A comparison of ocular melanocyte and uveal melanoma cell invasion and the implication of α1β1, α4β1 and α6β1 integrins

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Abstract

***Background/aims*—Posterior uveal melanoma is the most common intraocular tumour in adults, responsible for the death of approximately 35% of patients. Hepatic metastases are most frequent, and once diagnosed survival is usually less than 1 year. The β1 family of integrins, αvβ3 and MMP-2 and MMP-9 have been implicated in the metastasis of several types of tumour. To study their involvement in uveal melanoma we analysed the expression of the β1 integrins, αvβ3, MMP-2, and MMP-9 in 10 primary posterior uveal melanomas, and correlated expression with invasive potential in vitro. Comparable studies were undertaken on cultures of melanocytes.**

***Methods*—Expression of integrins was studied by immunohistochemistry, secretion of MMP-2 and MMP-9 by zymography, and the invasive potential was assessed using a transwell model.**

***Results*—MMP-2 was secreted by all uveal melanomas and seven of 10 secreted MMP-9. Among uveal melanoma, invasion levels of 4–25% were observed and the major integrins expressed were α1β1, α2β1, α3β1, α5β1, and avβ3. Melanocytes did not express α1β1, α4β1, and α6β1.**

***Conclusion*—The laminin binding α6β1 integrin was not expressed by either melanocytes or tumours with spindle morphology, which are considered to have a better prognosis. It is possible that expression of the α6β1 integrin may prove useful as a prognostic indicator.**

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Posterior uveal melanoma is the most common primary intraocular tumour in adults.[1] Despite new methods of treating the primary melanoma, the survival rate has remained unchanged because of the problem of detection and treatment of metastases.[2] Unlike cutaneous melanoma, which metastasises via lymphatic or haematogenous routes to various sites, uveal melanoma mainly disseminates haematogenously and preferentially establishes secondary disease in the liver.[3] At least 30% of patients will develop metastases within 10 years of successful treatment of the primary tumour,[4] but at initial presentation, only 1–2% of patients show evidence of hepatic involvement.[5] Detection of liver micrometastases is difficult with currently available clinical tests and it is thought that many patients have subclinical metastases at diagnosis.[5] Once metastatic disease has been detected, survival is usually less than 1 year.[2 6] Traditional prognostic indicators are based on assessment of the diameter and histological appearance of the tumour[7 8] but it remains difficult to determine which patients at initial presentation are at high risk of developing metastases.

Metastasis is a "multistep" process and tumour cells are required to invade through extracellular matrices (ECMs) at various times during the process. It is proposed that a number of cells detach from the primary tumour and attach via adhesion molecules to components of the ECM or vascular endothelium to be invaded.[9] ECM proteins are then degraded by proteolytic enzymes released from the primary tumour or the surrounding stroma.[9]

The process of cellular detachment and reattachment is facilitated by certain adhesion molecules such as cadherins, immunoglobulin-like adhesion molecules, selectins, and integrins.[10] Integrins are heterodimic glycoproteins consisting of α and β subunits and are the main transmembrane linkers between the cytoskeleton and ECM proteins and also act as cell-cell adhesion molecules.[11] Changes in integrin expression have been observed during the malignant progression of many tumours.[12] In cutaneous melanoma, the integrins α5β1 and αvβ3 have been strongly implicated in malignant progression.[13–15] However, it has recently been proposed that the acquisition of αvβ3 may not be required for the formation of metastases in uveal melanoma.[16] In general, integrin expression has been found to be inconsistent in uveal melanoma, and no correlation with cell type or aggressive behaviour has been made.[16–18]

Proteinases are also involved in invasion and metastasis, and among these, matrix metalloproteinases (MMPs) are often particularly implicated. MMPs degrade most ECM proteins and positive correlations between tumour malignancy and metalloproteinase secretion have been reported.[19 20] In particular, MMP-2 and MMP-9, which degrade the type IV collagen found in basement membranes, have been associated with malignant progression in a variety of human tumours.[21–24] Very little is known about the MMP profile of uveal melanoma; however, we have previously found that all uveal melanoma cell lines tested secreted MMP-2, and nine out of 15 secreted

MMP-9. There appeared to be a link between MMP-9 secretion and the presence of extrascleral spread, which is associated with poor prognosis, and it is possible that secretion of MMP-9 aids the dissemination of the tumour by speeding up the invasion process.[25]

