Human Melanoma Cells Derived from Lymphatic Metastases Use Integrin $\alpha_v\beta_3$ to Adhere to Lymph Node Vitronectin

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Abstract

Human melanoma is a highly metastatic cancer and the regional lymph nodes are generally the first site of metastasis. Adhesion to cryostat sections of human lymph nodes was therefore studied using two human melanoma models established from lymph node metastases, namely, MeWo cell lines of diverse metastatic potentials and a highly metastatic cell line of recent origin designated MIM/8. We found a good correlation between the metastatic potentials of the melanoma cells as measured in nude mice and their ability to adhere to cryostat sections of human lymph nodes. When adhesion to immobilized extracellular matrix proteins was measured, a significant increase in adhesion, which correlated with increased metastasis, was seen mainly on vitronectin and to a lesser extent on fibronectin. The adhesion to vitronectin and to the frozen sections were specifically blocked by an RGD-containing peptide, mAb 661 to vitronectin and mAb LM609 to integrin $\alpha_v\beta_3$. FACS® analysis revealed a significant and specific increase in cell surface expression of $\alpha_v\beta_3$ on the metastatic cells as compared to the parent line. Together these results suggest that the adhesion of melanoma cells to lymph node vitronectin via the $\alpha_v\beta_3$ receptor plays a role in the process of lymphatic dissemination. (*J. Clin. Invest.* 1992. 90:1406–1413.) Key words: lymphatic metastasis • adhesion • vitronectin receptor

Introduction

The regional draining nodes are the first site of metastasis for the majority of primary human neoplasms, including malignant melanoma. In melanoma patients, the appearance of regional and distant lymph node metastases is a major determinant of poor prognosis (1). Despite the importance of this process to the prognosis and treatment of human malignancies the underlying mechanisms remain virtually unexplored.

Cellular adhesion is now known to play a major role in the process of cancer dissemination. Tumor cell adhesion to various host cells such as lymphocytes, endothelial cells, or platelets (2–4) as well as to extracellular matrix proteins has been shown to regulate invasion and proliferation and promote tumor metastasis (for review see references 5 and 6). Integrins, a family of cell adhesion proteins that mediate cell–cell and cell–matrix interactions, some through recognition of the Arg-Gly-Asp peptide, have been implicated in the process of hematogeneous dissemination, as they mediate tumor cell attachment to vascular endothelial cells and to subendothelial matrix proteins such as laminin and fibronectin (5, 7). The involvement of integrins in the process of lymphatic metastasis has not, however, been investigated.

Previously, we have shown that tumor cell potential to metastasize lymphatically correlated well with tumor cell adhesion to frozen sections of lymph nodes in several carcinoma models of human, rat, and mouse origin (8–10). Here we extend these findings to two human melanoma models and show that adhesion of the melanoma cells to the frozen sections is $\alpha_v\beta_3$ mediated and that vitronectin is the primary adhesion ligand. The increased expression of $\alpha_v\beta_3$ on melanoma cells may therefore promote their potential to adhere in and metastasize to the lymph nodes.

Methods

Animals. 4–6-wk-old female nu/nu (CD-1)Br mice obtained from Charles River Canada (St. Constant, Quebec, Canada) were used to propagate the human melanoma line and to isolate lymph node–metastasizing cells. The mice were housed in Micro-isolator cages obtained from Lab Products Inc. (Maywood, NJ) and handled in a biological containment cabinet. Under these conditions, they remained disease free for ≥ 12 mon.

Tumor lines. The human melanoma cell line MeWo, which was originally established from a lymph node metastasis of a malignant melanoma patient (11), was obtained courtesy of Dr. R. S. Kerbel (Mt. Sinai Hospital, Toronto, Ontario, Canada). The cells were maintained as monolayer cultures in RPMI 1640 supplemented with 10% FCS, 1% gentamycin sulfate, 1% penicillin–streptomycin, and 2 mM glutamine (all reagents from Gibco Laboratories, Burlington, Ontario, Canada) (RPMI–FCS). Monolayers were dispersed twice weekly with Ca^{2+}- and Mg^{2+}-free PBS containing 0.02% EDTA and the cells were reseeded at a dilution of 1:4. All tumor cell cultures were incubated at 37°C in a 5% CO_2 incubator.

Selection and cloning of lymph node–metastasizing MeWo LNI 6I cells. Regional (inguinal, axillary, or brachial) lymph node metastases were detectable in some of the nude mice within 9 wk after the intradermal injection of 5×10^6 tumor cells when the primary tumor had reached a mean tumor diameter of ~ 1.0 cm. The animals were killed when the primary tumor was > 2.5 cm in diameter or when moribund. Pulmonary metastases were occasionally observed. The regional draining lymph nodes were aseptically removed and mechanically dispersed through an 85-μm filter (Nitex; B&SH Thompson, Town of Mount Royal, Canada). The cells were centrifuged and then seeded onto 25-cm^2 tissue culture flasks (Falcon; Fisher Scientific Co., Montreal, Canada) containing RPMI 1640 with 10% FCS. On the following day, the monolayers were washed with RPMI 1640 to remove nonadherent cells (mainly lymphocytes) and debris. This procedure was routinely

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carried out to confirm the presence of melanoma cells in the nodes. After one such procedure, confluent monolayers of lymph node–derived melanoma cells (designated MeWo LNI) were obtained. The monolayer was subsequently dispersed and the cells replated at low density leading to the isolation of individual clones. One of these clones, MeWo LNI 6I (6I),[1] was selected for further study as it had a high level of adhesion to frozen lymph node sections (see below). This clone was used for the studies described. For some of the experiments, tumor cells were also isolated from the primary MeWo tumor by enzymatic digestion with a trypsin/EDTA solution supplemented with DNAse (12). The dispersed tumor cells were passed through an 85-μm Nitex mesh, the filtrate spun down, and the cells plated in culture dishes. These cells were designated MeWo(v) to distinguish them from the MeWo cell line maintained in vitro only. MeWo(v) cells were cultured for $\leq$ 1 wk before use in the experiments.

