LABORATORY STUDY

Invasive and noninvasive uveal melanomas have different adhesive properties

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Abstract

Aims To establish if invasive and noninvasive uveal melanomas have differences in expression of adhesion molecules, and whether their adhesive interactions with the extracellular matrix (ECM) and endothelium vary.

Methods Cells from an invasive and noninvasive uveal melanoma cell line and hepatic and dermal microvascular endothelial cells were assessed by flow cytometry for adhesion molecule expression. Tumour cell adhesion to ECM substrates (collagens I and IV, fibronectin, laminin, and vitronectin) and endothelial cells was also investigated using a commercially available assay or a fluorescence-based *in vitro* assay, respectively. The significance of results comparing cell lines was determined using a Student's *t*-test, whereby *P*-values of less than 0.05 were taken as significant.

Results α1- and α4-integrins were not expressed by noninvasive cells, but were detected on invasive cells. The invasive cell line also expressed higher levels of other integrins than the noninvasive line. Correspondingly, invasive cells adhered in higher numbers to ECM substrates and endothelial cells, and for the latter, the difference was highly significant ($P < 0.001$). No preference in adhesion of invasive cells for the hepatic endothelium was observed.

Conclusions Successful attachment to and migration through the ECM, basement membrane, and endothelium are vital processes involved in malignant progression. Differential expression of α1- and α4-integrins by invasive and noninvasive cells infers a role for these receptors in invasion, while the ability of invasive cells to adhere more efficiently to the endothelium suggests that this is a critical factor in uveal melanoma invasion.

Introduction

During metastasis, tumour cells disperse via the vasculature and the lymphatic systems, undergoing intravasation and extravasation before finally invading and growing at secondary sites. For uveal melanoma however, dissemination via the vasculature is of primary importance as lymphatic vessels are absent from the eye. In addition, networks of extracellular matrix (ECM) channels have been identified in aggressive uveal melanomas, which could also assist in the dispersal of this malignancy.[1] To undertake successfully the stages required for dissemination, tumour cells must be able to adhere to, and subsequently detach from components of the ECM and basement membrane (BM) such as collagens, fibronectin, and laminin, and ultimately the vascular endothelium. Adhesion molecules, expressed by both the tumour and endothelium, mediate these adhesive interactions, and more specifically integrins are thought to be particularly important in cancer metastasis, forming a major group of adhesion molecules coordinating ECM and cell–cell interactions.[2]

Integrins are a family of heterodimeric glycoproteins consisting of α and β subunits, linking the cytoskeleton with the ECM or adjacent cells. Changes in integrin expression have been reported during the malignant progression of many tumours[3] and much evidence exists implicating their involvement in

cutaneous melanoma metastasis.[4] For example, altered $\alpha v\beta$ and $\alpha5\beta1$ expression has been described during the progression of metastatic melanoma cells,[5,6] while the presence of $\alpha4\beta1$ receptors, involved in attachment to the endothelium, promotes the accumulation of disseminated cells in distant organs.[7,8] Highly metastatic melanoma cells also adhere well to the ECM proteins type IV collagen and laminin, and blocking $\alpha3\beta1$ integrins prevents in vitro invasion through artificial BMs.[9–11] Together, this would suggest that the ability of melanoma cells to metastasise is reflected in their capacity to adhere to the ECM and endothelium.

Although both cutaneous and uveal melanomas are similarly derived from neuroectodermal tissue, their metastatic behaviour in the body differs greatly. In particular, while cutaneous melanomas metastasise widely, uveal melanomas commonly spread to the liver and other sites are less frequently affected. Uveal melanomas are reported to express a range of adhesion molecules, including integrins, and members of the immunoglobulin family members,[6,12–15] but only the expression of intercellular cellular molecule-1 (ICAM-1) has so far been shown to bear any relationship to clinical outcome.[15] Little is, however, known about how adhesion molecule expression in uveal melanomas may influence the invasion process, and it is unclear whether the same mechanisms important in cutaneous melanoma are equally relevant, or if different interactions are required. In this investigation, we therefore undertook to study the differences in the adhesive interactions between invasive and noninvasive uveal melanoma with ECM proteins and cells of the vasculature.

