed the protein species that reacts with mAb 4B9 (anti-VCAM-1) (Figure 1) and vice versa (not shown), suggesting that these mAbs recognize epitopes on the same structure. A third mAb, Hu8/4, raised against TNF-treated HEC, also recognized the same 110-kDa protein (INCAM-110/VCAM-1). Further evidence of the immunologic identity of INCAM-110 and VCAM-1 was obtained by the demonstration that the anti-INCAM-110 mAbs E1/6 and Hu8/4 reacted with COS cells transfected with a cDNA encoding a seven-Ig-like-domain construct of VCAM-1, the predominant endothelial surface form (Polte *et al.,* 1990; Cybulsky *et al.,* 1991; immunostaining, not shown).

Tumor cell surface expression of β_1 integrin subunits

Because endothelial INCAM-110/VCAM-1 mediates lymphocyte adhesion via the integrin $\alpha^4\beta_1$ (Elices *et al.,* 1990), we assessed the involvement of this and other β_1 integrin molecules in human tumor cell binding to vascular endothelium. The β_1 subunit can associate with several distinct α subunits as heterodimeric structures (Hemler *et al.,* 1990). Flow cytometric analysis using the anti-β_1 mAb A-1A5 demonstrated comparable levels of expression on the surface of melanomas SK-MEL-24 and Hs 294T, human melanocytes, the osteosarcoma MG-63, and the colon carcinoma HT-29 (not shown).

As shown in Table 2, human osteosarcoma cells expressed substantial amounts of the α^2, α^3, α^4, and α^5 integrin subunits, as did each of the melanomas, with the exception of SK-MEL-24, which expressed little or no α^5. Human melanocytes also expressed the α^2, α^3, and α^5 subunits, but, in contrast to the melanoma cells, did not express α^4. The colon carcinoma HT-29, which failed to recognize INCAM-110, expressed α^2 and α^3 but not α^4 or α^5.

Table 2. Tumor cell surface expression of integrin α subunits

	Mean fluorescence intensity			
Cell type	α^2	α^3	α^4	α^5
Melanoma SK-MEL-24	15.10	45.53	68.00	1.19
Melanoma Hs 294T	24.26	12.46	14.04	10.37
Melanocyte	6.99	72.41	−0.76	14.65
Osteosarcoma MG-63	8.33	59.90	19.07	16.83
Colon carcinoma HT-29	3.86	12.92	−3.26	−3.56

Cells were labeled with mAbs against α subunits followed by fluorescein-conjugated anti-immunoglobulins, and expression was assessed by flow cytometry. Primary antibodies were utilized as ascites at saturating concentrations (1/1000 dilution, anti-$\alpha^{2,3,5}$; 10 μg/ml, anti-α^4 mAb 163H.1). Data are expressed as relative mean fluorescence values after subtraction of background determined by use of a nonbinding primary antibody control.

Antibodies to the β_1 and α^4 integrin subunits inhibit the adhesion of melanoma and osteosarcoma cells to TNF-activated HEC

In tumor-endothelial adhesion assays, an anti-β_1 heteroserum inhibited SK-MEL-24 melanoma cells by 79 ± 6% (SE, 4 experiments) but failed to block the binding of the colon carcinoma HT-29. Hs 294T melanoma cells were inhibited by 55 ± 5% and MG-63 osteosarcoma cells by 48 ± 6%. Treatment with preimmune goat serum did not affect the adhesion of any of the tumor cell lines (not shown).

In studies of individual α subunits, the α^4 integrin was found to be active in tumor-endothelial interactions (Figure 2). mAb 163H.1 (anti-α^4) inhibited the adhesion of SK-MEL-24 melanoma (69 ± 9%), Hs 294T melanoma (56 ± 6%), and MG-63 osteosarcoma (57 ± 5%) to TNF-activated HEC (4–7 experiments). As with the anti-β_1 heteroserum, however, the anti-α^4 mAb did not significantly inhibit HT-29 colon carcinoma adhesion (7 ± 6%, 3 experiments). In addition, inhibition of these cell lines was not observed with mAbs to α^5 (Figure 2), α^2 or α^3 (2–5 experiments, not shown). Thus, antibodies to both β_1 and α^4 blocked the adhesion of multiple tumor cell lines that bind to endothelial INCAM-110 but not the colon carcinoma cell line HT-29, which binds to ELAM-1.