Melanoma line MIM/8. A cell line designated MIM was established from an inguinal lymph node metastasis of a 66-yr-old male melanoma patient obtained recently through the pathology laboratory of the Royal Victoria Hospital, Montreal, Canada. The node was minced and the cells passed through an 85-μm Nitex mesh membrane as described above for MeWo LNI 6I. A single-cell suspension was obtained and plated onto a culture dish precoated with 10 μg/ml type I collagen. The melanoma cells were grown to confluence and then cloned by the limiting dilution method. A pigmented clone, MIM/8, was selected for further study since it was found to be highly tumorigenic and metastatic, giving rise to tumors in 4/5 intradermally inoculated nude mice and to regional node metastases in 3/4 tumor-bearing mice. FACS® analysis (Becton Dickinson Co., Mountain View, CA) with mAb LM609 revealed that 95% of MIM/8 cells were $\alpha_v\beta_3$ positive.

Lymph node metastasis assay. Nude mice were inoculated intradermally in the right lateral flank with 5×10^6 melanoma cells. The mice were monitored twice weekly and the size of the primary tumor measured. A local tumor was observed $\sim$ 6 wk after inoculation. Positive regional axillary or brachial lymph nodes were palpable $\sim$ 3 wk after the appearance of the local tumors when the mean diameter measured $\sim$ 1.0 cm.

Anchorage-independent growth assay. To measure anchorage-independent growth of the melanoma cells, a modification of the standard soft agar cloning assay was used (13–15). Briefly, tumor cells were mixed with a solution of either 0.6 or 1.2% agar (Difco Laboratories Inc., Detroit, MI), added to an equal volume of a 2× concentrated complete RPMI–FCS medium, and plated at a concentration of 10^3 cells/well into six-well plates (Falcon; Fisher Scientific Co.) that were prelayered with a 1.2% agar solution. The overlay was allowed to solidify and covered with 1 ml RPMI–FCS. The medium was replenished on alternate days for 12 d, at which time the tumor colonies were fixed with 1% glutaraldehyde and stained with Coomassie Blue R-250 (16). The number and diameter of the colonies were determined using a microscope (Diaphot-TMD Inverted Nikon Canada) equipped with an ocular square millimeter grid.

Cell adhesion to cryostat sections of human lymph nodes. Human lymph nodes were obtained from the Surgical Pathology Laboratory of the Royal Victoria Hospital (Montreal, Canada). The nodes were removed from patients presenting with cervical node enlargement and were snap frozen in liquid N_2. They were routinely analyzed by electron microscopy and immunohistochemistry for evidence of malignancy and used when the pathology findings were negative. Cell adhesion to the cryostat sections was measured as described in detail elsewhere (8). In all the experiments 5-μm cryostat sections were used with 5×10^5 ^{51}Cr-labeled tumor cells suspended in a volume of 40 μl.

Cell adhesion to extracellular matrix proteins. Microtiter plates were coated with human fibronectin, mouse laminin, type IV collagen (all from Collaborative Research Inc., Bedford, MA), or human vitronectin (Sigma Chemical Co., St. Louis, MO). The proteins were added to the wells at concentrations ranging from 1 to 50 μg/ml and the wells allowed to dry in a laminar flow hood overnight. Before the adhesion assay, nonspecific protein-binding sites were blocked by the addition to each well of 0.05 ml of a 1% BSA solution in PBS for 1 h. The cell-adhesion assay was essentially that described elsewhere (17), with some modifications. Briefly, tumor cells (1×10^7 cells in 75-cm^2 flask) were metabolically labeled by incubation overnight with 250 μCi of ^{35}S-methionine (Tran ^{35}S-Label™, ICN Biomedicals Inc., Costa Mesa, CA). The following day, the cells were washed, added to the protein-coated wells, and incubated at 37°C for up to 60 min. The unbound tumor cells were aspirated and the wells were washed three times with PBS. Cells were lysed with 1 N NaOH and the radioactivity in the lysate measured in a liquid scintillation counter. Adhesion to BSA-coated wells was negligible (< 1%).

Antisera and peptides. The origin and specificity of mAb LM609 directed to the $\alpha_v\beta_3$ receptor were described in detail elsewhere (17). mAb P4C10 directed to a functional epitope on the β_1 integrin subunit (18) was a kind gift from Dr. E. A. Wayner (Cytel Co., La Jolla, CA). mAb W6/32 to human HLA class I, which recognizes an antigenic determinant common to products of the HLA-A, B, and C loci, was obtained from Cedarlane Laboratories Ltd. (Hornby, Ontario, Canada). mAb 661 to vitronectin was produced and characterized by Drs. D. J. Loskutoff and D. Cheresh. Balb/C mice were immunized with purified human vitronectin and the hybridomas initially tested by ELISA on vitronectin-coated wells. The supernatants of those hybridomas that were positive in the initial screening were subsequently analyzed for the ability to block the adhesion of human melanoma line M21 to vitronectin-coated dishes (17) and mAb 661 was found to be highly inhibitory. In initial tests this antibody blocked the adhesion of 6I cells to vitronectin but not to fibronectin (see Results, Fig. 5). mAb 3E3, which is directed to the cell attachment fragment of human fibronectin, was obtained from Boehringer Mannheim Canada (Laval, Quebec, Canada). Normal mouse immunoglobulin was obtained from Bio Can Scientific (Mississauga, Ontario, Canada). Rabbit anti-mouse IgG was obtained from Dakopatts A/S (Glostrup, Denmark). Peptides, Gly-Arg-Gly-Asp-Ser-Pro-Lys and Gly-Arg-Gly-Glu-Ser-Pro, were obtained from Hukabel Scientific (Longueuil, Quebec, Canada) and Peninsula Laboratories Inc. (Belmont, CA), respectively.

Inhibition assays. Tumor cells were incubated with the antibodies for 45 min at 4°C. Excess antibody was washed off before addition of the cells to lymph node sections or to vitronectin. Inhibition by peptides was measured after incubation of the cells with various peptide concentrations for 15 min at room temperature. Cells were added to the frozen sections or to vitronectin-coated wells without prior washing. When the effects of mAb 3E3 and 661 on cell adhesion were tested they were added to the frozen sections or to vitronectin-coated wells together with the radiolabeled cells.

