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### Research Article

Integrin alpha v beta 3 is a marker of progression in malignant melanoma. Previously we reported that human melanoma cells derived from regional lymph node metastases had increased alpha v beta 3-mediated adhesion to lymph node vitronectin. In the present study, the expression and function of alpha v beta 3 were further investigated with emphasis on the functional relationship between alpha v beta 3 and the urokinase-type plasminogen activator system of proteolysis. We found that metastases-derived melanoma MeWo LNI 6I (6I) and MIM/8 LNI cells had a markedly increased expression of alpha v mRNA transcripts relative to the parent lines which was reflected in significantly elevated levels of the alpha v beta 3 heterodimers on the cell surface. These cells also expressed elevated levels of urokinase plasminogen activator receptor (uPAR) mRNA and had higher levels of surface bound urokinase plasminogen activator as detected by immunolabeling. To determine whether the expression of uPAR and alpha v were linked, alpha v synthesis in the metastatic melanoma cells was suppressed using alpha v antisense phosphorothioate oligonucleotides. This resulted in a marked decrease in detectable alpha v mRNA and protein and a corresponding substratum-specific reduction in cell adhesion to vitronectin. When uPAR expression in these cells was subsequently analyzed, we found a reduction of approximately 50% in uPAR mRNA levels. On the other hand, [...]

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# Coordinated Expression of the Vitronectin Receptor and the Urokinase-type Plasminogen Activator Receptor in Metastatic Melanoma Cells

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## Abstract

Integrin  $\alpha_v\beta_3$  is a marker of progression in malignant melanoma. Previously we reported that human melanoma cells derived from regional lymph node metastases had increased  $\alpha_v\beta_3$ -mediated adhesion to lymph node vitronectin. In the present study, the expression and function of  $\alpha_v\beta_3$  were further investigated with emphasis on the functional relationship between  $\alpha_v\beta_3$  and the urokinase-type plasminogen activator system of proteolysis. We found that metastases-derived melanoma MeWo LNI 6I (6I) and MIM/8 LNI cells had a markedly increased expression of  $\alpha_v$  mRNA transcripts relative to the parent lines which was reflected in significantly elevated levels of the  $\alpha_v\beta_3$  heterodimers on the cell surface. These cells also expressed elevated levels of urokinase plasminogen activator receptor (uPAR) mRNA and had higher levels of surface bound urokinase plasminogen activator as detected by immunolabeling. To determine whether the expression of uPAR and  $\alpha_v$  were linked,  $\alpha_v$  synthesis in the metastatic melanoma cells was suppressed using  $\alpha_v$  antisense phosphorothioate oligonucleotides. This resulted in a marked decrease in detectable  $\alpha_v$  mRNA and protein and a corresponding substratum-specific reduction in cell adhesion to vitronectin. When uPAR expression in these cells was subsequently analyzed, we found a reduction of  $\sim 50\%$  in uPAR mRNA levels. On the other hand, ligation of the  $\alpha_v\beta_3$  receptor on the melanoma cells by immobilized antibody resulted in a twofold increase in uPAR mRNA. The results suggest that the expression of uPAR in metastatic melanoma cells is linked to the expression and function of the vitronectin receptor. (*J. Clin. Invest.* 1995; 95:2096–2103.) **Key words:** metastasis • melanoma • integrin • vitronectin receptor • urokinase plasminogen activator receptor.

## Introduction

Malignant melanoma is becoming increasingly prevalent worldwide (1) and its incidence is growing more rapidly than any other form of cancer (2). Metastasis to the draining regional

and distant lymph nodes precedes wide-spread dissemination of malignant cells and is normally associated with poor prognosis (3). Little is presently known about the molecular mechanisms involved in melanoma dissemination to regional nodes.

Tumor cell metastasis to secondary sites is a complex, multistep process involving cell detachment from the primary site, migration through lymph (or blood) vessels, cell adhesion to cellular and extracellular matrix (ECM)<sup>1</sup> elements of the invaded organ, proteolytic degradation of matrix proteins, and cell proliferation (4). Integrins, a family of cell adhesion receptors consisting of  $\alpha/\beta$  heterodimers, mediate cell–ECM as well as cell–cell interactions and are known to regulate cell migration and growth (5). Expression of the vitronectin receptor integrin  $\alpha_v\beta_3$  has been linked to malignant progression of melanoma (6) but its function in the process of invasion and metastasis are not presently clear. Recent studies have suggested that clustering of  $\alpha_v\beta_3$  by antibody or ligand can trigger increased expression of the metalloproteinase type IV collagenase thereby promoting invasion (7). Other studies suggested that  $\alpha_v\beta_3$  plays a role in regulation of melanoma cell growth in vitro and in vivo (8). We recently reported (9) that human melanoma cells derived from regional lymph nodes had an increased  $\alpha_v\beta_3$ -mediated adhesion to lymph node vitronectin.

In addition to the metalloproteinases, the serine protease urokinase plasminogen activator (uPA) and its membrane-linked receptor (uPAR) have also been repeatedly implicated in the metastatic process. Recently we have shown that increased skeletal metastasis of prostate cancer cells is directly related to uPA overproduction (10). The evidence is particularly strong for involvement of the uPA/uPAR system in melanoma invasion and dissemination (11, 12). This activity is regulated by plasminogen activator inhibitors 1 and 2. Binding of plasminogen activator inhibitor-1 to receptor-linked uPA has been shown to trigger internalization of the complex and subsequent processing and reexpression of the receptor at a second site providing a mechanism for receptor mobility as the cells migrate through degraded ECM (13). As the binding of uPA to its receptor occurs at focal adhesion contacts, and plasminogen activator inhibitor-1 is associated with ECM vitronectin and can stabilize vitronectin-mediated adhesion, it was of interest to investigate the relationship between  $\alpha_v\beta_3$  and the urokinase system in melanoma cells selected in vivo for increased metastasis to regional nodes. The present results show that uPAR transcription in malignant melanoma cells is linked to  $\alpha_v$  expression.