Because the β_1 integrins can function as receptors for matrix proteins such as fibronectin, the interaction of tumor cells with subendothelial matrix was also examined. All the tumor cell lines showed β_1 integrin-dependent binding to subendothelial matrix. Anti-β_1 heteroserum inhibited the melanoma cell lines SK-MEL-24 and Hs 294T by 87 ± 3% (6 experiments) and 52 ± 1% (2 experiments), respectively. MG-63 osteosarcoma adhesion was inhibited by 55% (single experiment). Although anti-β_1 heteroserum failed to block the adhesion of HT-29 colon carcinoma cells to activated endothelium, it did inhibit the binding of these cells to subendothelial matrix by 46 ± 6% (6 experiments). Unlike adhesion to activated endothelial monolayers, the anti-α^4 mAb 163H.1 caused little or no inhibition of melanoma Hs 294T adhesion to subendothelial matrix (8 ± 2%, 3 experiments). Furthermore, this anti-α^4 mAb did not block the binding of Hs 294T cells to purified fibronectin (not shown). Anti-α^5 mAb did inhibit Hs 294T adhesion to both subendothelial matrix (24 ± 5%, 3 experiments) and purified fibronectin (87 ± 4%, 2 experiments). Anti-α^4 mAb 163H.1 did block the adhesion of SK-MEL-24 cells to subendothelial matrix 46 ± 13% (5 experiments), but did not affect the binding of osteosarcoma MG-63 (not shown).

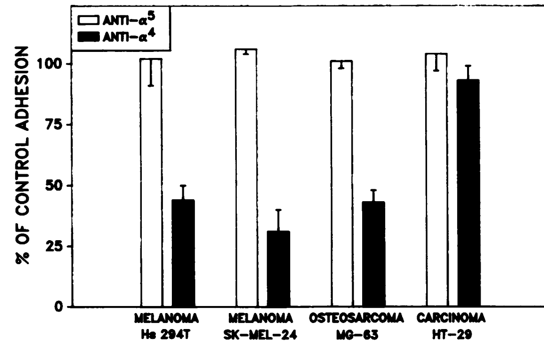

Figure 2. **Anti-α^4 monoclonal antibody inhibits tumor cell adhesion to activated HEC.** Tumor cells pretreated with anti-α^4 or α^5 mAbs were applied to TNF-activated HEC monolayers for a 30-min adhesion assay as described in Methods. Results are expressed as percent adhesion of untreated (no antibody) tumor cells to TNF-activated endothelial cell monolayers. Bars represent mean ± SE of 2–4 experiments, each performed with quadruplicate wells.

On TNF-activated endothelial monolayers, the effect of combinations of mAbs directed against tumor cell surface α^4 and endothelial cell surface INCAM-110/VCAM-1 was also assessed. Little or no enhancement of activity was observed when mAb E1/6 (anti-INCAM-110) and mAb 163H.1 (anti-α^4) were tested in combination (not shown), consistent with a direct interaction between tumor cell $\alpha^4\beta_1$ and endothelial INCAM-110/VCAM-1.

N-terminal domains of INCAM-110/VCAM-1 are active in tumor cell adhesion

To further evaluate the interaction of tumor cell surface $\alpha^4\beta_1$ with INCAM-110/VCAM-1, a cDNA clone was constructed that encodes the three *N*-terminal Ig-like domains of INCAM-110/VCAM-1 fused to the Fc portion of human IgG1. This dimeric receptor:immunoglobulin fusion protein was recognized by anti-INCAM-110 mAb E1/6 (immunostaining, not shown) and secreted from transfected COS cells. When captured onto plastic, the *N*-terminal INCAM-110/VCAM-1 receptor:Ig supported the adhesion of human melanoma cells (Figure 3). Adhesion was inhibited by mAb E1/6 (anti-INCAM-110), or by pretreatment of the melanoma cells with anti-α^4 mAb 163H.1 (Figure 3). In addition, treatment of Hs 294T melanoma cells with anti-β_1 serum blocked adhesion to the INCAM-110/VCAM-1 fusion protein 92 ± 4% (single experiment). A similarly constructed ELAM-1 receptor:immunoglobulin (Walz *et al.,* 1990) did not support

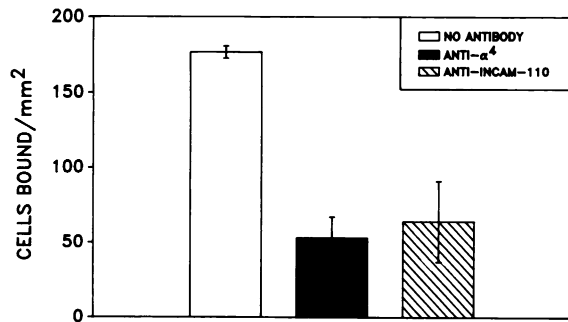

***Figure 3. N*-terminal domains of INCAM-110/VCAM-1 support adhesion of Hs 294T melanoma.** A fusion protein consisting of the three N-terminal Ig-like domains of INCAM-110/VCAM-1 coupled to the Fc portion of human Ig was captured onto wells precoated with goat antibody directed against human Fc. Certain wells were treated with anti-INCAM-110 or control mAb supernatants. Control mAb did not inhibit adhesion (not shown). Tumor cells were treated with anti-α^4 mAb 163H.1, anti-α^3 mAb, or without antibody for 30 min (25°C) before adhesion. Bars represent mean ± SE of quadruplicate wells, after subtraction of background adhesion (wells coated with goat whole serum; 58 ± 17 cells/mm^2). Hs 294T melanoma adhesion was unaffected by treatment with anti-α^3 mAb (5 ± 10% inhibition, 3 experiments). In contrast, colon carcinoma HT-29 adhesion was supported by the ELAM-1 receptor:Ig (803 ± 69 cells/mm^2) but not the INCAM-110/VCAM-1 fusion protein (−51 ± 67 cells/mm^2).