Indirect immunofluorescence assay. Tumor cells were incubated for 2 h at 37°C on eight-chamber slides (Lab-Tek; Nunc, Inc., Naperville, IL) that had been precoated with 10 μg vitronectin. The cells were fixed with 1% glutaraldehyde/PBS and permeabilized with 0.4% Triton X-100 in PBS containing 50 mM glycine HCl. The chambers were washed with RPMI and mAb LM609 added at a dilution of 1:25 for 1 h at 4°C, followed by a second incubation with FITC-conjugated goat anti-mouse IgG (Bio/Can Scientific) at a dilution of 1:100. The slides were dried and mounted with a glycine–glycerol solution (19).

Flow cytometry. For flow cytometric analysis, 1×10^6 tumor cells were incubated for 1 h on ice first with 65 μg/ml of the mAb and then with FITC-conjugated goat anti-mouse IgG (150 μg/ml). After washing, the cells were resuspended in 1 ml of RPMI 1640 and analyzed using a cell analyzer (FACSTAR; Becton Dickinson, Mountain View, CA) equipped with an argon-ion (15 MW) laser at 488 nm with a 530/30 DF filter. The program used to analyze the data was Consort 30™ Version E 12/86 (Becton Dickinson). Control cells were incubated with the second antibody only. For each cell line $\sim$ 5,000 cells were analyzed.

1. *Abbreviations used in this paper:* ECM, extracellular matrix; MIF, mean intensity of fluorescence; RGD, Arg-Gly-Asp; 6I, line MeWo LNI6I.

Statistics. Student's *t* test was used to analyze adhesion data and Fisher's Exact test was used to analyze results of in vivo studies (20).

Results

Metastatic MeWo cells were isolated from the regional lymph nodes of tumor-bearing nude mice, cultured in vitro for ~ 2 wk and their adhesion to cryostat sections of human lymph nodes measured and compared with that of the parental MeWo line. The results shown in Fig. 1 are consistent with previous findings we obtained using a breast carcinoma model (9) and demonstrate that the lymph node metastases–derived tumor cells were significantly more adherent to lymph node cryostat sections than the parent line. The metastatic cells were subsequently cloned and clone 6I was selected for further study as it was found to be highly adherent to lymph node cryostat sections (Fig. 1). When the tumorigenic and metastatic properties of clone 6I were subsequently analyzed by inoculation of the tumor cells intradermally in nude mice, we found that tumor-bearing animals inoculated with 6I cells had a significantly higher incidence of lymph node metastases than animals inoculated with the MeWo cells, whereas the incidence and rates of growth of the primary tumors were not markedly different (Table I). Furthermore, 6I cells had a significantly higher cloning efficiency in semisolid agar (Table II), a property previously shown to correlate with the metastatic potential of melanoma cells (13).

The adhesion of MeWo and 6I cells to extracellular matrix proteins that were previously identified in human lymph nodes (21, 22), namely, fibronectin, laminin, type IV collagen, and vitronectin, was subsequently investigated using matrix protein-coated dishes. As shown in Fig. 2, a significant difference in adhesion between MeWo and 6I cells at all time intervals tested was seen only on vitronectin-coated plates. Attachment of 6I cells to other matrix proteins was lower than that to vitronectin and differences between the adhesion of the two cell lines, where seen, were of a considerably lower magnitude. Adhesion to BSA was < 1%.

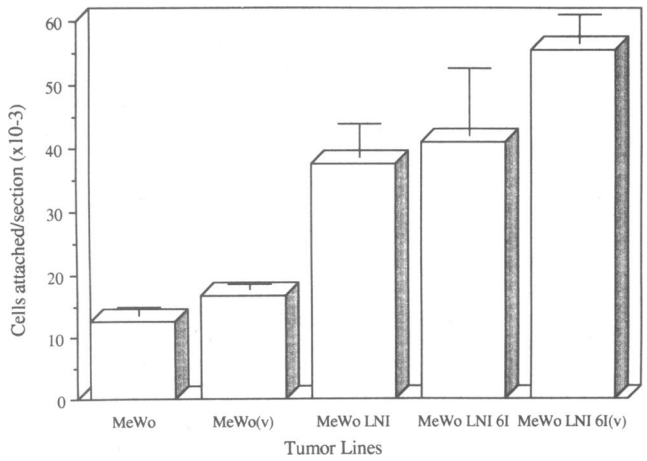

Figure 1. Adhesion of MeWo cells to cryostat sections of human lymph nodes. Sublines that have been passaged in vivo and readapted to culture in vitro are denoted with a (v). Results are means and SD of quadruplicate samples. The proportion of the 6I and MeWo LNI cells that adhered to the sections was significantly higher than that of MeWo cells ($P < 0.007$ and 0.039, respectively). There was no significant difference between the adhesion of MeWo LNI and 6I cells ($P > 0.1$).

Table I. Incidence of Primary Tumors and Lymph Node Metastases in Animals Inoculated with Tumor Lines MeWo and MeWo LNI 6I Cells

Cells injected	Incidence of primary tumors	Incidence of regional lymph node metastases
MeWo	14/19*	2/14‡
MeWo LNI 6I	10/10	6/10

Nude mice were injected intradermally in the right lateral region with 5×10^6 cells. Tumors developed within 5–6 wk and lymph node metastases observed when the mean diameter of the tumors measured 1.0 cm. * $P = 0.134$. ‡ $P = 0.032$ (Fisher's Exact Test).

That this differential adhesion to vitronectin was not an artifact of the prolonged in vitro culture of MeWo cells was established by reinoculation of the melanoma cells into nude mice to obtain–MeWo(v). These tumor cells were maintained in culture for only 1 wk to deplete the host cell infiltrate and their adhesion to cryostat sections was then measured. Results shown in Fig. 1 demonstrate that there was no significant difference ($P > 0.12$) between the adhesion of MeWo or 6I cells propagated in vivo and those maintained in vitro for an extended period of time, confirming that the increased adhesion of 6I cells was not due to the in vitro–in vivo selection process per se. Similar results were obtained when the adhesion of these lines to vitronectin-coated dishes was measured (data not shown).