## Methods

**Animals and tumor lines.** Female nu/nu (CD-1)Br mice, 4–6 wk old (Charles River Canada; St-Constant, Canada) were used for propagation

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1. **Abbreviations used in this paper:** ECM, extracellular matrix; GFD, growth factor–like domain; uPA, urokinase plasminogen activator; uPAR, urokinase plasminogen activator receptor.

of the human melanoma cell lines and for isolation of the metastatic variants. The mice were housed in Microisolator cages (Lab Products Inc., Maywood, NJ) and handled in biohazard cabinets. They remained disease free for up to 1 yr.

Cell line MIM was established from an inguinal lymph node metastasis of a male melanoma patient as we described previously (9). A pigmented clone-MIM/8 was derived by serial dilution cloning of MIM cells. Subsequent intradermal injection of  $5 \times 10^6$  MIM/8 cells into the right lateral flanks of nude mice gave rise to palpable primary tumors at 3 wk and regional lymph node metastases at 12 wk, after inoculation. The lymph node-invading melanoma cells were isolated by mincing the lymph nodes and mechanically dispersing the cells through an 85- $\mu$ m nylon filter (Nitex; B&SH Thompson, Town of Mont Royal, Canada). Cells were washed and seeded onto 25-cm<sup>2</sup> tissue culture flasks (Falcon Labware; Fisher Scientific Co., Montreal, Canada) containing RPMI-1640 supplemented with 5% FCS, 1% gentamycin sulfate, 1% penicillin-streptomycin, and 2 mM glutamine (all reagents were from Gibco Laboratories, Burlington, Canada) (RPMI-1640-FCS). Nonadherent cells and cellular debris were removed 24 h later and the adherent melanoma cells designated MIM/8 LNI, were maintained in culture until a confluent monolayer was obtained. The tumorigenicity and metastatic properties of the melanoma cells were determined after intradermal injection of  $5 \times 10^6$  cells into nude mice. Of five animals inoculated with MIM/8 cells, only two developed regional lymph node metastases whereas four/four animals bearing MIM/8 LNI tumors developed regional lymph node metastases.

The origin and metastatic properties of cell line MeWo LNI 6I (6I) were described in detail previously (9).

All cell lines were maintained as monolayer cultures in RPMI-1640-FCS. Cell lines were subcultured twice weekly using Ca<sup>2+</sup> and Mg<sup>2+</sup>-free PBS containing 0.02% EDTA to disperse the cells.

**Antibodies.** mAb W6/32 directed 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 (Hornby, Canada). Normal mouse immunoglobulin was from Bio-Can Scientific (Mississauga, Canada), rabbit anti-mouse IgG was from Dakopatts A/S (Glostrup, Denmark), peroxidase-conjugated goat anti-rabbit IgG from Sigma Chemical Co. (St. Louis, MO), and peroxidase-conjugated rabbit anti-mouse IgG from Dimension Laboratories (Mississauga, Canada). The following antibodies were kind gifts; mAb's LM142 and LM609 to the  $\alpha_v$  subunit, and  $\alpha_v\beta_3$  complex (14), respectively, were from Dr. D. Chersesh (Scripps Research Clinic, LaJolla, CA), mAb P3G8 to  $\alpha_v$  (15) and mAb A3-IIF5 to  $\alpha_3$  (16) from Dr. M. Hemler (Dana-Farber Cancer Institute, Boston, MA), mAb AP-3, directed to the  $\beta_3$  integrin subunit, from Dr. P. J. Newman (Blood Research Institute, Milwaukee, WI), and rabbit antibody to uPA, which recognizes the growth-factor-like domain (GFD) (17), from Dr. Andrew Mazar (Abbott Laboratories, Chicago, IL).

**cDNA probes and oligomers.**  $\alpha_v$  cDNA (18) was obtained from Gibco Laboratories. The mouse 72-kD type IV collagenase cDNA was a kind gift from Dr. Ann F. Chambers (London Regional Cancer Centre, London, Ontario, Canada). An antisense oligonucleotide corresponding to the first 15 amino acids of the uPA receptor (19) was synthesized by Sheldon Biotechnology (McGill University, Montreal, Canada). uPA cDNA (pHUK-1) was obtained from the American Type Culture Collection (Rockville, MD). Antisense phosphorothioate oligonucleotides directed to the  $\alpha_v$  subunit of human vitronectin receptor were chosen on the basis of the BLAST alignment program (20) to have no homology with other known human DNA sequences including integrin  $\alpha$  subunits such as  $\alpha_5$  (6). They include AS2 (5'-TCAGCATCAATATCTTGT-3'), complementary to bases 563 to 580 of the human vitronectin receptor sequence; AS3 (5'-AAGCCATCGCCGAAGTGC-3'), complementary to bases 31 to 48 of the human sequence; and AS4 (5'-GACTGTCCACGTCTAGGT-3'), complementary to bases 136 to 153. As a control, a sense sequence S1 (5'-GCACTTCGGCGATGGCTT-3'), corresponding to AS3 was used. The oligonucleotides were synthesized on a DNA/RNA synthesizer (392; Applied Biosystems, Foster City,

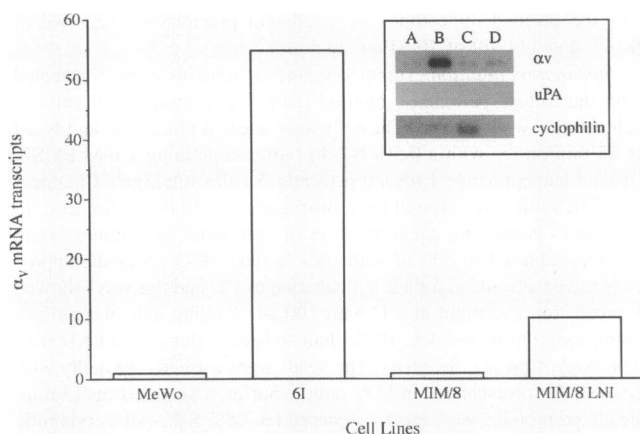
CA) and purified three times using ethanol precipitation (2.5 vol of ethanol and 1/4 vol of 10 M ammonium acetate).