Materials and methods

Cell culture

The invasive (SOM 196B) and noninvasive (SOM 157d) uveal melanoma cell lines were derived from samples obtained from primary posterior uveal melanomas upon enucleation, and were confirmed for melanoma status and maintained as previously described.[16,17] Levels of invasion had been previously quantified using an in vitro Matrigel invasion assay.[17] Ethical approval was obtained prior to study, and protocols adhered to the principles of the Declaration of Helsinki. Human dermal microvascular endothelial cells (adult) (HDMECAs) were obtained commercially (TCS Cellworks Ltd, UK), while human liver sinusoidal endothelial cells (HuLiSECs) were freshly extracted from liver resections and maintained as detailed before.[17,18] The invasive cutaneous melanoma cell line A375[19] was maintained in Dulbecco's minimal medium (Invitrogen, UK) containing 10% foetal calf serum.

ECM adhesion assays

Adhesion of uveal melanoma cells to ECM proteins (collagen type I, collagen type IV, fibronectin, laminin, and vitronectin) was assessed using a CytoMatrix™ screening kit (Chemicon International, CA, USA). Briefly, wells coated with human ECM protein were rehydrated with phosphate-buffered saline (PBS) (Oxoid Ltd, Hampshire, UK), and nonspecific binding was blocked with 1% bovine serum albumin (BSA). Cells were nonenzymatically disaggregated from culture flasks using cell dissociation solution (Sigma-Aldrich, Dorset, UK), and resuspended at a concentration of 5×10^5 cells/ml in RPMI-1640 with 0.1% BSA. Cell suspension (100 μl) was added to each well, and the plate was incubated at 37°C for 1 h. Plates were carefully washed by hand three times with PBS, and stained with 0.2% crystal violet in 10% ethanol for 5 min. Wells were manually washed a further three times before adding a solubilisation buffer of equal volumes of 0.1 M NaH_2PO (pH 4.5) and 50% ethanol. Levels of adhesion were determined by assessing the absorbance at 540 nm on a microplate reader (Dynex Technologies Inc., VA, USA). The data were collected and analysed using Revelation™ software (Dynex Technologies Inc., VA, USA). Triplicate wells were assessed for each treatment; experiments were repeated three times and the mean value was calculated. In all cases, adhesion to wells coated with BSA acted as negative controls, and levels of adhesion to ECM substrates were assessed relative to the controls.

Inhibition of adhesive interactions with the ECM using integrin-blocking antibodies

To block integrin-mediated adhesion to the ECM, uveal melanoma cells were preincubated with the relevant anti-integrin blocking mouse monoclonal antibodies (mAb) (Table 1), diluted in RPMI-1640 supplemented with 0.1%. BSA at a range of concentrations, for 30 min at room temperature prior to the ECM adhesion assay (as described above). With the exception of the negative control antibody, all mAbs were purchased from Chemicon International Inc. (Harrow, UK). A negative IgG$_1$ isotype control antibody (DakoCytomation, Ely, UK) was included in each assay, and results were expressed as a percentage reduction in adhesion when compared with negative controls. As ICAM-1 and $\alpha4$-integrins are reported to be primarily involved in tumour–tumour and tumour–endothelial attachment, respectively, the effect of blocking these adhesion molecules on ECM binding was not assessed. Inhibition experiments were also only carried out for integrins for which levels of expression were deemed sufficiently high to enable successful antibody blocking.

Table 1 Source and reference list for mAbs used in adhesion and flow cytometry experiments.

Antigen	Antibody	Clone
$\alpha 1$	MAB1973	FB12
$\alpha 2$	MAB1998	BHA2.1
$\alpha 3$	MAB2056	ASC-1
$\alpha 4$	MAB16983	P1H4
$\alpha 5$	MAB1986	NKI-SAM-1
$\alpha 6$	MAB1378	NKI-GoH3
$\alpha v \beta 3$	MAB1976	LM609
ICAM-1	MAB2147	P2A4
E-selectin	MAB2150	P2H3
VCAM-1	MAB2144	P3C4
FITC-labelled goat anti-mouse IgG	AP124F	—
FITC-labelled goat anti-rat IgG	AP136F	—
IgG$_1$ isotype control	X-0931	—

All mAbs were purchased from Chemicon International Inc. (Harrow, UK), with the exception of the IgG$_1$ isotype control (DakoCytomation, Ely, UK). When used for blocking, cells were preincubated with anti-integrin mAbs prior to the assay, inhibiting their function in ECM adhesion.