To investigate the role of vitronectin in the adhesion of line 6I cells to lymph node sections, the tumor cells were treated before the adhesion assay with antibody LM609 directed to integrin $\alpha_v\beta_3$. Results shown in Fig. 3, *A* and *B* demonstrate a dose-dependent inhibition by the antibody of attachment to the sections and to vitronectin-coated dishes. Maximal inhibition ranged between 70 and 80% of control levels. A mAb directed to the functional domain of the β_1 subunit (P4C10) also inhibited adhesion to the sections with maximal inhibition ranging between 40 and 45% whereas a mAb directed to an HLA class I determinant (W6/32) had no measurable effect on the adhesion (Fig. 3 *C*).

Table II. Cloning Efficiency of MeWo Cells in Semisolid Agar

	Cloning efficiency*		Colony size‡	
Cell line	0.3%§	0.6%	0.3%	0.6%
			μm	
MeWo	0.27±0.047	0.15±0.05	19.5±2.6	53.3±14.0
MeWo LNI 6I	0.73±0.28	0.37±0.13	113.4±13.3	110.0±46.9

The cloning method is described in Methods. The results are means and SD of triplicate samples. The difference in cloning efficiency and colony size between MeWo and 6I cells was significant ($P < 0.05$) in both agar concentrations. * Cloning efficiency was calculated by counting the number of colonies 12 d after plating 10^3 tumor cells. ‡ The diameters of all the colonies were measured using a Nikon inverted microscope equipped with an ocular grid. § Final concentration of agar in the overlay.

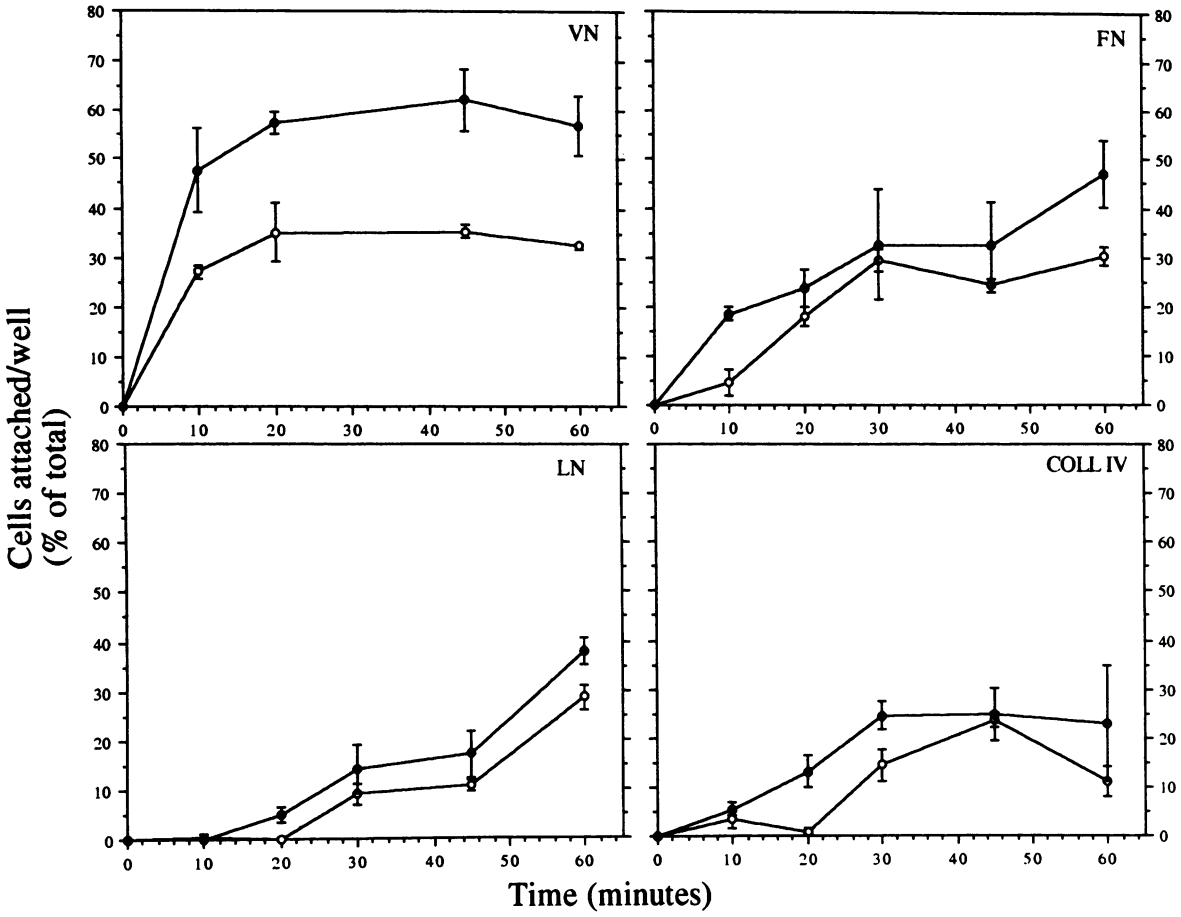

Figure 2. Adhesion of MeWo and 6I cells to ECM protein–coated plates. For coating, 50 μl of a 10 μg/ml solution of the proteins were added to each well of a microtiter plate. Results shown are means and SD of triplicate samples. The background adhesion to BSA-coated wells (< 1%) was subtracted. 6I (●) cells were significantly more adherent to vitronectin than MeWo cells (○) at all time intervals ($P < 0.04$ at 10 and 20 min and $P < 0.001$ at 40 and 60 min). Differences in attachment to fibronectin and laminin were significant only at 60 min ($P < 0.04$ and < 0.01, respectively) and to type IV collagen at 20 and 30 min ($P < 0.05$).