**Immunoprecipitation.** Tumor cell-surface proteins were <sup>125</sup>I-labeled using the lactose peroxidase method (21). Equal numbers of cells of each cell line were labeled. The cells were washed repeatedly and lysed for 15 min on ice with a 0.5% NP-40 buffer containing 1 mM PMSF, 1 mg/ml leupeptin, and 1 mg/ml pepstatin A (all from Sigma Chemical Co.). The lysate was cleared by centrifugation at 10,000 g for 15 min. To aliquots containing equal numbers of cpm from each tumor lysate (volumes adjusted to 500  $\mu$ l with lysis buffer), 1–5  $\mu$ g of the appropriate mAb were added for a 2-h incubation at 4°C and this was followed by incubation overnight at 4°C with 100  $\mu$ l of rabbit anti-mouse IgG complexed to protein-A CL-4B Sepharose beads (Pharmacia LKB Biotechnology, Uppsala, Sweden). The beads were washed repeatedly with 0.1% NP-40, resuspended in SDS sample buffer, and boiled for 15 min. The eluted proteins were electrophoresed on 7.5% SDS-polyacrylamide gels under nonreducing conditions. For radiolabeled proteins, gels were dried and exposed to x-ray film (X-OMAT AR; Eastman Kodak Co., Rochester, NY) for 7–14 d. The relative intensity of the integrin bands on the autoradiographs was analyzed by laser densitometry using an Ultrosan XL Enhanced Laser Densitometer (LKB Instruments Inc., Bromma, Sweden).

**RNA isolation and Northern blot analysis.** Total RNA was isolated using the acid guanidium thiocyanate-phenol-chloroform method of Chomczynski and Sacchi (22). Poly (A)<sup>+</sup>RNA was isolated using an mRNA purification kit obtained from Stratagene Inc. (La Jolla, CA). The RNA (30  $\mu$ g of total RNA or 5  $\mu$ g of poly (A)<sup>+</sup>RNA) was separated by electrophoresis through a 1.1% agarose gel containing 2 M formaldehyde. The RNA was transferred to a nylon membrane (Hybond N; Amersham International, Oakville, Canada) by capillary action and hybridized with cDNA probes radiolabeled by random primer extension with [ $\alpha$ -<sup>32</sup>P]dCTP (23) or with oligomers which were end-labeled with [ $\gamma$ -<sup>32</sup>P]dATP using T4 polynucleotide kinase (24) for 48 h at 42°C. The membranes were washed twice for 30 min at room temperature with 1  $\times$  SSC containing 0.1% SDS and twice for 30 min at 55°C with 0.1  $\times$  SSC containing 0.1% SDS. The blots were exposed for 2–7 d at –70°C. As a control for RNA loading, the blots were subsequently probed with a <sup>32</sup>P-labeled, 800-bp BamHI restriction fragment of rat cyclophilin cDNA (25). The relative amounts of the mRNA transcripts were analyzed by laser densitometry using an Ultrosan XL Enhanced Laser Densitometer and normalized relative to the internal cyclophilin controls.

**Reverse transcriptase-PCR.** 1  $\mu$ g of total RNA extracted as described was used for the reverse transcription reaction. Subsequent amplification of the resultant first strand cDNA in a GeneAmp PCR System 9600 (Perkin-Elmer Cetus Instruments, Emeryville, CA) thermal cycler was performed using established procedures (26). 30 cycles of amplification were performed each consisting of denaturation at 94°C for 2 min, reannealing at 55°C for 1 min, and extension at 72°C for 1 min. The PCR product was analyzed on a 1% Tris-borate/EDTA agarose gel. Based on the known number of basepairs between the specific sense and antisense primers used for the amplification, the size of the human uPA DNA product was predicted to be 750 bp. For adequate detection of uPA DNA, a second amplification of 5  $\mu$ l of the first reaction product was necessary.

**Indirect immunocytochemistry.** Tumor cells were cultured overnight at 37°C on eight-chamber slides (Lab-Tek; Nunc, Inc., Naperville, IL). The cells were fixed with 2% formaldehyde/PBS at room temperature for 30 min and permeabilized with 100% acetone for 2 min at –20°C. After rehydration with PBS, rabbit anti-human uPA (anti-GFD) antibody was added at a dilution of 1:250 for 45 min at room temperature followed by a peroxidase-conjugated goat anti-rabbit IgG diluted 1:100. The slides were developed with 100  $\mu$ l/chamber of a 0.1-M Tris-HCl (pH 7.6) solution containing 400  $\mu$ g/ml 4-chloro-1-naphthol (Bio-Rad Laboratories, Richmond, CA) and 0.03% H<sub>2</sub>O<sub>2</sub> (Fisher Scientific Co., Montreal, Canada). The chamber slides were dried, mounted with Immuno-Fluore (ICN Biomedicals Inc., Costa Mesa, CA), and visualized by light microscopy.



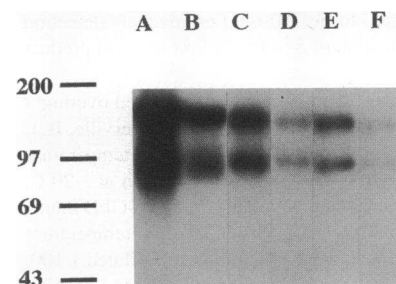
**Figure 1.** Northern blot analysis of  $\alpha_v$  mRNA transcripts in melanoma sublines. Poly (A)<sup>+</sup> RNA derived from MeWo, 6I, MIM/8, and MIM/8 LNI cells (inset, lanes A, B, C, and D, respectively) were size-fractionated on 1.1% formaldehyde-agarose gels after loading of 5  $\mu$ g of mRNA onto each lane. The blots were probed sequentially with <sup>32</sup>P-labeled  $\alpha_v$  and uPA cDNA. Laser densitometry was used to quantitate the levels of  $\alpha_v$  mRNA, relative to control cyclophilin mRNA. Results of this analysis are shown in the bar graph and are expressed as the ratios of  $\alpha_v$  to cyclophilin mRNA in the metastatic cells relative to the parental cells, which were assigned an arbitrary value of 1.0.