melanoma adhesion (−2 ± 3 cells/mm^2). These data indicate an interaction between the tumor cell surface $\alpha^4\beta_1$ integrin and the three *N*-terminal Ig-like domains of INCAM-110/VCAM-1.

Comparison of the anti-INCAM-110 mAbs E1/6 and Hu8/4 provided further evidence of this interaction. Unlike mAb E1/6, mAb Hu8/4 failed to inhibit the adhesion of melanoma Hs 294T cells to either the *N*-terminal INCAM-110/VCAM-1 receptor:Ig (single experiment, not shown) or to TNF-activated HEC monolayers (−12 ± 7% inhibition, 3 experiments). Further, although mAb Hu8/4 did recognize COS cells transfected with a seven-Ig-like-domain form of INCAM-110/VCAM-1, it did not bind the *N*-terminal domains of this molecule. Taken together, these data suggest that the interaction of the three *N*-terminal Ig-like domains of endothelial INCAM-110/VCAM-1 and the tumor surface $\alpha^4\beta_1$ integrin is sufficient to promote adhesion.

Discussion

In cancer patients, endothelial activation may occur in a variety of settings. A known response to malignancy is the production of cytokines such as TNF by host inflammatory cells (e.g., macrophages) (Old, 1985; Beutler and Cerami, 1987). Tumor cells themselves also produce cytokines such as TNF (Spriggs *et al.,* 1987) that could be released into the systemic circulation or act locally when blood-borne cells are arrested mechanically in the microvasculature. Tissue damage, localized infection, or sepsis also involve cytokine production and endothelial activation. The expression of endothelial INCAM-110 and ELAM-1 as a result of cytokine-mediated activation could promote tumor cell interactions with the vessel wall and thereby affect the frequency and distribution of metastatic lesions.

The interaction of tumor cell $\alpha^4\beta_1$ and endothelial INCAM-110 provides a novel and selective mechanism for tumor cell arrest and extravasation during hematogenous metastasis. Because INCAM-110 is also expressed by certain nonvascular cells, such as follicular dendritic cells within organized lymphoid tissues (Freedman *et al.,* 1990; Rice *et al.,* 1990), this molecular interaction could also support several extravascular adhesive events. α^4 is found in distinct structural forms (Hemler *et al.,* 1990) and can bind an alternatively spliced domain of fibronectin (Wayner *et al.,* 1989; Guan and Hynes, 1990) through a site different from that which recognizes INCAM-110/VCAM-1 (Elices *et al.,* 1990). Rapidly emerging information suggests the importance of $\alpha^4\beta_1$ in multiple immunological events, including human T-cell activation (Groux *et al.,* 1989; Nojima *et al.,* 1990), cytolytic function and homotypic aggregation (Bednarczyk and McIntyre, 1990; Campanero *et al.,* 1990), the binding of natural killer cells to activated endothelium (Allavena *et al.,* 1991), and the adhesion of murine lymphocytes to Peyer's patch high endothelial venules (Holzmann *et al.,* 1989). By analogy, additional roles for tumor cell α^4 in cancer should be assessed.

In the present study, tumor cell surface $\alpha^4\beta_1$ integrin was found to mediate the increased adhesion of melanoma and osteosarcoma cells to cytokine-activated endothelium via an interaction with INCAM-110/VCAM-1. However, the anti-α^4 mAb 163H.1 inhibits neither the adhesion of melanoma Hs 294T or osteosarcoma MG-63 to subendothelial matrix nor Hs 294T binding to purified fibronectin. In an earlier study, Hs 294T and SK-MEL-24 melanomas demonstrated increased adhesion to cytokine-activated endothelial cells in suspension (Rice *et al.,* 1988). In addition, quantitative immunoassays have revealed that TNF-activation of endothelial monolayers does not result in an increase in exposed fibronectin (not shown),

consistent with the finding that short-term cytokine treatment does not cause endothelial cell retraction (Rice *et al.,* 1988). Taken together, these observations strongly suggest that the increased adhesion of tumor cells to activated endothelium in this investigation does not involve a molecular interaction of $\alpha^4\beta_1$ and matrix fibronectin. The $\alpha^4\beta_1$/fibronectin interaction may, however, play a role in the binding of certain tumor cells to matrix (Mould *et al.,* 1990), consistent with our findings with the melanoma SK-MEL-24.