Consistent with the role of vitronectin as the ligand on the lymph node frozen sections was also our finding that an RGD-containing peptide significantly and specifically blocked the adhesion of 6I cells to the sections (shown in Fig. 4). Furthermore, a mAb to human vitronectin (mAb 661), which blocked the binding of 6I cells to vitronectin-coated dishes (Fig. 5 *B*) but not to fibronectin-coated dishes (Fig. 5 *C*), also inhibited the adhesion of these cells to the cryostat sections by 80% (Fig. 5 *A*). In contrast a mAb, directed to the cell attachment fragment of human fibronectin (mAb 3E3), inhibited adhesion to the sections by only 19%.

Cell surface expression of $\alpha_v\beta_3$ on the MeWo cells was subsequently measured by flow cytometric analysis using indirect immunofluorescence staining. As shown in Table III and Fig. 6, the proportion of positively labeled cells was significantly higher in the 6I subline relative to MeWo. This was also reflected in an increase in the mean intensity of fluorescence (MIF) (from 133.2 for MeWo to 163.2 for 6I). When the MIF measured with MeWo cells was used as an arbitrary lower limit (Fig. 6 *A, solid vertical bar*), to estimate the proportion of "brightly" fluorescing cells in the two cell lines, as many as 40% of 6I cells but only 8.1% of MeWo cells ($P < 0.001$) were found to be brightly stained. These results were in contrast to fluorescence profiles obtained with antibodies P4C10 and W6/32, where no increase in the proportion of labeled cells or in MIF for 6I cells was observed (Fig. 6 *B* and *C* and Table III). Fluorescein-labeled 6I cells cultured on vitronectin-coated dishes are shown in Fig. 7. The cells were virtually indistinguishable morphologically from MeWo cells.

To determine whether our findings with the MeWo line were relevant to other melanomas, in particular melanoma cells of more recent origin, a second melanoma model recently developed from a human lymph node metastasis was used. A pigmented clonal subline, designated MIM/8, was obtained and found to be highly tumorigenic and metastatic, giving rise to primary tumors in four of five intradermally inoculated nude mice and to regional lymph node metastases in three of four tumor-bearing mice.

When adhesion of MIM/8 cells to cryostat sections of human lymph nodes was measured, it was found to be as high as that of the 6I cells (i.e., 5–6 $\times$ 10^4 cells attached/section). Results of inhibition studies with the mAb are shown in Table IV. Similar to the results seen with 6I cells, the adhesion to the sections could be significantly inhibited by mAb LM609, whereas mAb P4C10 and mAb W6/32 did not significantly reduce binding. A significant inhibition of binding was also seen when either mAb 661 or mAb 3E3 were added to the sections together with the MIM/8 cells.

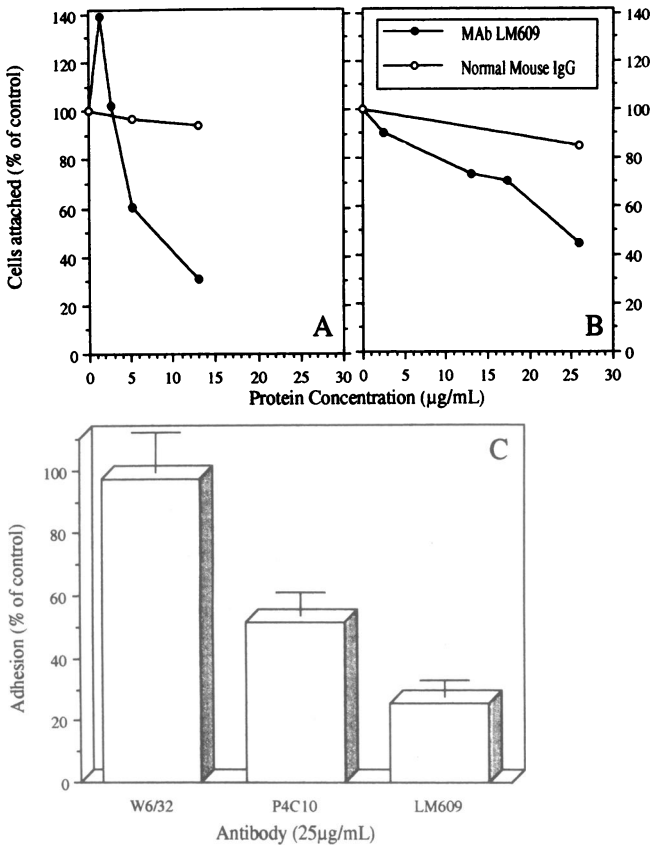

Figure 3. mAb LM609 blocks the adhesion of 6I cells to the lymph node sections. Cells were pretreated with the antibody and washed three times before their addition to the frozen sections (*A*) or to vitronectin (*B*). The results shown are the means and SD of quadruplicate samples. Adhesion to BSA-coated wells (< 1%) was subtracted. The effects of mAb P4C10 (directed to the β_1 subunit of integrins) and mAb W6/32 (directed to HLA class I determinant) on adhesion to the sections are compared with the effect of LM609 in *C*. There was no significant difference between the adhesion of untreated cells and cells treated with mAb W6/32.

Discussion

Our previous work has shown that tumor cell adhesion to frozen lymph node sections was an in vitro correlate of tumor cell potential for lymphatic metastasis in several animal and human carcinoma models (8–10). The present study extends these findings and establishes the relevance of this adhesion system to human melanoma. Our findings with the MeWo and MIM/8 lines are particularly significant as both lines were established from lymph node metastases and the major site of metastasis for these cells in nude mice after intradermal inoculation is the regional lymph node (11). The increased attachment to frozen sections seen with melanoma cells that originated from a lymph node metastasis in a nude mouse and similar findings obtained with a human breast carcinoma sub-

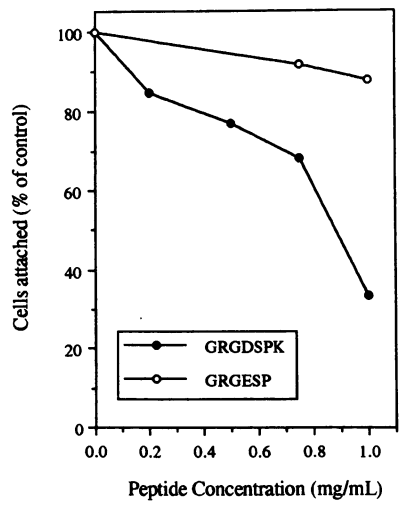

Figure 4. An RGD-containing peptide blocks the adhesion of 6I cells to lymph node sections. The cells were incubated with the peptide for 15 min before addition to the sections. The results are means and SD of quadruplicate samples.