**Cell treatment with oligonucleotides.** Melanoma cells were plated in 6- or 96-well plates (Falcon Labware; Fisher Scientific Co.). To each well 100 (96 wells) or 700 (6 wells)  $\mu$ l of the indicated concentrations of oligonucleotides diluted in RPMI-1640-FCS medium was added every 24 h for a period of 3 d. Cells were analyzed on day 4.

**Detection of cell surface  $\alpha_v$  by ELISA.** Oligonucleotide-treated cells plated in the 96-well plates were analyzed by ELISA (16). After removal of the oligonucleotide-containing medium, the cells were washed and incubated with PBS containing 1% BSA for 30 min. Anti- $\alpha_v$  mAb LM142 was used at a dilution of 1:500 followed by an alkaline phosphatase-conjugated rabbit anti-mouse IgG diluted 1:1,000. ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)) was used as a substrate and the colorimetric reaction measured with a microplate reader (3550; Bio-Rad Laboratories) at a wavelength of 415 nm.

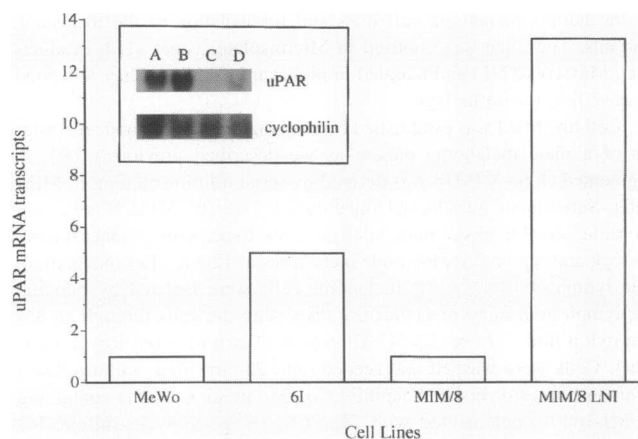
**Cell adhesion assays.** Microtiter plates were coated with 5  $\mu$ g/ml of human vitronectin (Gibco Laboratories), human fibronectin, or mouse laminin, (both from Collaborative Research Inc., Bedford, MA). Cell adhesion was measured as we previously described using  $4 \times 10^4$ , <sup>51</sup>Cr-labeled tumor cells/well and a 30 min incubation at 37°C (9).

**mAb-mediated ligation of  $\alpha_v$  complexes.** One million MeWo LNI 6I



**Figure 2.** Analysis of cell surface  $\alpha_v\beta_3$  by immunoprecipitation. <sup>125</sup>I-radio-labeled proteins were precipitated from NP-40 extracts of 6I (lane A), MeWo (lane B), MIM/8 LNI (lanes C and E), and MIM/8 (lanes D and F) cells with mAbs LM142 (lanes A-D), or AP-3 (lanes E and F).

The immunoprecipitated proteins were separated by electrophoresis on 7.5% polyacrylamide gels under nonreducing conditions and the protein bands visualized by autoradiography of the dried gels. The positions of prestained molecular weight standards are indicated by the bars.



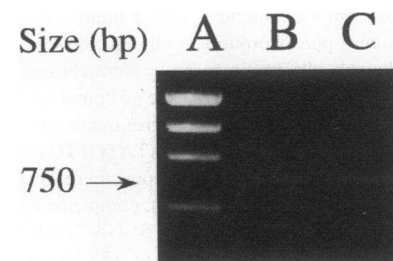
**Figure 3.** Northern blot analysis of uPAR mRNA transcripts. Total RNA derived from MeWo (A), 6I (B), MIM/8 (C), and MIM/8 LNI (D) cells (inset) was size-fractionated by electrophoresis on 1.1% formaldehyde-agarose gels. 30  $\mu$ g of RNA were loaded in each lane. The blots were probed with a <sup>32</sup>P-labeled uPAR antisense oligomer. Laser densitometry was used to quantitate the intensity of the bands relative to control bands of cyclophilin mRNA. Results of this analysis are shown in the bar graph and are expressed as the ratios of uPAR to cyclophilin mRNA in the metastatic cells relative to the parental cells, for which the uPAR:cyclophilin ratio was assigned a value of 1.0.

cells were seeded in 6-well culture plates precoated with 10  $\mu$ g/ml of mAb P3G8 for a 24-h incubation. RNA was extracted and uPAR mRNA analyzed as described above. As controls, wells precoated with mAb A3-IIF5 to human  $\alpha_3$  or mAb W6/32 to MHC class I antigens were used.