Although integrins and metalloproteinases play an important part in the metastatic process, few studies have investigated their relation to each other. In human osteogenic sarcoma cell lines, the number of α2 integrins found on the tumour cell surface dictated the level of MMP-1 gene expression.[26] In addition, comparison between integrin expression and MMP-2 and MMP-9 secretion has been undertaken in cutaneous melanoma, with elevated levels of αvβ3 correlating with increased in vitro invasiveness and secretion of MMP-2 and MMP-9.[27] No study has explored the relation between invasion, MMP secretion, and integrin expression in uveal melanoma. In this study we present the results of combined analysis of in vitro invasion, MMP secretion, and integrin expression in a series of 10 uveal melanomas.

Materials and methods

TISSUE CULTURE

Fresh samples were obtained from 10 primary posterior uveal melanomas at enucleation and were immediately transferred to tissue culture. Each sample was chopped finely, washed in PBS, spun down at 1000 rpm for 10 minutes, and resuspended in RPMI-1640 (Gibco/BRL (Paisley, UK). The cells were grown in monolayer cultures and were maintained by serial passages in RPMI-1640 supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), fungizone (5 μg/ml) (Gibco/BRL, Paisley, UK), epidermal growth factor (0.2 μg/ml), fetal calf serum (20%), and glucose (0.2%) (Sigma, Poole, Dorset, UK) at 37°C in a 5% carbon dioxide/95% air atmosphere. Sufficient numbers of cells were obtained from all 10 tumours to perform all aspects of the study. All cells were used within five passages of the primary cultures, to provide the best approximation of the in vivo situation. The clinicopathological details of the patients are presented in Table 1. Choroidal melanocytes were cultured from eyes obtained through the Bristol Eye Bank, from normal donors. Short term cultures were obtained as described previously.[28]

INVASION ASSAY

Uveal melanoma invasion was determined in vitro, using a method previously described by Dewhurst *et al.*[28] Transwell inserts with polycarbonate filters were obtained from Costar UK (High Wycombe, Bucks). For each short term culture to be tested, three filters with 8 μm pores were coated with 50 μl of human fibronectin solution (Sigma, Poole, Dorset) at 10 μg/ml. The filters were incubated for 1 hour to allow the fibronectin to adhere and the inserts were placed into a 24 well plate containing 600 μl of serum-free RPMI medium. Uveal melanoma cells were removed from tissue culture flasks using 0.5% trypsin solution (Sigma, Poole, Dorset) and centrifuged at 250 *g* for 5 minutes, after which they were resuspended in serum-free medium supplemented with 0.1% bovine serum albumin (Sigma, Poole, Dorset).

Cell suspensions (100 μl), with 1.2×10^5 cells per ml, were added to each transwell insert and incubated for 20 hours at 37°C in a 5% carbon dioxide/95% air atmosphere. A positive control melanoma cell line was included in each assay and maintained the same invasive level throughout. The assay was repeated three times for each culture. After incubation, the medium/cells solution was collected from the upper and lower chambers and replaced by an equal volume of cell dissociation solution (Sigma, Poole, Dorset) to remove any remaining cells. The number of cells per ml in the upper and lower chambers was determined using a haemocytometer. Invaded cells were those that had been removed from the underside of the filter, floating in the media of the 24 well plate or attached to the bottom of the 24 well plate. Non-invaded cells were any remaining in the upper chamber or attached to the upper surface of the polycarbonate filter. The percentage of cells that had invaded through the fibronectin over 20 hours was calculated using the formula:

(Number of invasive cells (lower chamber)/ Total number of cells) × 100

ZYMOGRAPHY

The identification of proteolytic enzymes expressed was performed by electrophoresis of serum-free conditioned medium taken from confluent uveal melanoma cells growing in tissue culture, based on the method of Laemmli.[29] A gelatin/SDS polyacrylamide gel was used to detect the presence of the gelatinolytic 72 kD metalloproteinase (MMP-2) and the 92 kD metalloproteinase (MMP-9). Electrophoresis was carried out at room temperature at a constant voltage of 100 V.