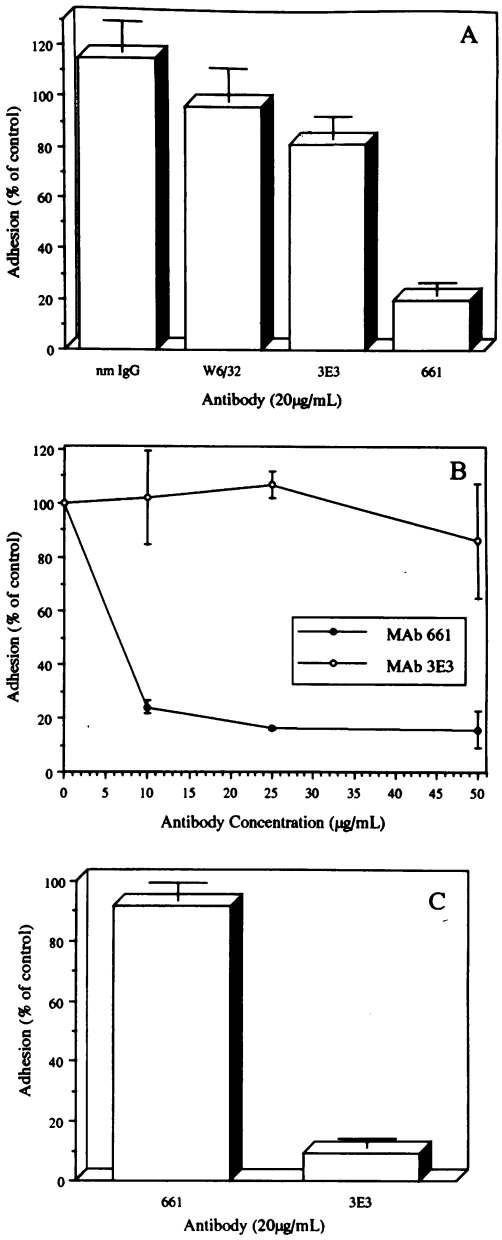

Figure 5. mAb 661 to human vitronectin blocks adhesion of 6I cells to frozen sections. Shown are the effects of mAb 661 on adhesion of 6I cells to frozen lymph node sections (*A*), vitronectin-coated dishes (*B*), and fibronectin-coated dishes (*C*). Cells were added to the different substrata together with the antibody. The results shown are the means and SD of triplicate samples. Adhesion to the sections in the presence of mAb 661 was significantly lower ($P < 0.02$) than that of any of the control groups.

Table III. Flow Cytometric Analysis of mAb-stained Melanoma Cells

Antibody	Cell surface determinant	Cell type analyzed	Positive cells
			%
LM609	$\alpha_v\beta_3$	MeWo	62.6
		MeWo LNI 6I	95.9
P4C10	β_1	MeWo	99.9
		MeWo LNI 6I	99.8
W6/32	HLA class I	MeWo	100
		MeWo LNI 6I	100

Cells were labeled using an indirect immunofluorescence assay and analyzed using a FACSTAR Cell Analyser. Control levels were determined by treating the cells with the second antibody only. Maximal staining under control conditions was used as a lower limit for gating positive cells.

line (10) suggest that cellular mechanisms that mediate lymphatic dissemination in this animal model are relevant to the clinical process. It should be noted in this context that there was no significant difference between the adhesion of MeWo and MeWo LNI 6I cells to cryostat sections of human kidneys (adhesion levels were 2.2 and 2.6%, respectively), suggesting that the adhesion ligand on the lymph node sections may be expressed in an organ-selective manner.

Our results show that binding of 6I cells to the sections was RGD dependent and mediated in large part by the receptor $\alpha_v\beta_3$. Adhesion studies on extracellular matrix-coated dishes confirmed that this metastatic subline was significantly more adherent to vitronectin than the parental line MeWo whereas the differences in attachment to collagen IV, fibronectin, and laminin were more minor. This increased adhesion was not the result of the in vivo selection process per se as no differences were found in either the levels of adhesion or in the expression of $\alpha_v\beta_3$ (as assessed by FACS; results not shown) between MeWo cells maintained in culture or derived from a solid tumor growing in vivo. Moreover, in recently published studies by Albelda et al. (23), it has been shown that adaptation of melanoma biopsy-derived cells to growth in vitro augmented rather than reduced their expression of $\alpha_v\beta_3$. It appears therefore that melanoma cells metastatic to the lymph node represent a subpopulation of tumor cells with increased adhesiveness to the lymph node extracellular matrix (ECM).

The ability of mAb LM609 to block the adhesion of 6I and MIM/8 cells to frozen sections confirmed that it was mediated by integrin $\alpha_v\beta_3$. The differences in the levels of inhibition by mAb LM609 seen on the frozen sections (Fig. 3 *A*) and on vitronectin-coated dishes (Fig. 3 *B*) suggest that although $\alpha_v\beta_3$ may be the main receptor mediating adhesion to vitronectin on the frozen sections, other receptors, possibly $\alpha_v\beta_5$, may play a role in the adhesion of the 6I cells to vitronectin-coated dishes.