Anti-INCAM-110 mAb E1/6, shown to block both lymphocyte (Rice *et al.,* 1990) and tumor cell adhesion (Rice and Bevilacqua, 1989), recognized a recombinant fusion protein containing the three N-terminal Ig-like domains of INCAM-110/VCAM-1, whereas the nonblocking mAb Hu8/4 did not. Further, this portion of INCAM-110/VCAM-1 was sufficient to support adhesion by an α^4 integrin-dependent mechanism, although additional domains of this molecule may further enhance tumor-endothelial interaction. A similar interaction of another integrin with a member of the immunoglobulin gene superfamily has been demonstrated with CD11a/CD18 (LFA-1) and intercellular adhesion molecule 1 (ICAM-1) (Staunton *et al.,* 1990). The location of the active sites of both INCAM-110 and ICAM-1 at their distal extracellular domains may increase accessibility to their counterreceptors and thereby enhance adhesive function (Staunton *et al.,* 1990).

The utilization of several distinct adhesive mechanisms could help stabilize tumor cell arrest at the vessel wall during metastatic spread. Although the increased adhesion to activated endothelium of the melanoma, osteosarcoma, and kidney carcinoma cells studied here appears to be largely INCAM-110 dependent, other molecular interactions may also contribute. Although these cells do not appear to be affected by anti-ELAM-1 mAb, nor do they express its known carbohydrate ligand sialyl-Le^x (flow cytometric analysis, not shown), the potential synergism of INCAM-110 and other (perhaps yet undefined) molecules in supporting tumor adhesion deserves further study. It has previously been shown that colon carcinoma cells can adhere to ELAM-1 (Rice and Bevilacqua, 1989) and can express sialyl-Le^x (Walz *et al.,* 1990; Corless and Bevilacqua, unpublished observation), suggesting the involvement of this endothelial adhesive mechanism in colon carcinoma metastasis. Interestingly, small cell lung cancer cells have been reported to express the $\alpha_L\beta_2$ (LFA-1) and $\alpha_M\beta_2$ (Mo1) integrins (Feldman *et al.,* 1991), counterreceptors for another cytokine-inducible endothelial surface protein, ICAM-1. The potential utilization of individual or combinations of endothelial adhesive mechanisms may be a common route by which several tumors escape the bloodstream.

The acquisition (or loss) of specific adhesion molecules may facilitate tumor spread. Tumor cells can express antigens not found on their normal counterparts, an observation well documented with melanomas (Houghton *et al.,* 1987). For example, molecules immunologically related to the IIb-IIIa integrin complex, normally involved in platelet aggregation, are present on human metastatic melanomas but not benign melanocytes (Boukerche *et al.,* 1989; McGregor *et al.,* 1989). Melanoma expression of ICAM-1, a protein normally involved in a number of leukocyte adhesive interactions, also correlates with metastatic potential (Johnson *et al.,* 1989), as can the expression of specific lectin structures by several tumor types (Raz and Lotan, 1987; Raz *et al.,* 1989). As demonstrated in this and other studies (Albelda and Buck, 1990), human melanocytes do not express the α^4 subunit. In addition, recent observations suggest that the $\alpha^4\beta_1$ integrin is found in a greater percentage of human metastatic melanomas *in situ* than in benign melanocytic lesions (Albelda *et al.,* 1990). Similarly, neither osteoclasts nor osteoblasts express the α^4 subunit (Horton and Davies, 1989), whereas we found that osteosarcoma cells express this integrin and adhere to endothelial INCAM-110. These observations support the hypothesis that tumor cell acquisition of the α^4 integrin subunit can accompany malignant transformation.

Increased tumor cell adhesion to vascular endothelium may promote their escape into target organs. In a recent study, interleukin-1 treatment of athymic nude mice was shown to increase the number of metastases after injection with human melanoma cells (Giavazzi *et al.,* 1990). We have demonstrated a similar effect utilizing C57BL/6 mice and B16-F10 melanoma cells (Fidler, 1975; Taichman and Bevilacqua, unpublished observation), which bind endothelial INCAM-110 (Rice and Bevilacqua, 1989). Taken together, these observations suggest that localized or widespread expression of endothelial adhesion molecules, such as INCAM-110 and ELAM-1, may support tumor metastasis. Knowledge of the expression of these endothelial molecules and their tumor cell counterreceptors, such as the $\alpha^4\beta_1$ integrin, in cancer patients may provide insight into the settings and patterns of metastasis.