Table 1 Clinicopathological details of the 10 patients with primary posterior uveal melanoma

Tumour number	*Age*	*Sex*	*Location*	*Diameter (mm)*	*Cell type*	*Metastatic disease*	*Status**
Mel 204	65	F	choroid	12.65	mixed	liver	alive
Mel 205	74	M	ciliary body	13.25	mixed	no	dead, unrelated cause
Mel 206	86	F	ciliary body	9.01	spindle B	no	alive
Mel 209	65	F	ciliary body and choroid	14.65	mixed	liver	dead
Mel 211	71	M	choroid	15.20	spindle A	no	alive
Mel 212	45	M	ciliary body and choroid	15.55	mixed	liver	dead
Mel 213	72	F	ciliary body and choroid	16.05	mixed	no	alive
Mel 214	72	M	ciliary body	8.36	mixed	no	alive
Mel 218	43	M	choroid	12.35	mixed	no	alive
Mel 222	36	F	choroid	12.85	spindle B	no	alive

*Patient status at 1.5 years post-enucleation.

Table 2 Monoclonal antibodies used to study integrin expression in a series of 10 primary posterior uveal melanomas

Antigen	*Antibody*	*Purchased from*
α1β1	MCA1133	Serotec, Oxford, UK
α2β1	NCL-CD49b	Novocastra, Newcastle upon Tyne, UK
α3β1	NCL-CD49c	Novocastra, Newcastle upon Tyne, UK
α4β1	P4G9	Dako Corporation, CA, USA
α5β1	P1D6	Dako Corporation, CA, USA
α6β1	NCL-CD49f	Novocastra, Newcastle upon Tyne, UK
αvβ3	MCA757G	Serotec, Oxford, UK
β1	MCA532	Serotec, Oxford, UK
MMP-2	42-5D11	Chemicon International Inc, USA
MMP-9	56-2A4	Chemicon International Inc, USA

When the tracking dye reached the bottom of the gel, it was washed in a 2% solution of Triton-X-100 (Sigma, Poole, Dorset) and incubated overnight in a buffer appropriate for enzyme activity. The gels were subsequently stained with Coomassie brilliant blue R250[30] (Sigma, Poole, Dorset). Areas of gelatinolytic degradation appeared as transparent bands on the blue stained background of the gel. A wide range molecular weight marker (M4038, Sigma, Poole, Dorset) was used to estimate the apparent molecular weights for bands of substrate degradation and a negative sample of serum-free media was run in one lane.

IMMUNOHISTOCHEMISTRY

Short term cultures of 10 posterior uveal melanoma cells and six melanocytes, cultured from normal donors, were analysed by immunohistochemistry for expression of integrins using a panel of monoclonal antibodies directed against specific integrin heterodimers (Table 2). Uveal melanoma cells were grown on sterilised glass slides for subsequent immunostaining and washed with PBS, air dried, and fixed with acetone for 10 minutes, then stored at −20°C until used. Six melanocyte cultures from normal donors were prepared using the same method. Slides were placed on staining racks in a humidified chamber and divided into separate areas using a Dako pen (Dako Corporation, CA, USA). The slides were incubated with the primary antibody diluted in TRIS buffered saline (TBS), for 60 minutes at room temperature. After rinsing with TBS, a three stage ABC immunoperoxidase technique was used.[31] Bound antibody was detected using an AEC substrate kit (3-amino-9-ethyl carbazole) (Vector Laboratories Ltd, Peterborough) which produced a red chromagen. After rinsing, the slides were mounted with Dako Faramount aqueous mounting medium (Dako Corporation, CA, USA). Negative controls were processed by the same method, replacing the primary antibody with TBS. The intensity of positive staining of the melanoma cells was scored semiquantitatively by two independent observers.

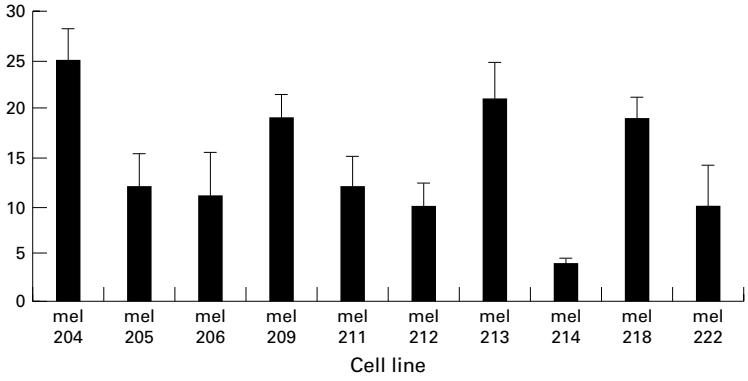

Figure 1 Percentage invasion of 10 cultures of primary posterior uveal melanoma as determined by invasion assay. All cultures were tested within the first five passages. Values shown are means (SEM) for three combined assays.