Flow cytometry

Levels of integrin and ICAM-1 expression by uveal melanoma cells and VCAM-1 and E-selectin by endothelial cells were assessed using flow cytometry (FACSort Vantage) (Beckton Dickinson, NJ, USA) (Table 1). Expression was detected by the use of appropriate FITC-labelled secondary antibodies (Table 1) with a band pass filter (BP530/30). The data were collected and analysed using Cell Quest™ software (Beckon Dickinson, NJ, USA), assessing the median values of fluorescence. Test samples were run against a control sample, labelling cells with a negative IgG$_1$ isotype control antibody. Results were expressed as the relative median fluorescent intensity (MFI), comparing test with control samples. Relative MFI values of greater than 2 were taken as positive, as expression levels were considered to have doubled. Experiments with SOM 157d and SOM 196B were repeated three times and a mean calculated.

Endothelial adhesion assays

To study the adhesive interactions between endothelial and SOM 157d and SOM 196B, HDMECAs or HuLiSECs $(2 \times 10^4/\text{well})$ were grown to confluence in a 96-well plate, precoated with gelatin, for 24 h. Prior to setting up the adhesion assay, the growth medium was removed from the wells and the cells were washed twice with PBS. Adhesion of A375 cells was used as a comparison with a cutaneous melanoma cell line.

Tumour cells were labelled with carboxy-fluorescein diacetate, succinimidyl ester (CFDA-SE) (5 μM) (Molecular Probes Inc., OR, USA) (fluorescing at 492–

517 nm)[17] following the manufacturer's instructions. Cells were then resuspended at a concentration of $5 \times 10^5/\text{ml}$ in RPMI-1640 with 0.1% BSA, and 50 μl of the cell suspension $(25 \times 10^3/\text{well})$ was pipetted into each well containing the endothelial cell layer. Plates were incubated at 37°C for 4 h. At 5 min prior to the end of the incubation period, endothelial cells were counterstained with Hoescht (33342) (10 μM) (Sigma-Aldrich, Dorset, UK) and replaced in the incubator. Any nonadherent cells were carefully removed through gentle pipetting and plates were washed three times manually with PBS. Cells were fixed with 4% formaldehyde/PBS for 15 min, before rinsing in distilled water and air-drying overnight in a dark, dry atmosphere. Levels of adhesion were determined by assessing the absorbance at 510 nm on a microplate reader (Denley, Stevenage, UK). The data were collected and analysed using Wellfluor Data Capture™ software (Denley, Stevenage, UK). Six wells were analysed for each condition and experiments were repeated three times and a mean calculated. In all cases, adhesion to wells coated with fibronectin (15 μg/ml) acted as positive controls, while for negative controls no tumour cells were added to endothelial cell monolayers. Levels of adhesion to endothelial cells were assessed relative to the controls.

Statistical analysis

A Student's t-test was used to compare population means of adhesion assay data, analysing treatment values against an appropriate control. Due to the presence of low absorbance units when assessing the adhesion to ECM proteins, the variance in the data obtained was found to be heterogeneous, and consequently a square root transformation was applied $(\sqrt{(x+0.5)})$. Comparisons between results with SOM 196B and SOM 157d and between results with HDMECAs and HuLiSECs were similarly statistically assessed using the Student's t-test. In all cases, P-values of less than 0.05 were taken as significant and were used to establish the fact that cellular adhesion was significantly increased, or decreased, compared to the respective control level. As experiments were repeated three times assessing triplicate values, there is only a small possibility that the significant differences observed would have occurred by chance.

Results

Adhesion molecule expression

Basal levels of expression of adhesion molecules by SOM 196B and SOM 157d assessed by flow cytometry are shown in Table 2. In summary, both uveal melanoma cell

Table 2 Levels of expression of adhesion molecules by SOM 196B and SOM 157d cells and the effect of blocking antibodies on adhesion to ECM.

SOM	Adhesion molecule	Level of expression (MFI±SEM)	% reduction in adhesion to ECM substrates using blocking antibodies				
			Collagen type I	Collagen type IV	Fibronectin	Laminin	Vitronectin
196B	α1	9.98±0.787	5.2	26.3	—	10.9	—
	α2	29.9±2.68	51.5	53.3	—	2.8	—
	α3	170±4.82	14.5	22.5	38.7	14.2	—
	α4	2.48±0.328	—	—	—	—	—
	α5	53.4±4.00	—	—	13.3	—	—
	α6	13.8±0.784	—	—	—	33.6	—
	αvβ3	2.60±0.0713	3.8	—	2.2	—	13.4
	ICAM-1	1.90±0.248	—	—	—	—	—
157d	α1	1.67±0.0353	—	—	—	—	—
	α2	5.27±0.0845	60.9	70.0	—	0	—
	α3	18.7±1.79	4.9	20.5	—	16.6	—
	α4	1.24±0.0258	—	—	—	—	—
	α5	5.05±0.142	—	—	—	—	—
	α6	2.99±0.191	—	—	—	24.7	—
	αvβ3	2.17±0.0522	—	—	—	—	—
	ICAM-1	1.02±0.0397	—	—	—	—	—