Methods

Cells

HEC were grown to confluence in microtiter wells (Rice *et al.*, 1988). Human osteosarcoma (MG-63, R. Taichman, Harvard Dental School, Boston, MA), colon (HT-29), and kidney carcinoma (CAKI-1) (American Type Culture Collection, Rockville, MD) and melanoma (SK-MEL-24 and Hs 294T, American Type Culture Collection; SK-MEL-23 and SK-MEL-131 clones; A. Houghton, Memorial Sloan-Kettering Cancer Center, New York, NY) cell lines as well as human newborn foreskin melanocytes (R. Byers, Massachusetts General Hospital, Boston, MA) were maintained as adherent cultures.

Antibodies

mAbs E1/6 (IgG1; Rice and Bevilacqua, 1989), H18/7 (IgG2a; Bevilacqua *et al.*, 1987), and H4/45 (IgG1; Bevilacqua *et al.*, 1987) recognize INCAM-110, ELAM-1, and p96 (a 96-kDa surface molecule constitutively expressed on HEC), respectively. mAbs recognizing the human integrin subunits α^2 (clone P1E6; IgG1), α^3 (clone P1B5; IgG1), and α^5 (clone P1D6; IgG3) were obtained from Telios Pharmaceuticals (San Diego, CA). mAb A-1A5 and a goat heteroserum react with the human β_1 integrin subunit (Elices *et al.*, 1990) (M. Hemler, Dana Farber Cancer Institute, Boston, MA). mAb 163H.1 (IgG1) was raised against a human T-cell leukemia (by B.M. Longenecker) and blocks its adhesion to bone marrow stromal cells. mAb 163H.1 was shown to bind α^4 integrin positive (but not negative) tumor cells and to precipitate the α^4 subunit in association with β_1 from lysates of K-562 erythroleukemia cells transfected with a cDNA encoding α^4 (Elices *et al.*, 1990; not shown). mAb Hu8/4 (IgG1), raised against TNF-activated endothelium, immunoprecipitated the same 110-kDa polypeptide as anti-INCAM-110 mAb E1/6. mAb 4B9 (IgG1) recognizes VCAM-1 (Carlos *et al.*, 1990) (J. Harlan, Univ. of Washington, Seattle, WA).

Flow cytometry and immunostaining

Flow cytometry was performed on tumor cells exposed to primary mAbs (45 min, 4°C), followed by fluorescein-conjugated anti-mouse immunoglobulin (45 min, 4°C) and fixed (1% paraformaldehyde in phosphate-buffered saline; Rice *et al.*, 1990). Immunoperoxidase staining was done on fixed COS cells (50:50 vol/vol acetone:methanol; 5 min, 4°C), exposed to mAb (1h, 25°C), followed by biotinylated horse anti-mouse IgG (1h, 25°C), avidin-biotin peroxidase complexes (Vector Labs, Burlingame, CA), and 3-amino-9-ethylcarbazole as chromogen.

Immunoprecipitation

Lysates of ^{125}I-labeled TNF-activated HEC, precleared with a nonbinding mAb, were incubated with primary mAb (4h, 4°C), followed by goat anti-murine IgG coupled to Sepharose-4B (Organon Teknika, West Chester, PA; 2–4h), which had been pretreated with unlabeled lysates. Antigens specifically bound to sepharose beads were extensively washed and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 5–12% linear gradient; Cybulsky and Gimbrone, 1991).

Construction of soluble INCAM-110/VCAM-1

cDNA sequences encoding the three N-terminal Ig like domains of VCAM-1 (Osborn *et al.*, 1989) were amplified by the polymerase chain reaction (PCR) and the use of a cDNA library from interleukin-1-stimulated HEC linearized with Mlu 1 (Aruffo *et al.*, 1990). A forward primer, encoding sequences upstream of the signal peptide and including an *Xho* I site (5'-GCG ACT AGT CTC GAG CTT AAA ATG CCT GGG AAG ATG GTC-3'), and a reverse primer, containing a *Hin*cII restriction site (5'-CAC CGT CGA CTC ACC TGC TTC AAC AAT TAA TTC CAC CTC-3'), were synthesized. PCR products were digested with *Xho* I and *Hin*cII and ligated to *Xho* I-*Sca*I cut vector containing the genomic sequence of the hinge, CH2, and CH3 domains of human IgG1 (Aruffo *et al.*, 1990). The resulting construct was transfected into COS cells and the fusion protein, consisting of two chains of the three N-terminal Ig-like domains of VCAM-1 complexed to the Fc portion of human IgG1, was secreted into culture supernatant (Aruffo *et al.*, 1990), which was concentrated (~3×, Centriprep Concentrator; Amicon, Danvers, MA; typically 0.3–1.5 μg/ml fusion protein) for use in adhesion assays (described below). SDS-PAGE analysis of metabolically labeled (^{35}S cysteine/methionine), protein A-purified proteins secreted from transfected COS cells revealed a single major band at ~80 kDa under reducing conditions, and at ~170 kDa under nonreducing conditions. These findings suggest the formation of homodimeric structures. Approximately 10% of the total protein in serum-free conditions was the INCAM-110/VCAM-1 receptor globulin.