Results

INVASION OF POSTERIOR UVEAL MELANOMA CELLS

The results of melanoma invasion assays are summarised in Figure 1. All cultures of posterior uveal melanoma contained cells that were able to invade through the fibronectin layer. Uveal melanoma cells were found to have a mean invasion of 14.5% (SD 2.1%) (n = 10) and intertumour variation in invasion levels was observed, ranging from 4%–25%. Previously recorded data using the same methods, by Dewhurst *et al*[28] had found a mean invasion of 5.1% (1.1%) (n = 11) for melanocytes, which was significantly lower than those reported for either cutaneous of ocular melanomas.

ZYMOGRAPHIC ANALYSIS OF MMP-2 AND MMP-9

The qualitative results of zymography are summarised in Table 3. All tumour cultures (10/10) secreted the 72 kD MMP-2 enzyme and seven of 10 expressed the 92 kD MMP-9 enzyme. Of the three cultures that expressed MMP-2 alone, two thirds were of higher than average invasion. Secretion of MMP-2 and MMP-9 was compared with invasiveness but no association was found. The highest invader did not secrete MMP-9 and the lowest invader secreted both enzymes. There was no increase in invasion seen in the small number of tumours that secreted both MMP-2 and MMP-9. Only one tumour (Mel 218) secreted the active forms of MMP-2 (62 kD) and MMP-9 (82 kD) and was of higher than average invasion (Fig 2). Zymographic analysis of two additional cultures of melanocytes showed that neither secreted MMP-2 or MMP-9, while immunohistochemistry performed on the six melanocyte cultures assessed for expression of integrins, using antibodies for MMP-2 and MMP-9, confirmed that neither was expressed.

Table 3 Zymographical analysis of MMP-2 and MMP-9 secretion in a series of 10 primary posterior uveal melanomas

Tumour number	*Passage number*	*pro-MMP-2 (72 kD)*	*pro-MMP-9 (92 kD)*
Mel 204	3	+ve	−ve
Mel 205	2	+ve	+ve
Mel 206	2	+ve	+ve
Mel 209	3	+ve	+ve
Mel 211	3	+ve	−ve
Mel 212	1	+ve	+ve
Mel 213	2	+ve	−ve
Mel 214	2	+ve	+ve
Mel 218*	2	+ve	+ve
Mel 222	2	+ve	+ve

*Mel 218 also secreted the active forms of both MMP-2 (62 kD) and MMP-9 (84 kD).

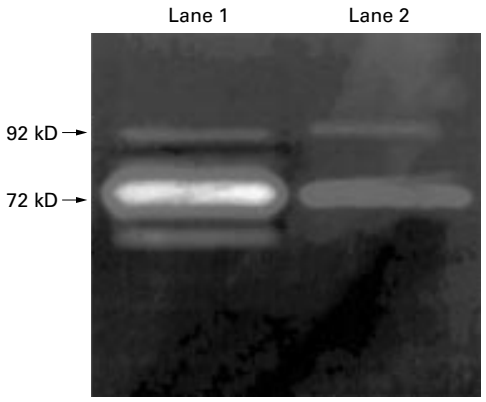

Figure 2 Gelatin zymogram showing examples of MMP-2 (72 kD) and MMP-9 (92 kD) secretion by cultured posterior uveal melanoma cells. A molecular weight marker was run on each gel (not shown). Mel 218 (lane 1) also secreted the active forms of both enzymes. Mel 222 (lane 2).

IMMUNOHISTOCHEMICAL ANALYSIS OF INTEGRIN EXPRESSION IN POSTERIOR UVEAL MELANOMA AND MELANOCYTES

The results of integrin expression in the 10 uveal melanomas are summarised in Table 4. The integrins α1β1, α2β1, α3β1, α5β1, and αvβ3 were expressed by all 10 tumours, seven of 10 expressed α4β1, and six of 10 expressed α6β1. Two of the four tumours with spindle or mixed morphology did not express α4β1 integrin and four of four did not express α6β1 integrin. The intensity of the immunoreaction and percentage of cells positive for integrin expression varied from tumour to tumour (Fig 3). A series of short term cultures of melanocytes was also analysed for integrin expression. Melanocyte staining was homogeneous and expression of α2β1, α3β1, α5β1, and αvβ3 integrins was observed in all melanocytes. None of the melanocytes expressed α1β1, α4β1, or α6β1 integrins (data not shown). Loss of staining for α4β1 or α6β1 in the tumours tested showed no association with invasive ability or expression of MMPs.