Expression levels were assessed by flow cytometry and are presented as the mean relative MFI (±SEM) for three experiments. Test samples were run against a negative IgG$_1$ isotype control and expressed as the relative MFI, comparing test with control samples. Relative MFI values of greater than 2 were taken as positive. Levels of adhesion to ECM substrates were assessed as detailed in Figure 1; the effect on blocking antibodies on ECM substrate adhesion is expressed as a percentage reduction when compared with negative controls and represents the mean of three experiments. A '—' signifies that the experiment was not performed due to an inappropriate substrate or low or absent basal levels of expression.
SOM: Sheffield ocular melanoma.

lines were negative for ICAM-1 expression. Invasive SOM 196B cells expressed high levels of the integrin subunits α1, α2, α3, α5, and α6, while only being weakly positive for α4 and αvβ3. The noninvasive SOM 157d similarly expressed α2, α3, α5, and α6, and low levels of αvβ3, and in contrast to SOM 196B, was negative for both α1 and α4 expression. For those integrins detected, the levels of expression were notably higher for SOM 196B when compared with SOM 157d, apart from αvβ3 that was comparable. Neither E-selectin nor VCAM-1 was detected on dermal or hepatic endothelial cells (data not presented in Table 2).

Adhesion to ECM proteins and inhibition studies

Similar patterns of adhesion to ECM proteins were seen for both cell lines (Figure 1), and with the exception of vitronectin, invasive SOM 196B adhered at higher levels than noninvasive SOM 157d, but levels did not reach significance ($P > 0.05$). When compared with negative controls, both cell types preferentially adhered to fibronectin ($P < 0.01$). Preincubation with anti-integrin-blocking antibodies established that attachment was primarily via α3-integrins (Table 2). Blocking the α5-subunit and the αvβ3 receptor on SOM 196B cells also partially decreased adhesion (Table 2). Both uveal melanoma cell lines also attached in significant numbers to laminin ($P < 0.01$), and vitronectin ($P < 0.05$) via α6- and

αvβ3-integrins, respectively (Table 2), when comparing with control levels to BSA. In addition, SOM 196B but not SOM 157d adhered to both collagens I and IV ($P < 0.01$) and this proved to be principally mediated through α2-subunits, and to a lesser extent by means of the α1- and α3-subunits (Table 2).