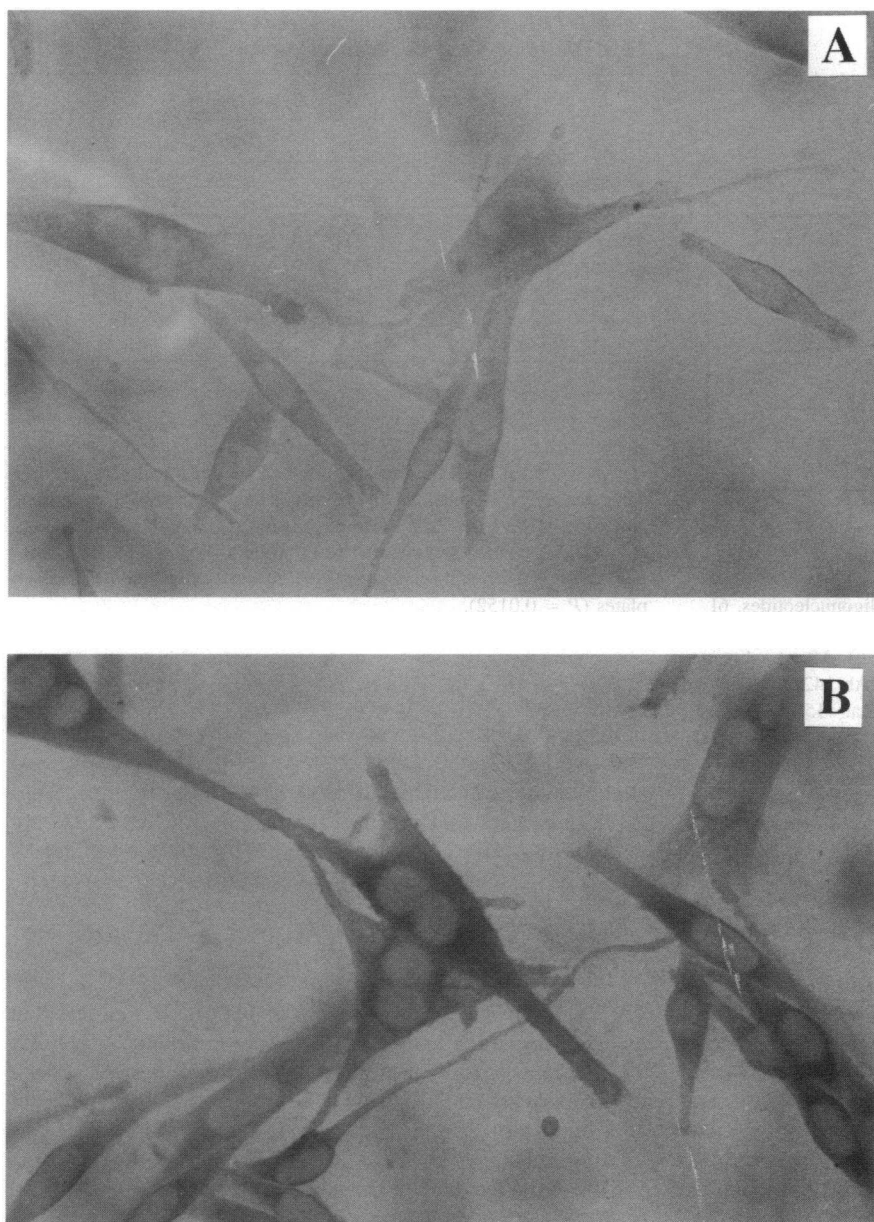
**Statistics.** The Student's *t* test was used to analyze differences in cell adhesion to ECM proteins.

## Results

To measure  $\alpha_v\beta_3$  expression in the parental and lymph node metastases-derived cells, Northern blot analysis and immunoprecipitation with monoclonal antibodies to  $\alpha_v$  (mAb LM142) and  $\beta_3$  (mAb AP-3) were used. The results were in accord with our previous observations based on flow cytometry (9). They show that the expression of  $\alpha_v$  mRNA in the metastatic cells was significantly increased as compared to the parent lines. When normalized against control cyclophilin mRNA levels and analyzed by densitometry, MIM/8 LNI and 6I cells were found to express 10- and 55-fold more  $\alpha_v$  mRNA than MIM/8 and MeWo cells, respectively, (Fig. 1). This was reflected in in-



**Figure 4.** Detection of uPA message by RT-PCR. 1  $\mu$ g of total cellular RNA derived from MeWo (B) and 6I (C) cells was reverse transcribed and the subsequent cDNA was amplified using specific uPA primers. The cDNA product was electrophoresed on a 1% TBE-agarose gel. The expected size of the PCR product was 745 bp. Size standards are shown in lane A.



**Figure 5.** Immunocytochemical analysis of cell-surface-associated uPA. Melanoma cells were cultured on 8-well chamber slides and incubated first with a polyclonal antiserum to uPA (anti-GFD) and then with a peroxidase-conjugated goat antiserum to rabbit IgG. Photomicrographs of representative areas from MIM/8 (A) and MIM/8 LNI (B) cultures are shown.  $\times 400$ .

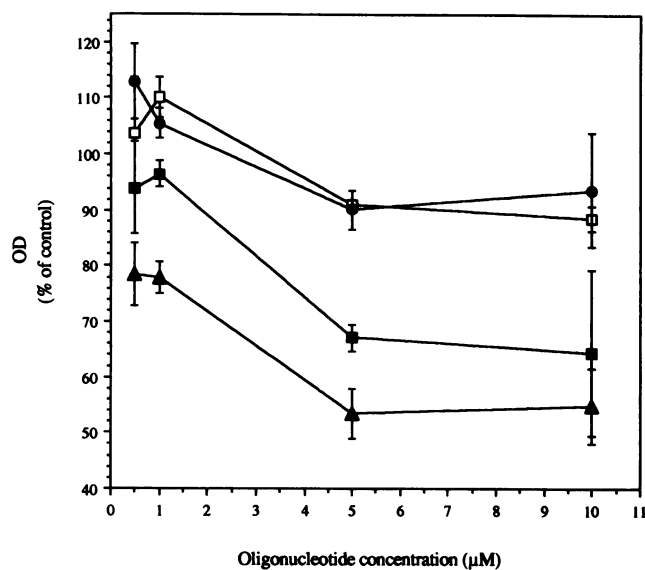
creased cell surface expression of both  $\alpha_v$  and  $\beta_3$  as shown in Fig. 2. mAb LM142 and mAb AP-3 each immunoprecipitated two bands with molecular weights corresponding to  $\alpha_v$  and  $\beta_3$ . Densitometric analysis revealed increases of 5- and 90-fold, respectively, in immunoprecipitable  $\alpha_v$  on 6I and MIM/8 LNI cells and a 47-fold increase in immunoprecipitable  $\beta_3$  on MIM/8 LNI cells, as compared to the respective parent lines.