Adhesion assays

Adhesion of tumor cells to confluent HEC monolayers or subendothelial matrix was performed as described (Rice *et al.*, 1988). Briefly, 2–10 × 10^4 fluorescent-labeled tumor cells were applied to TNF-treated (200 U/ml complete medium; 4–6h, 37°C) HEC or subendothelial matrix (exposed by nonenzymatic removal of endothelial cells with 2 mM EDTA, 30 min, 37°C) for 30 min at 25°C. Wells were inverted and centrifuged (removing nonadherent cells), and bound cells were quantified. In certain studies, HEC and/or tumor cells were preincubated with mAbs (30 min, 25°C).

The recombinant INCAM-110/VCAM-1 coupled to human Ig (described above) was captured onto HL-A tissue culture wells (Nunc, Naperville, IL) precoated with goat anti-human IgG (Fc fragment; Cat. #0101-0121) or goat whole serum as control (Organon Teknika, Malvern, PA; 10 μg/ml in 50 mM tris(hydroxymethyl)aminomethane-HCl [Tris-HCl], pH 8.2; 1h, 25°C) and washed (50 mM Tris-HCl). Coated wells were incubated with supernatant containing the INCAM-110/VCAM-1 receptor:Ig fusion protein (1h, 25°C) and washed with medium, 1 × 10^5 tumor cells were applied (10 min, 25°C), wells were washed to remove nonadherent cells, and bound cells were fixed (2.5% glutaraldehyde, 5 min) and counted by microscopy. Similarly, adhesion to fibronectin was studied with HL-A bacteriologic plastic wells (Nunc) coated with purified human serum fibronectin (Telios Pharmaceuticals; 100 μg/ml, 2–3 h, washed with 50 mM Tris-HCl).

Acknowledgments

We thank Drs. R. Byers, W.M. Gallatin, M.A. Gimbrone, Jr., J. Harlan, M. Hemler, A. Houghton, and R. Taichman for the provision of cell lines and antibodies; K. Case for cell culture; A. Olivier for assistance in flow cytometry; V. Davis for technical assistance in the production and immunocharacterization of mAb Hu8/4; and J. Kim for general laboratory assistance. We also thank Drs. C. Corless, M. Elices, and M. Hemler for critical review of the manuscript, and Drs. R.S. Cotran, S.K. Lo, and B. Seed for helpful discussions.

This work was supported by grants HL-36028, HL-07727 and GM-38903 from the National Institutes of Health. D.B. Taichman is a Merck Fellow in the Stanley J. Sarnoff Society for Research in Cardiovascular Science. S. Aruffo is a Fellow of the Leukemia Society of America. M.P. Bevilacqua is a Pew Foundation Scholar in the Biomedical Sciences.

Received: January 31, 1991.
Revised and accepted: March 20, 1991.

References

Albelda, S.M., and Buck, C.A. (1990). Integrins and other cell adhesion molecules. FASEB J. *4,* 2868–2880.

Albelda, S.M., Mette, S.A., Elder, D.E., Stewart, R., Damjanovich, L., Herlyn, M., and Buck, C.A. (1990). Integrin distribution in malignant melanoma: association of the β_3 subunit with tumor progression. Cancer Res. *50,* 6757–6764.

Alby, L., and Auerbach, R. (1984). Differential adhesion of tumor cells to capillary endothelial cells in vitro. Proc. Natl. Acad. Sci. USA *81,* 5739–5743.

Allavena, P., Paginin, C., Martin-Padura, I., Peri, G., Gaboli, M., Dejana, E., Marchisio, P.C., and Mantovani, A. (1991). Molecules and structures involved in the adhesion of natural killer cells to vascular endothelium. J. Exp. Med. *173,* 439–448.

Aruffo, A., Stamenkovic, I., Melnick, M., Underhill, C.B., and Seed, B. (1990). CD44 is the principal cell surface receptor for hyaluronate. Cell *61,* 1303–1313.

Bednarczyk, J.L., and McIntyre B.W. (1990). A monoclonal antibody to VLA-4 α-chain (CDw49d) induces homotypic lymphocyte aggregation. J. Immunol. *144,* 777–784.

Beutler, B., and Cerami, A. (1987). Cachectin: more than a tumor necrosis factor. N. Engl. J. Med. *316,* 379–385.

Bevilacqua, M.P., Pober, J.S., Mendrick, D.L., Cotran, R.S., and Gimbrone, M.A., Jr. (1987). Identification of an inducible endothelial-leukocyte adhesion molecule. Proc. Natl. Acad. Sci. USA, *84,* 9238–9242.

Bevilacqua, M.P., Stengelin, S., Gimbrone, M.A., Jr., and Seed, B. (1989). Endothelial leukocyte adhesion molecule 1: an inducible receptor for neutrophils related to complement regulatory proteins and lectins. Science *243,* 1160–1165.