Adhesion to endothelial cells

Due to the preferential binding of both SOM 157d and SOM 196B to fibronectin observed in ECM adhesion assays (Figure 1), this substrate was used as a positive control in the endothelial adhesion assays, allowing comparison with attachment to the endothelium. In common with adhesion to ECM substrates (Figure 1), the invasive SOM 196B line was more adherent than the noninvasive SOM 157d line ($P < 0.001$) and the cutaneous melanoma A375 line to both microvascular endothelial cell types (Figure 2). No preference for hepatic endothelial cells (HuLiSECs) was, however, shown by the invasive SOM 196B cells, and cells attached in similar numbers to both hepatic and dermal (HDMECAs) endothelial cells ($P > 0.05$) (Figure 2). All melanoma cells studied nevertheless adhered in higher numbers to the ECM substrate fibronectin than to the endothelium ($P > 0.05$) (Figure 2).
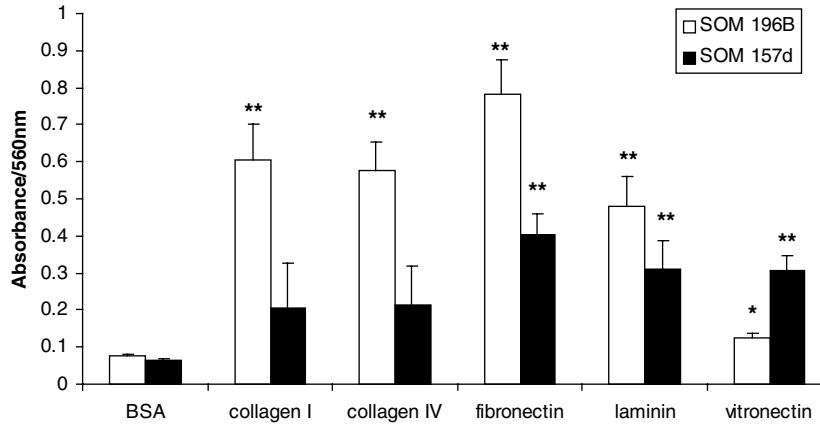
Figure 1 Adhesion of SOM 196B and SOM 157d to ECM proteins. The levels of cell adhesion were measured using 0.2% crystal violet staining. Following staining and washing, the retained dye was solubilised and measured at an absorbance at 560 nm. Wells coated with BSA acted as negative controls. The bars show the mean (±SEM) absorbency at 560 nm for three experiments. The *x*-axis shows the substrate and the *y*-axis shows the absorbance at 560 nm. $^{**}P < 0.01$ and $^{*}P < 0.05$ when comparing adhesion to ECM proteins with negative controls. SOM: Sheffield ocular melanoma; ECM: extracellular matrix.
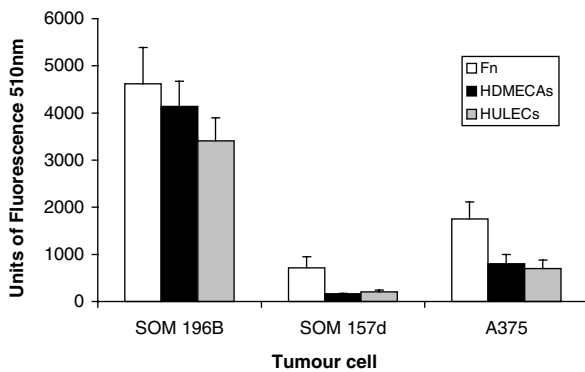
Figure 2 Adhesion of the uveal melanoma cell lines SOM 196B and SOM 157d and the cutaneous melanoma cell line A375 to fibronectin (Fn), HDMECAs, and (HuLiSECs). Mean (± SEM) number of tumour cells adhering to Fn or different endothelial cell types. Results represent the mean of three experiments. Units of fluorescence at 510 nm are measured on the *y*-axis. No significant differences in adhesion of SOM 196B, SOM 157d and A375 cells to fibronectin were observed ($P > 0.05$) when compared with adhesion to either HDMECAs or HuLiSECs. When comparing adhesion to either endothelial cell types between tumour cell types, significantly higher levels of SOM 196B cells adhered to both HDMECAs and HuLiSECs, when compared with SOM 157d ($P < 0.001$).

Discussion

Uveal melanomas are highly aggressive tumours predominantly metastasising to the liver,[20] with the most aggressive tumours possessing the ability to invade through the surrounding matrix, the underlying BM and into the vasculature and establish secondary disease. Similarly *in vitro*, uveal melanoma cells also demonstrate differences in invasive abilities through BM and

endothelial barriers[17] and we have correspondingly shown in this present study that invasive cells express higher integrin levels and are more adherent than their noninvasive counterparts to both the ECM and cells of the vasculature (Figures 1 and 2).

A previous study carried out in this laboratory associated expression of α1-, α4- and α6-integrins by uveal melanomas with the more aggressive phenotype.[13] In this present study, a similar pattern of α1- and α4-expression was observed between invasive and noninvasive cells (Table 2), while lower levels of α6-expression were detected on noninvasive cells when compared with their invasive counterparts (Table 2). Together, these data support an involvement of these receptors with malignant progression and the invasive phenotype previously demonstrated.[6,13,17] In this current study, inclusion of blocking antibodies in ECM adhesion assays identified that α1- and α6-integrins on SOM 196B cells are involved with attachment to collagens I and IV and laminin (Table 2), with these cells correspondingly adhering at higher levels to these substrates than noninvasive SOM 157d (Figure 1). As both laminin and collagen type IV are found in high levels in the BM, and premetastatic uveal melanoma cells, associated with a better prognosis, lack laminin receptors,[21] this would similarly infer an involvement of α1- and α6-integrins with uveal melanoma invasion. Also in view of the phenomenon of networks of nonendothelial channels in uveal melanoma that are rich in laminin,[1] increased expression by aggressive uveal melanomas of laminin-binding integrins may facilitate dispersal by such means.