Urokinase has been strongly implicated in melanoma metastasis (11, 12, 27) and ECM vitronectin in its regulation (13). It was therefore of interest to determine whether expression of urokinase and its receptor were also modulated in the metastatic melanoma cells. When mRNA transcripts for uPAR and uPA were measured, we found that the patterns of uPAR expression on the metastatic sublines paralleled those seen for  $\alpha_v$  mRNA, namely the expression was significantly increased for 6I (X5) and MIM/8 LNI (X13) cells as compared to the respective parent lines (Fig. 3). No uPA mRNA could be detected by the Northern blot assay when as much as 5  $\mu$ g poly(A)<sup>+</sup> RNA of

each cell type were analyzed (Fig. 1), although under similar conditions, uPA transcripts were detectable in RNA extracted from a uPA-rich human prostate carcinoma cell line, PC-3 (data not shown). When analyzed by the reverse transcriptase-PCR method, urokinase cDNA was detectable in all melanoma cell lines suggesting that low levels of the enzyme were expressed (Fig. 4).

When a rabbit anti-uPA antibody (anti-GFD) was subsequently used to immunolabel the cells, we found that significantly higher levels of uPA could be detected on the metastatic cells than on the parental cells (Fig. 5), suggesting that the former could more efficiently bind the enzyme on the cell surface.

To address the question of a possible coordination at the transcriptional level in expression of the vitronectin and urokinase receptors, two approaches were used. First, expression of  $\alpha_v$  in 6I cells was suppressed by treatment with  $\alpha_v$  antisense oligonucleotides. This treatment had no apparent effect on cell



**Figure 6.** Inhibition of  $\alpha_v$  synthesis by antisense oligonucleotides. 6I cells were treated with antisense oligonucleotides, AS2 (●), AS3 (■), and AS4 (▲); or with the sense oligonucleotide, S1 (□). After treatment, cell surface expression of  $\alpha_v$  was measured with mAb 142 using an ELISA. Results shown are expressed as absorbance at 415 nm relative to control untreated 6I cells and represent means  $\pm$ SD of triplicate wells.

morphology or the integrity of the monolayer as confirmed by phase contrast microscopy and did not reduce cell viability as assessed by trypan blue exclusion dye. A reduction in cell surface  $\alpha_v\beta_3$  expression was confirmed by ELISA (Fig. 6) and was reflected in a marked and substratum-specific decrease in cell adhesion to vitronectin (up to 70% inhibition;  $P = 0.009$ ) in the antisense-treated cells (Table I). When  $\alpha_v$  expression was analyzed, a nine-fold decrease in  $\alpha_v$  mRNA expression was seen as assessed by Northern blot analysis (Fig. 7 A). In these cells, a significant decrease was also seen in uPAR mRNA. In a total of three experiments performed, uPAR mRNA transcripts were decreased 1.78–2.5 (mean 2.2)-fold relative to control cells. A representative experiment is shown in Fig. 7 B. In all the experiments, treatment with 10–20  $\mu$ M sense oligonucleotides failed to reduce uPAR mRNA and in fact caused a slight increase (up to 21%) in some of the experiments. The reduction in uPAR mRNA was specific, as no decrease was seen in the mRNA levels of another related proteinase expressed by these melanoma cells, namely, MMP-2 (Fig. 7 C).

As a second approach and to determine whether uPAR expression was linked to vitronectin receptor function, we investigated the effect on uPAR mRNA expression of antibody-mediated ligation of the vitronectin receptor. Melanoma 6I cells were plated on culture dishes precoated with mAb P3G8 and their RNA extracted and analyzed. mAb W6/32 directed to an MHC determinant and mAb A3-IIF5 directed to integrin subunit  $\alpha_3$ , which is also expressed by 6I cells (unpublished data), were used as controls. Results of a representative experiment shown in Fig. 8, demonstrate an antibody-specific 2.4-fold increase in uPAR message in cells attached to P3G8-coated plates (Fig. 8, lane C). In three experiments performed, the increases observed in uPAR mRNA ranged from 1.54- to 2.48-fold in cells plated on P3G8-coated dishes (mean stimulation was  $1.97 \pm 0.47$ ). On

**Table I.** Reduced Adhesion to Vitronectin in Melanoma Cells Treated with  $\alpha_v$  Antisense Oligonucleotides

ECM protein	Adhesion (percentage of control)	
	Oligonucleotide	
	AS3	S1
Vitronectin	42.7 $\pm$ 9.4	118.8 $\pm$ 14.1
Laminin	145.6 $\pm$ 11.1	
Fibronectin	139.5 $\pm$ 5	
None	189 $\pm$ 20	

6I cells were treated with 25  $\mu$ M of the oligonucleotides for 3 d and then dispersed and used to measure adhesion to microtiter plates precoated with 5  $\mu$ g/ml of the ECM proteins. Adhesion of untreated cells was measured at the same time and used as control to calculate relative adhesion levels. Results shown are means $\pm$ SD of triplicate samples. Adhesion of AS3-treated cells to vitronectin was significantly reduced ( $P = 0.023$ ) relative to untreated cells, whereas it was unchanged for laminin ( $P = 0.187$ ), fibronectin ( $P = 0.124$ ), and increased for uncoated plates ( $P = 0.0152$ ).

the other hand, cells plated on mAb A3-IIF5 showed no increase in uPAR mRNA with levels ranging from 0.76 to 1.06 of controls while in cells plated on mAb W6/32 the levels were slightly reduced and ranged from 0.79 to 0.94 of controls. Similar findings (2.2-fold specific stimulation of uPAR) were obtained with MeWo cells in which the  $\alpha_v\beta_3$  receptor was ligated with mAb LM609 under the same conditions (not shown).