With the exception of the association between integrin expression and cell type, there was no relation between expression of integrins, invasive ability, and secretion of MMPs and other clinical parameters

Discussion

Invasion through extracellular matrices and subsequent metastasis are dependent on cooperation between adhesive and proteolytic systems. Integrins and metalloproteinases are important mediators of the metastatic process and have been implicated in the metastasis of many malignant tumours.[12 19] Limited investigation has been carried out in cutaneous melanoma, correlating levels of αvβ3 with increased invasion and secretion of MMP-2[27] but no such study has been undertaken in uveal melanoma.

Relative overexpression of MMP-2 and MMP-9 has been detected in a variety of human tumours.[21 22] Further evidence that MMPs play a part in invasion is observed in experiments in which MMP inhibitors block tumour cell invasion of the ECM.[32] In the present study, we compared secretion of MMP-2 and MMP-9 with invasiveness and other prognostic indicators. No correlation was found between secretion of either MMP and invasiveness, although recent evidence has suggested that MMP-2 is correlated with indicators of poor prognosis in uveal melanoma.[33] One explanation for the lack of correlation is that MMP-2 and MMP-9 degrade mainly type IV collagenase and gelatin, and fibronectin was used as a barrier to invasion in this study, as previous investigations had established fibronectin as the preferred substrate for uveal melanoma cell attachment.[34] Furthermore, other proteolytic systems, such as plasminogen activators, work synergistically with metalloproteinases to degrade matrix proteins.[19]

Heterogeneity in integrin expression has been found in both cutaneous and uveal melanoma cell lines[14 16] and it has been observed that integrin expression cannot be correlated with cell type or invasiveness in uveal melanoma.[17] All uveal melanoma cells in this study were positive for α1β1, but all ocular melanocytes were negative, and previous evidence also indicates that cutaneous melanocytes do not express α1.[35] This may suggest that this integrin is involved in the progression of uveal melanoma cells to a more invasive phenotype. There is little information available on the expression of the α1β1 integrin in tumours, although one study observed that progression from dysplastic naevi to thick melanoma was accompanied by a sevenfold

Table 4 Expression of integrins as detected by immunohistochemistry in a series of 10 cultured primary posterior uveal melanomas, and relation to in vitro cell morphology

Tumour	*Passage*	*α1β1*	*α2β1*	*α3β1*	*α4β1*	*α5β1*	*α6β1*	*αVβ3*	*β1*	*In vitro cell type*
Mel 204	5	h-50/++	+++	+/++	+/++	+++	+/++	+	+	epithelioid
Mel 205	3	h-50/++	h-60/+++		h-70/+	++/+++	+/++	++/+++	+	epithelioid
Mel 206	4	++/+++	+	++	h-30/+	+++	++	++/+++	+	epithelioid
Mel 209	4	h-65/++	+/++	++	h-50/+	+	++	+/++	+	epithelioid
Mel 211	3	+	++/+++	++/+++	++	+++	−ve	+/++	+/++	spindle
Mel 212	3	h-20/+	++	++	h-20/+	h-60/+/++	−ve	++	+++	mixed
Mel 213	3	+++	++/+++	++/+++	+	++	++	+/++	+	epithelioid
Mel 214	3	+++	+	++/+++	h-60/+	+++	++	++	++	epithelioid
Mel 218	3	+	+	++/+++	−ve	++	−ve	++	+	mixed
Mel 222	4	h-70/+	+/++	+++	−ve	+	−ve	h-70/+	++	spindle

The percentage of cells staining was considered homogeneous if 75% of cells were stained. The expression was considered to be heterogeneous if the percentage of cells staining was between 5% and 75%, and the relevant percentage is indicated (h5–h75). Less than 5% of cells stained was considered negative. Staining intensity was scored as + = weak, ++ = medium, or +++ = strong.