On migrating through the surrounding ECM and BM, malignant cells further invade through the vessel wall ('transendothelial migration'), ultimately facilitating

dissemination. To establish secondary colonies successfully, this process must be accomplished at both the primary and secondary sites.[22] As described earlier, using an *in vitro* transendothelial invasion assay, we have shown that invasive SOM 196B cells invade through the BM and endothelium in greater numbers than noninvasive SOM 157d.[17] Correspondingly in this present study, SOM 196B was significantly more adherent to both endothelial cells types than SOM 157d ($P < 0.001$) (Figure 2); similar interactions with endothelial cells have also been reported for lymphoma cells *in vitro*.[23]

Both uveal melanoma cell types were, however, less adherent to the endothelium than to the ECM substrate fibronectin. This observation was reflected in the previous invasion study whereby comparison of BM invasion with transendothelial invasion identified a significant reduction in numbers of cells invading by inclusion of the endothelial layer ($P > 0.05$).[17] Together with the current findings of a highly significant difference between the number of invading and noninvading cells adhering to the endothelium ($P < 0.001$), these results would suggest that adhesion to the endothelium is a more specific event, requiring a more malignant phenotype than attachment to the ECM. As a consequence, the ability of invasive uveal melanoma cells to adhere efficiently to the endothelium could be a critical factor in invasion. In support, studies on prostate carcinoma, a tumour frequently metastasising to the bone, have shown that adhesion of tumour cells to bone is regulated by direct attachment to the endothelium in preference to the ECM,[24] similarly implying that adhesion to the vasculature is a crucial stage in prostate carcinoma metastasis.

As prostate carcinoma cells *in vitro* have also been reported to adhere to primary bone marrow endothelium in higher numbers than to other endothelial cell types,[25,26] this present study also investigated the possibility that uveal melanoma cells preferentially attach to hepatic endothelial cells. Despite the selective targeting of uveal melanoma cells to the liver *in vivo*,[19] uveal melanoma cells, however, had no significant preference for hepatic endothelial cells (HuLiSECs) ($P > 0.05$). Instead, the invasive uveal melanoma cell line adhered in greater numbers to HDMECAs ($P > 0.05$) (Figure 2); a direct explanation for this preference remains uncertain, but did indicate differential attachment to the two endothelial cell types. Adhesion molecules involved in tumour–endothelial interactions can, however, vary depending upon static or hydrodynamic conditions *in vivo*[27,28] and the microenvironment[29–31], and as the *in vitro* assay system used in this study was static, this may have affected the patterns of adhesion observed.

With the exception of α4-integrins, ICAM-1, E-selectin, and VCAM-1, all adhesion molecules studied are primarily involved with tumour attachment to the ECM. In contrast, the aforementioned adhesion molecules all mediate either tumour–endothelial or tumour–tumour interactions. Expression of ICAM-1 has been associated with an increased risk of uveal melanoma metastasis[15] and worse prognosis in cutaneous melanoma,[4,32] and similarly invasive cells studied here expressed higher levels than their noninvasive counterparts (Table 2), also implicating an involvement of ICAM-1 in invasion. As described previously, both uveal melanocytes[13] and noninvasive cells do not express α4-integrins, while increased levels were detected on invasive cells (Table 2). Although little is known about α4-integrins and uveal melanoma metastasis, ligation of α4β1 with its endothelial ligand VCAM-1 is involved with cutaneous melanoma metastasis,[7] and the pattern of spread is proposed to relate to organ-specific VCAM-1 expression.[8] Moreover, E-selectin is reported to mediate colorectal tumour cell adhesion to the hepatic endothelium.[29,33] Both endothelial cell types studied here nevertheless lacked expression of E-selectin and VCAM-1, but as inflammatory regulators upregulate their expression,[29,30] their involvement in uveal melanoma attachment to the endothelium cannot be excluded. Further studies are nevertheless required to validate the importance of these adhesion molecules in uveal melanoma metastasis.

In summary, we have demonstrated here that invasive uveal melanoma cells adhere in higher numbers to ECM substrates and cells of the vasculature than noninvasive cells. The highly significant difference ($P < 0.001$) in numbers of invasive and noninvasive cells attaching to the endothelium would suggest that adhesion to the vasculature is vital in determining the invasive phenotype. The absence of α1- and α4-integrins on noninvasive cells and uveal melanocytes,[13] mediating the adhesive interactions studied, would infer a vital role for these receptors in uveal melanoma invasion. Further investigation into the factors regulating these interactions is required to improve an understanding of uveal melanoma metastasis.

Acknowledgements

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