Although receptor $\alpha_v\beta_3$ is also known to mediate attachment to fibrinogen and von Willebrand factor (17), these proteins are unlikely to play a role in the adhesion of the melanoma cells to the lymph node sections. In a recent study, fibrinogen could not be detected on lymph node cryostat sections whereas von Willebrand factor was found to be localized in the blood vessels (D. A. Cheresh, unpublished observations). Tumor cell attachment, on the other hand, has been localized to the lymph node stroma and the subcapsular sinus (10). Furthermore, we found that mAb 661 to human vitronectin, which inhibited binding of 6I cells to vitronectin but not fibronectin-coated dishes, also blocked adhesion of these cells to the frozen sections. A second mAb (3E3) directed to the cell-binding domain of fibronectin had only a minor inhibitory effect on the adhesion of 6I cells but could significantly reduce adhesion of MIM/8 cells, suggesting that for some melanoma cells $\alpha_v\beta_3$ may mediate adhesion to multiple ECM ligands in the node.

Flow cytometric analysis using indirect immunofluorescence labeling revealed that a significantly larger proportion of 6I cells compared with MeWo cells labeled intensely with antibody LM609. This increased expression was specific to $\alpha_v\beta_3$

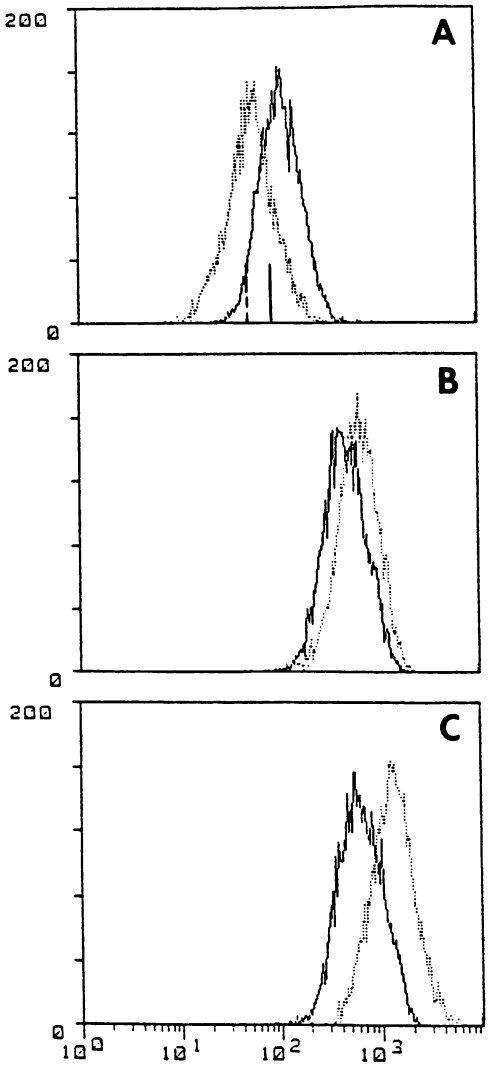

Figure 6. Flow cytometric analysis of immunofluorescence-labeled MeWo and 6I cells. An indirect staining method was used to label the cells with mAb LM609 (*A*), P4C10 (*B*), and W6/32 (*C*). Control cells that were incubated only with the second antibody were used for gating. The dotted vertical bar represents the maximum staining of control cells. The solid vertical bar in *A* denotes the mean intensity of fluorescence measured for MeWo cells. This was used as an arbitrary lower limit to determine the proportion of cells in the two cell lines for which cell surface expression of $\alpha_v\beta_3$ exceeded this level. Each profile is based on the analysis of ~ 5,000 cells. The ordinate in each graph represents the number of cells and the abscissa shows the relative intensity of fluorescence.