Boukerche, H., Berthier-Vergnes, O., Bailly, M., Dore, J.F., Leung, L.L.K., and McGregor, J.L. (1989). A monoclonal antibody (LYP18) directed against the blood platelet glycoprotein IIb/IIIa complex inhibits human melanoma growth in vivo. Blood *74,* 909–912.

Campanero, M.R., Pulido, R., Ursa, M.A., Rodriguez-Moya, M., de Landazuri, M.O., and Sanchez-Madrid, F. (1990). An alternative leukocyte homotypic adhesion mechanism, LFA-1/ICAM-1-independent, triggered through the human VLA-4 integrin. J. Cell Biol. *110,* 2157–2165.

Carlos, T.M., Schwartz, B.R., Kovach, E.Y., Rosso, M., Osborn, L., Chi-Rosso, G., Newman, B., Lobb, R., and Harlan, J.M. (1990). Vascular cell adhesion molecule-1 mediates lymphocyte adherence to cytokine-activated cultured human endothelial cells. Blood *76,* 965–970.

Cybulsky, M.I., Fries, J.W.U., Williams, A.J., Sultan, P., Davis, V.M., Gimbrone, M.A., Jr., and Collins, T. (1991). Alternative splicing of human VCAM-1 in activated vascular endothelium. Am. J. Pathol. *138,* 815–820.

Cybulsky, M.I., and Gimbrone, M.A., Jr. (1991). Endothelial expression of a mononuclear leukocyte adhesion molecule during atherogenesis. Science *251,* 788–791.

Dejana, E., Bertocchi, F., Bortolami, M.C., Regonesi, A., Tonta, A., Breviario, F., and Giavazzi, R. (1988). Interleukin 1 promotes tumor cell adhesion to cultured human endothelial cells. J. Clin. Invest. *82,* 1466–1470.

Elices, M.J., Osborn, L., Takada, Y., Crouse, C., Luhowskyj, S., Hemler, M.E., and Lobb, R. (1990). VCAM-1 on activated endothelium interacts with the leukocyte integrin VLA-4 at a site distinct from the VLA-4/fibronectin binding site. Cell *60,* 577–584.

Feldman, L.E., Shin, K.C., Natale, R.B., Todd, R.F. III. (1991). β_1 integrin expression on human small cell lung cancer cells. Cancer Res. *51,* 1065–1070.

Fidler, I.J. (1975). Biological behavior of malignant melanoma cells correlated to their survival in vivo. Cancer Res. *35,* 218–224.

Freedman, A.S., Munro, J.M., Rice, G.E., Bevilacqua, M.P., Morimoto, C., McIntyre, B.W., Rhynhart, K., Pober, J.S., and Nadler, L.M. (1990). Adhesion of human B cells to germinal centers in vitro involves VLA-4 and INCAM-110. Science *249,* 1030–1033.

Giavazzi, R., Garofalo, A., Bani, M.R., Pietro, G., Boraschi, D., Mantovani, A., and Dejana, E. (1990). Interleukin-1-induced augmentation of experimental metastases from a human melanoma in nude mice. Cancer Res. *50,* 4771–4775.

Groux, H., Huet, S., Valentin, H., Pham, D., and Bernard, A. (1989). Suppressor effects and cyclic AMP accumulation by the CD29 molecule of $CD4^+$ lymphocytes. Nature *339,* 152–154.

Guan, J.-L., and Hynes, R.O. (1990). Lymphoid cells recognize an alternatively spliced segment of fibronectin via the integrin receptor $\alpha^4\beta_1$. Cell *60,* 53–61.

Hemler, M.E., Elices, M.J., Parker, C., and Takada, Y. (1990). Structure of the integrin VLA-4 and its cell-cell and cell-matrix adhesion functions. Immunol. Rev. *114,* 45–65.

Holzmann, B., McIntyre, B.W., and Weissman, I.L. (1989). Identification of a murine Peyer's patch-specific lymphocyte homing receptor as an integrin molecule with an α chain homologous to human VLA-4α. Cell *56,* 37–46.

Horton, M.A., and Davies, J. (1989). Perspectives: adhesion receptors in bone. J. Bone Miner. Res. *4,* 803–808.

Houghton, A.N., Real, F.X., Davis, L.J., Cordon-Cardo, C., and Old, L.J. (1987). Phenotypic heterogeneity of melanoma: relation to the differentiation program of melanoma cells. J. Exp. Med. *164,* 812–829.

Hynes, R.O. (1987). Integrins: a family of cell surface receptors. Cell *48,* 549–554.

Johnson, J.P., Stade, B.G., Holzmann, B., Schwable, W., and Riethmuller, G. (1989). *De novo* expression of intercellular-adhesion molecule 1 in melanoma correlates with increased risk of metastasis. Proc. Natl. Acad. Sci. USA *86,* 641–644.