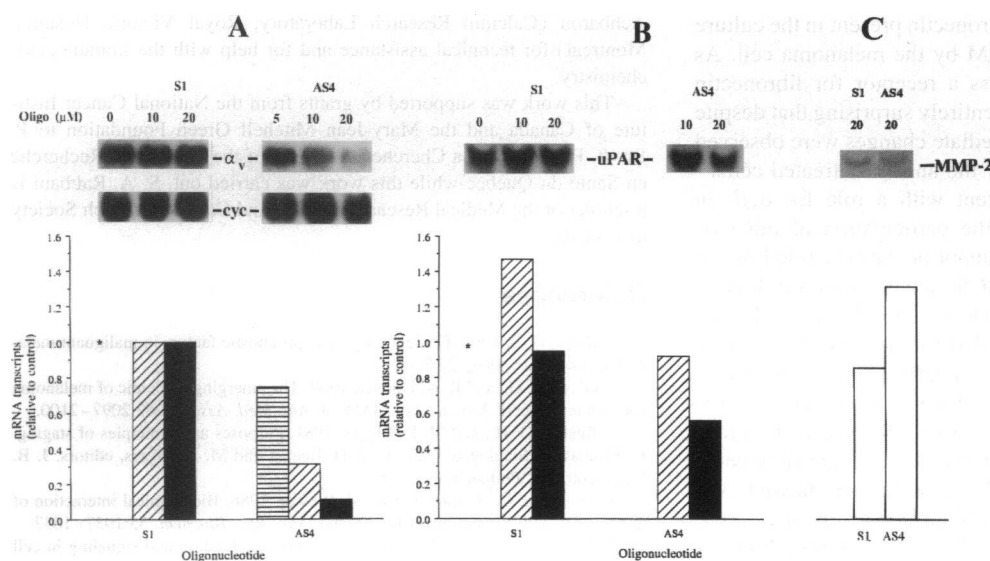
## Discussion

The evidence for the involvement of both  $\alpha_v\beta_3$  and uPAR in melanoma invasion and metastasis is compelling (6, 7, 11, 12, 27). Recent findings also suggest that the two receptors are functionally linked (13, 28). In the present study, the possibility of a regulatory link in the expression of these receptors was investigated.

We found that human melanoma cells selected for increased metastasis to regional lymph nodes expressed higher levels of  $\alpha_v$  mRNA transcripts and increased levels of cell surface  $\alpha_v$  and  $\beta_3$  as compared to the parent cell lines. This confirms and extends our previous finding that metastatic melanoma cells have an increased  $\alpha_v\beta_3$ -mediated adhesion to lymph node vitronectin (9). In addition, our results show that the highly metastatic cells expressed significantly more uPAR mRNA transcripts than the parental cells. These cells provide therefore a unique experimental model for analysis of the functional relevance of  $\alpha_v\beta_3$  to melanoma metastasis on one hand and the link between expression and synthesis of these two receptors on the other. In this context, it should be noted that the increases observed in expression of the  $\alpha_v\beta_3$  complex in the two melanoma models were not proportional to the increases in  $\alpha_v$  mRNA transcripts (Fig. 1). This may be due to differences either in the levels of the  $\beta_3$  subunit available for complex formation or in posttranscriptional processing of the  $\alpha_v$  and  $\beta_3$  subunits in these cells.

While the expression of uPAR transcripts was increased in the metastatic cells, expression of uPA mRNA was relatively low. However, higher levels of cell surface uPA could be de-





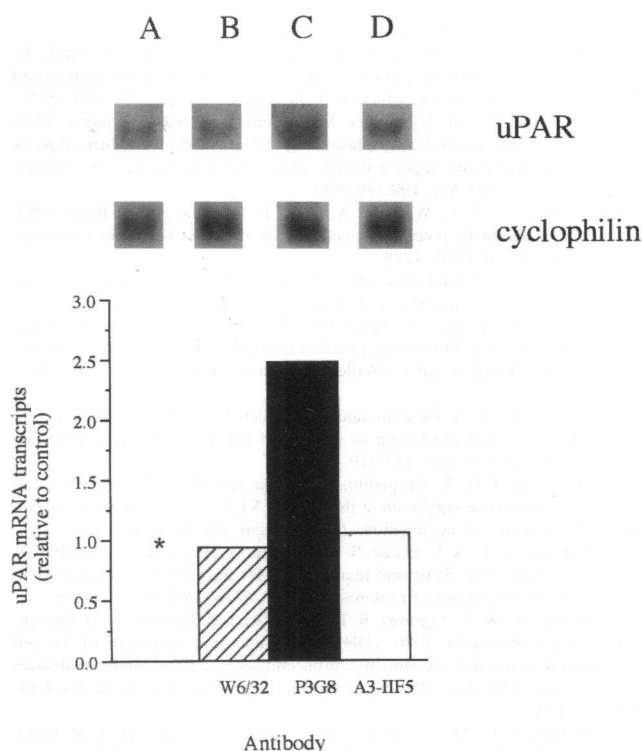
**Figure 7.** Northern blot analysis of  $\alpha_v$  and uPAR mRNA transcripts in cells treated with antisense oligonucleotides. 15  $\mu$ g of total RNA extracted from cells treated with oligonucleotides AS4 or S1, were electrophoresed on a 1.1% agarose gel, blotted to nylon membranes, and probed as described in Methods (top, panels A, B, and C). Results of the densitometric analysis are shown (bottom, panels A, B, and C). All values have been normalized relative to the respective untreated cells which were assigned a value of 1.0 (indicated by \*).

tected on these cells by immunoperoxidase labeling suggesting that they could more efficiently immobilize uPA from the extracellular environment (13). In a recent study of colon adenocarcinoma tissue, high levels of uPAR were observed in association

with the malignant cells while uPA was detected only on the adjacent stromal cells (29, 30). Other findings also suggest that the uPA/uPAR system can function either in an autocrine fashion or it may involve the cooperation of several different cell types, each contributing a different component of the system (13, 29, 30). It is conceivable that in the present model, the invasive and metastatic melanoma cells could utilize either endogenous and/or exogenous uPA produced and secreted into the microenvironment of the lymph node by stromal or monocytic cells (31).