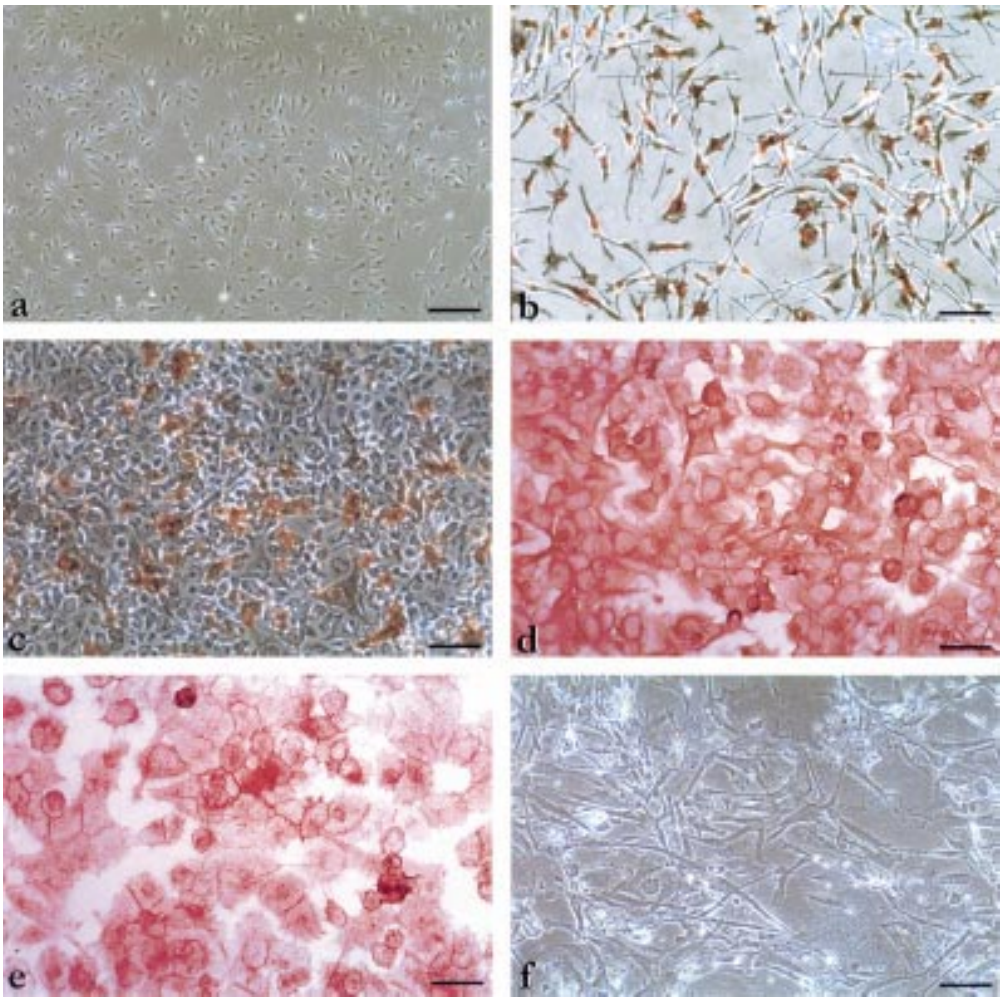

Figure 3 Photomicrographs showing examples of immunohistochemical staining for integrin expression in cultures of posterior uveal melanoma and melanocytes. (a) Negative staining for α1β1 in melanocytes (×10) (scale bar 100 μm). (b) Homogeneous staining for α3β1 in melanocytes (×40) (scale bar 400 μm). (c) Positive heterogeneous staining of x% of cells for α1β1 in melanoma cells with epithelioid morphology (×20) (scale bar 200 μm). (d) Homogeneous staining for αvβ3 in melanoma cells with epithelioid morphology (×40) (scale bar 400 μm). (e) Homogeneous staining for α6β1 in melanoma cells with epithelioid morphology (×40) (scale bar 400 μm). (f) Negative staining for α6β1 in melanoma cells with spindle-like morphology (×40) (scale bar 400 μm).