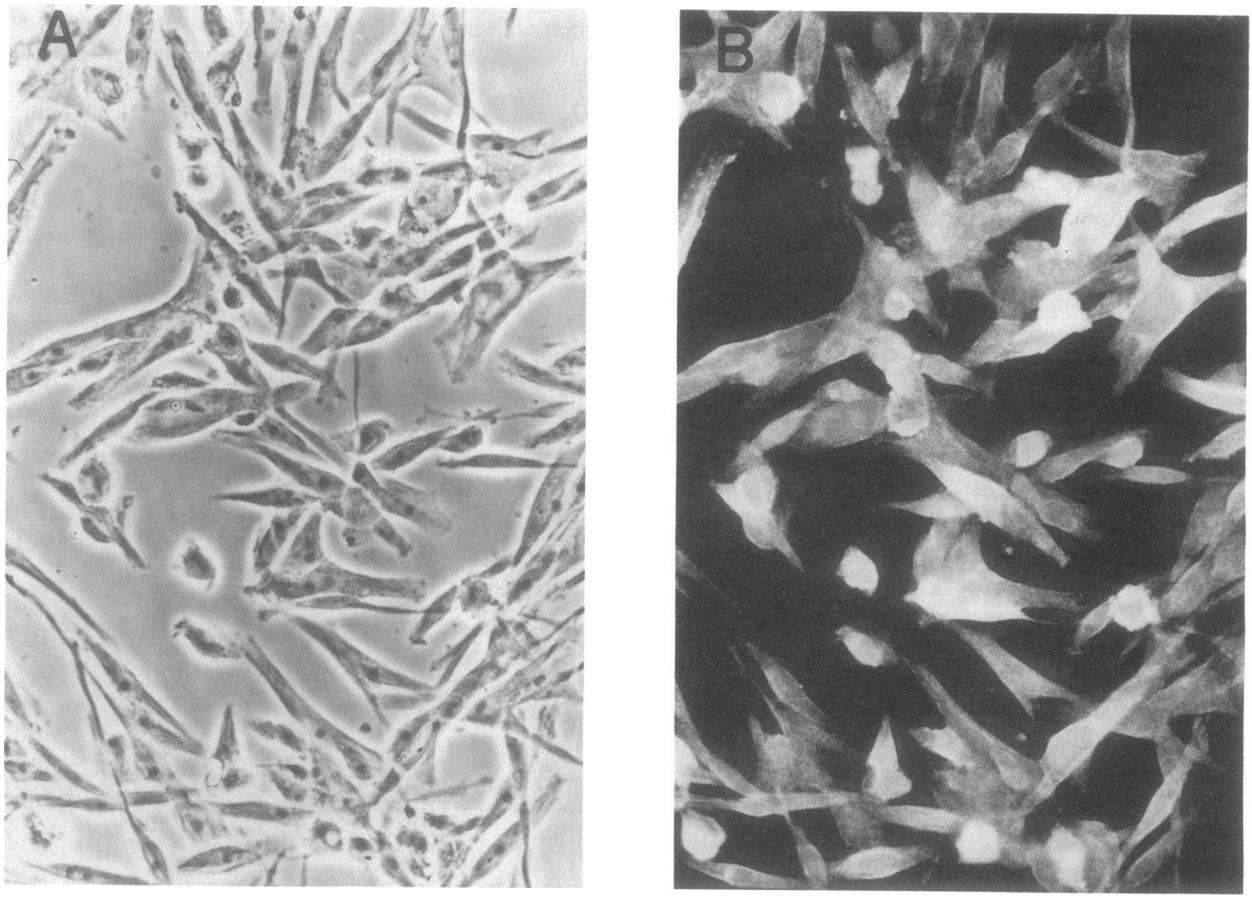

Figure 7. Immunofluorescence-labeled 6I cells. Cells were cultured on vitronectin-coated chamber slides and labeled with mAb LM609 and an FITC-conjugated goat anti-mouse IgG antibody. Positively stained cells are seen in *B*. The same microscopic field viewed by phase contrast microscopy is shown in *A*. ×400.

and did not reflect a general upregulation of integrins or differentiation antigens as neither β_1 nor MHC expression were increased in these cells. Together our results suggest that melanoma cells expressing high levels of $\alpha_v\beta_3$ may have a selective advantage with respect to metastases formation in the lymph node. As adhesion to vitronectin in the present tumor model is rapid (with maximal binding seen at 20 min) and since differences between adhesion of the two cell lines were apparent as early as 10 min after incubation with the ligand, it is likely that these "high expressor" cells also represent the major subpopulation mediating attachment in the in vitro assays.

Table IV. The Effect of mAb Treatment on the Adhesion of MIM/8 Cells to Frozen Sections of Human Lymph Nodes

mAb used	Adhesion
	% of control
661	51±10
3E3	50±11
LM609	62±8
P4C10	88±11
W6/32	97±20
mmIgG	137±23

Treatment with mAb was as described in the legends to Figs. 3 and 5. Antibody concentrations used were 20–25 μg/mL. Shown are means ±SD of two to three experiments. Mean adhesion of control (nontreated) cells was 5.3×10^4 cells/section (10% of total).

Our results with antibody P4C10 suggest that β_1 integrins also play a role in the adhesion of 6I cells to the frozen sections. However the failure to demonstrate a consistent difference in the attachment of MeWo and 6I cells to fibronectin, laminin, and type IV collagen and the results of the FACS analysis showing a decrease in the intensity of labeling with P4C10 in 6I cells compared with MeWo cells (Fig. 6 *B*) when taken together argue that the preferential attachment to vitronectin through $\alpha_v\beta_3$ plays a more decisive role in this tumor model. It is possible that adhesion through β_1-containing complexes represents a secondary event that may be triggered by $\alpha_v\beta_3$-mediated attachment and may play a stabilizing role.

Although changes in integrin-mediated adhesion of tumor cells to the extracellular matrix have been shown to regulate the growth and hematogenous dissemination of cancer cells (5), this report provides the first indication that an integrin receptor molecule may play an important role in lymphatic metastasis. In this context our findings are consistent with two recent reports that implicated the $\alpha_v\beta_3$ receptor in the malignant progression of human melanoma. Using a histochemical analysis to measure the expression of various integrin molecules on frozen sections derived from melanoma biopsies, Albelda et al. (23) have recently shown that a significant increase in the cell surface expression of this receptor in situ is associated with progression of the disease from benign nevus to vertical growth

phase-malignant melanoma. Marshall et al. (24), using immunohistochemistry and immunoprecipitation with an anti-VnR-specific mAb, have shown that $\alpha_v\beta_3$ expression is significantly augmented in human melanoma cells compared with foreskin-derived normal melanocytes.

The functional significance of cell adhesion to vitronectin in the process of lymph node metastasis requires further investigation. Important in this respect are recent reports by Kramer et al. (21) and by Reilly and Nash (22), the first demonstrating that basement membrane components such as fibronectin, laminin, and type IV collagen are associated with the reticular fibers found throughout the lymph node parenchyma, including the lymphatic sinuses, and the second showing a codistribution of vitronectin and fibronectin to the reticulin stroma of human lymph nodes (reference 22; see also references 25 and 26). We have confirmed these findings and found that these ECM proteins can be localized to the reticular fibers and germinal centers on lymph node cryostat sections (unpublished observation). As light-microscopy analysis revealed that some of the cells adhering to the frozen sections can be found in the marginal sinus (8, 10), it is possible that vitronectin and fibronectin provide a site of anchorage for cancer cells infiltrating the node and reaching the marginal sinus via the afferent lymphatic channels. The observation by Reilly and Nash (22) that synthesis of vitronectin and fibronectin in the node is upregulated after invasion by the tumor cells suggests that the local environment in the node may become increasingly favorable to cancer cells that express high levels of the VnR.

Our finding that 6I cells had a significantly increased metastatic potential in vivo compared with MeWo cells is consistent with the notion that $\alpha_v\beta_3$ plays a role in the increased metastatic potential of these tumor cell in vivo. The increased cloning efficiency of 6I cells in semisolid media suggests that, in addition, changes in other, growth-related properties of these cells (possibly indirectly related to $\alpha_v\beta_3$ expression) also contribute to their enhanced metastatic potential.

Boukerche et al. (27) have shown that the local growth of a human melanoma in nude mice could be inhibited by pretreatment of the cells with a mAb reactive against the platelet glycoprotein $\alpha_{IIb}\beta_3$. This glycoprotein complex is known to mediate platelet aggregation and shares the β_3 subunit with the $\alpha_v\beta_3$. Taken together with these observations our results strongly suggest that reagents such as mAb and peptides that can interfere with melanoma adhesion may provide potent therapeutic tools in the management of malignant melanoma during the early stages of dissemination.

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