In an attempt to determine whether the increased expression of uPAR in the metastatic cells was linked to the elevated expression of  $\alpha_v\beta_3$ , two approaches were used. First, antisense phosphorothioate oligonucleotides corresponding to unique sequences in the  $\alpha_v$  subunit were used to suppress synthesis of the vitronectin receptor. Our results show that treatment with antisense oligonucleotides significantly reduced cell surface expression of the  $\alpha_v$  integrin complex and cell adhesion to vitronectin. It also resulted in a concomitant specific reduction in uPAR message. This suggests that uPAR synthesis in these melanoma cells is regulated by  $\alpha_v$  levels.

Several mechanisms may be invoked in interpreting these results. It is conceivable that uPAR synthesis is transcriptionally regulated through a feedback mechanism involving cellular  $\alpha_v$  or the  $\alpha_v$ -containing integrin complexes. This could provide a mechanism for coordinating cell adhesion, matrix degradation, and motility since  $\alpha_v\beta_3$  mediates adhesion to matrix vitronectin, and the uPAR/uPA complex is involved in localized matrix degradation at the cell-substratum interphase during cell migration. Alternatively, ligand binding by  $\alpha_v\beta_3$  may trigger a signaling cascade leading to upregulation of uPAR synthesis. This may be mediated through upregulation of autocrine growth factors which are produced by melanoma cells and can modulate uPA and uPAR expression (32, 33) such as basic fibroblast growth factor (34) and TGF- $\beta$  (35). The latter mechanism is supported by our finding that mAb-mediated ligation of the vitronectin receptor caused a specific increase in cellular levels of uPAR mRNA. In cells whose  $\alpha_v$  production has been suppressed by treatment with antisense  $\alpha_v$  oligonucleotides, the decline in uPAR expression may be a consequence of the re-



**Figure 8.** Increased uPAR mRNA expression in response to ligation with an mAb to  $\alpha_v$ . RNA was extracted from 6I melanoma cells grown overnight on uncoated (inset, lane A), mAb W6/32- (B), mAb P3G8- (C), or mAb A3-IIF5- (D), coated wells. Northern blot analysis and laser densitometry were performed as described in the legend to Fig. 3. The results of the densitometry are shown in the bar graph. The values have been normalized relative to the respective untreated cells which were assigned a value of 1.0 (indicated by \*). Results shown in the inset are from a representative experiment of three performed.

duced adhesion of these cells to vitronectin present in the culture medium or deposited into the ECM by the melanoma cell. As these melanoma cells also express a receptor for fibronectin ( $\alpha_5\beta_1$ , results not shown) it is not entirely surprising that despite the reduction in  $\alpha_v$  levels, no immediate changes were observed in the adhesiveness or viability of the antisense-treated cells.

While our results are consistent with a role for  $\alpha_v\beta_3$  in regulation of uPAR expression, the participation of other  $\alpha_v$  integrins such as  $\alpha_v\beta_5$  and  $\alpha_v\beta_1$  cannot be entirely ruled out. It should be noted however, that FACS® analyses with mAb IA9 to subunit  $\beta_5$  (36), (a gift from Dr. M. Hemler, Dana-Farber Cancer Institute, Boston, MA) failed to show increases in  $\beta_5$  expression on the metastatic cells relative to parental lines. MIM/8 LNI cells showed in fact a slight reduction (63% positive cells; mean intensity of fluorescence 588) in cell-surface  $\beta_5$  as compared to the parent MIM/8 cells (76% positive cells, mean intensity of fluorescence 620) (results not shown). We have also previously shown that cell surface levels of  $\beta_1$  were not altered in the metastatic cells (9). These findings suggest that the other  $\alpha_v$  complexes if involved, may play more minor roles.

Antibody- or ligand-mediated cross-linking of integrins has been shown to trigger signal transduction mechanisms leading to protein tyrosine phosphorylation (37), changes in calcium influx (38), gene transcription (39), and altered cell growth. Several lines of evidence suggest that  $\alpha_v\beta_3$  is also involved in signal transduction mechanisms. Thus,  $\alpha_v\beta_3$  was implicated in the regulation of processes such as cell migration (40), differentiation, angiogenesis (41), and tumorigenicity (8). Cross-linking of  $\alpha_v\beta_3$  was shown to increase melanoma cell invasiveness (7) and several  $\alpha_v\beta_3$ -associated proteins have been described, at least one of which, a 190-kD protein, is phosphorylated in the presence of PDGF (42). As several of these processes such as cell migration and invasion also involve uPA-mediated proteolysis (43), it is conceivable that some of the effects attributed to  $\alpha_v\beta_3$  were actually mediated through upregulation and cell-surface localization of the uPAR/uPA complex. In a recent series of reports (32, 44, 45) it has been shown that uPA and uPAR expression in cultured endothelial cells is regulated by basic fibroblast growth factor and that uPA in turn can catalyze the proteolytic conversion of the precursor of TGF $\beta$  into its biologically active form. As TGF $\beta$  may enhance melanoma cell proliferation (46), the induction of uPAR synthesis in response to  $\alpha_v\beta_3$  ligand binding may also provide a mechanism for control of melanoma cell growth. In the regional lymph node, stromal cell-derived or autocrine basic fibroblast growth factor (47) may initiate this cascade when tumor cells adhere to stromal vitronectin (9) resulting in increased melanoma cell invasion and migration.

The transcriptional regulation of uPAR synthesis is poorly understood (13). Yet the evidence is strong that this receptor is of major importance to the invasion and metastasis of several malignancies, including melanoma (11, 12, 27). A better understanding of the regulatory role that integrin  $\alpha_v\beta_3$ -mediated adhesion plays in uPAR synthesis and function will shed light on the mechanisms regulating this receptor and can potentially provide a basis for the development of specific biological reagents designed to inhibit the aggressive behavior of malignant melanoma.

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