increase in α1 expression.[13] More recently, a 50% increase in expression of α1β1 was observed in a highly metastatic pancreatic carcinoma cell line when compared with a non-metastatic line.[36] The α1 and α6 integrins have in common the fact that they bind laminin. Invasion of tumour cells through basement membranes is a critical step in metastasis and laminin is the most abundant constituent of basement membranes but is sparse in other matrices.[10 37] Therefore, cells in contact with basement membranes, such as epithelial cells are more likely to express laminin receptors.[38] The laminin receptor, α6β1 was not expressed by spindle and mixed morphology tumours in vitro, and all melanocytes were negative for α6β1. Pure spindle cell tumours have a better prognosis, with a 5 year survival rate of 80% compared with only 40% in tumours which contain a large number of epithelioid type cells.[39] It is possible that the more metastatic epithelioid cells require the α6β1 receptor to attach to subendothelial basement membranes—for example, in choroidal capillary endothelium. In a previous study, premetastatic uveal melanoma cells with mostly spindle morphology have also been shown to lack the α6β1 integrin,[38] and evidence suggests that laminin receptor expression is associated with invasive potential.[35 37]

In cutaneous melanoma, α6β1 integrin expression was increased in the highly metastatic B16-F10 subline relative to the less metastatic B16-F1 subline[40] and blocking of laminin receptors with a synthetic laminin peptide was found to inhibit experimental metastasis formation.[37] Recently, α6β1 was found to be overexpressed in a highly metastatic pancreatic carcinoma cell line compared to a non-metastatic line.[36] Uveal melanoma frequently metastasises to the liver and it is of interest that large amounts of α6β1 have been detected on colon carcinoma cells and have been implicated in promoting hepatic metastases in vivo.[41] In this study, expression of the laminin receptor did not correlate with invasiveness, though this is not surprising as fibronectin was used as a barrier to invasion and is found in ECMs and not in basement membranes.

The α4β1 integrin was expressed by most tumours; however, two of four of the spindle/mixed morphology tumours did not express α4 and none of the melanocytes expressed α4. Under normal conditions the α4β1 integrin is expressed on the surface of B and T lymphocytes, monocytes and eosinophils. During the inflammatory process, immune cells expressing the α4β1 integrin are able to attach to blood vessels via the VCAM-1 adhesion molecule which is expressed on the surface of activated endothelial cells.[42] It has been suggested that tumour cells use the same mechanism to intravasate into blood vessels. Expression of α4β1 has been observed on many cell lines derived from malignant tumours. For example, in cutaneous melanoma, increased expression of α4β1 correlates with the more advanced stages of tumour development and the increased presence of metastases.[43 44] Other integrins implicated in melanoma progression include α5β1 and αvβ3 and increased expression of these integrins is strongly associated with tumour progression to a more malignant phenotype.[13 43] In this study, expression of α5β1 and αvβ3 did not correlate with invasiveness and melanoma cells and melanocytes expressed both integrins, implying, as previously suggested, that they are of less importance in uveal melanoma.[16 17]

Metastatic cells often express a wider range of adhesion molecules than non-transformed cells[45 46] and this was also observed in the present study, with uveal melanoma cells expressing more integrins than melanocytes. We also found that expression of some integrins in tissue culture differed from the in situ expression found in previous studies,[16 17] and it is possible that such variations may result from regulation by the natural environment, with the constraints placed upon tumour cells absent in tissue culture.[47]

Integrins and metalloproteinases are now thought to work synergistically in the invasion process. Signal transduction by integrins can lead to MMP secretion in melanoma and other tumour types.[48] For example, the αvβ3 integrin has been found to induce MMP-2 expression by cutaneous melanoma cells.[48] Integrins may also be involved in the invasive process by localising MMPs at the tumour cell surface. Expression of αvβ3 on cultured melanoma cells enabled them to bind active MMP-2[49] and, recently, the αvβ6 integrin was found to induce MMP-9 secretion by colon carcinoma cells.[50] Although integrins are involved in the attachment to ECM step of the invasive process and possibly the localisation of MMPs at the tumour cell surface, other proteolytic enzymes such as plasminogen activators are thought to be required for invasion.

In uveal melanoma, tumour size remains the single most important indicator of prognosis, with tumours exceeding 10 mm having a poorer prognosis.[7] All tumours in this study were from enucleation patients and were therefore relatively large, and no link between tumour size and invasiveness was found. However, a link between integrin expression and cell type was found. Tumours with a spindle morphology (in vitro), which is associated with better prognosis,[39] appear to lack the α6β1 integrin. Expression of the α6β1 integrin may be useful as a marker for invasive potential in posterior uveal melanoma but a larger study of the importance of this integrin is required, examining its expression in vivo and in secondary lesions.

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