Luscinskas, F.W., Cybulsky, M.I., Kiely, J.M., Peckins, C.S., Davis, V.M., and Gimbrone, M.A., Jr. (1991). Cytokine-activated human endothelial monolayers support enhanced neutrophil transmigration via a mechanism involving both endothelial-leukocyte adhesion molecule-1 and intercellular adhesion molecule-1. J. Immunol. *146,* 1617–1625.

McGregor, B.C., McGregor, J.L., Weiss, L.M., Wood, G.S., Hu, C-H., Boukerche, H., and Warnke, R.A. (1989). Presence

of cytoadhesins (IIb-IIIa-like glycoproteins) on human metastatic melanomas but not on benign melanocytes. Am. J. Clin. Pathol. *92,* 495–499.

Mould, A.P., Wheldon, L.A., Komoriya, A., Wayner, E.A., Yamada, K.M., and Humphries, M.J. (1990). Affinity chromatographic isolation of the melanoma adhesion receptor for the IIICS region of fibronectin and its identification as the integrin $\alpha^4\beta_1$. J. Biol. Chem. *265,* 4020–4024.

Nojima, Y., Humphries, M.J., Mould, A.P., Komoriya, A., Yamada, K.M., Schlossman, S.F., Morimoto, C. (1990). VLA-4 mediates CD-3-dependent $CD4^+$ T cell activation via the CS1 alternatively spliced domain of fibronectin. J. Exp. Med. *172,* 1185–1192.

Old, L.J. (1985). Tumor necrosis factor (TNF). Science *230,* 630–632.

Osborn, L., Hession, C., Tizard, R., Vassallo, C., Luhowskyj, S., Chi-Rosso, G., and Lobb, R. (1989). Direct expression cloning of vascular cell adhesion molecule 1, a cytokine-induced endothelial protein that binds to lymphocytes. Cell *59,* 1203–1211.

Pauli, B.U., and Lee, C. (1988). Organ preference of metastasis: the role of organ-specifically modulated endothelial cells. Lab. Invest. *58,* 379–387.

Polte, T., Newman, W., and Gopal, T.V. (1990). Full length vascular cell adhesion molecule 1 (VCAM-1). Nucleic Acids Res. *18,* 5901.

Raz, A., and Lotan, R. (1987). Endogenous galactoside-binding lectins: a new class of functional tumor cell surface molecules related to metastasis. Cancer Metastasis Rev. *6,* 433–452.

Raz, A., Pazerini, G., and Carmi, P. (1989). Identification of the metastasis-associated, galactoside-binding lectin as a chimeric gene product with homology to an IgE-binding protein. Cancer Res. *49,* 3489–3493.

Rice, G.E., and Bevilacqua, M.P. (1989). An inducible endothelial cell surface glycoprotein mediates melanoma adhesion. Science *246,* 1303–1306.

Rice, G.E., Gimbrone, M.A., Jr., and Bevilacqua, M.P. (1988). Tumor cell-endothelial interactions: increased adhesion of human melanoma cells to activated vascular endothelium. Am. J. Pathol. *133,* 204–210.

Rice, G.E., Munro, J.M., and Bevilacqua, M.P. (1990). Inducible cell adhesion molecule 110 (INCAM-110) is an endothelial receptor for lymphocytes: a CD11/CD18-independent adhesion mechanism. J. Exp. Med. *171,* 1369–1374.

Rice, G.E., Munro, J.M., Corless, C., and Bevilacqua, M.P. (1991). Vascular and nonvascular expression of INCAM-110: a target for mononuclear leukocyte adhesion in normal and inflamed human tissues. Am. J. Pathol. *138,* 385–393.

Ruoslahti, E., and Pierschbacher, M.D. (1987). New perspectives in cell adhesion: RGD and integrins. Science *238,* 491–497.

Spriggs, D., Imamura, C., Rodriguez, C., Horiguchi, J., and Kufe, D.W. (1987). Induction of tumor necrosis factor expression and resistance in a human breast tumor cell line. Proc. Natl. Acad. Sci. USA *84,* 6563–6566.

Staunton, D.E., Dustin, M.L., Erickson, H.P., and Springer, T.A. (1990). The arrangement of the immunoglobulin-like domains of ICAM-1 and the binding sites for LFA-1 and rhinovirus. Cell *61,* 243–254.

Walz, G., Aruffo, A., Kolanus, W., Bevilacqua, M.P., and Seed, B. (1990). Recognition by ELAM-1 of the Sialyl-Le^x determinant on myeloid and tumor cells. Science *250,* 1132–1135.

Wayner, E.A., Garcia-Pardo, A., Humphries, M.J., McDonald, J.A., Carter, W.G. (1989). Identification and characterization of the T-lymphocyte adhesion receptor for an alternative cell attachment domain (CS-1) in plasma fibronectin. J. Cell Biol. *109,* 1321–1330.