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Cell adhesion receptor expression during melanoma progression and metastasis

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Summary

Many steps in melanoma metastasis involve cell-cell or cell-matrix adhesive interactions. The surface molecules which mediate these processes therefore play an important role in regulating melanoma dissemination and their level of expression may alter during the course of tumor progression. Human melanocyte strains and melanoma cell lines have been characterised with regard to levels of cell surface receptors of the integrin family. Increased amounts of at least two integrins, VLA-4 ($\alpha_4\beta_1$) and VnR ($\alpha_v\beta_3$), appeared to correlate with progression in this tumor, type. A novel VnR composed of an $\alpha_v\beta_1$ association has been observed in one melanoma cell line and there is the possibility that heterogeneity of integrin composition could affect biological behavior of these tumors.

CD44, a cell surface glycoprotein which functions as the major receptor for hyaluronate, is another molecule whose expression increases in transformed cells of the melanocytic lineage. Iterative sorting on the FACS for stable variants, of both human and murine melanomas, expressing low and high levels of CD44 established that lack of expression of this molecule correlated with impaired ability to form pulmonary tumor nodules subsequent to i.v. injection into appropriate recipient mice. These findings illustrate that an understanding of the regulation of melanoma adhesion receptors could provide insights into the process of tumor spread.

Introduction

Curative treatment for cutaneous malignant melanoma is surgical excision; this approach is effective for thin (< 0.8 mm) but not thick (> 1.7 mm) lesions because of the association of deeper tumors with the occurrence of tumor dissemination [1]. Accordingly, melanoma is a neoplasm where success or failure of treatment depends almost completely upon the phenomenon of cancer metastasis. It would seem therefore that an understanding of melanoma metastasis might be important for developing new therapeutic modalities and that this tumor type might serve as a paradigm for other tumors with the propensity to metastasize.

Many of the steps in the metastatic sequence involve cell-cell and cell-matrix interactions [2, 3] and thus the cell surface molecules regulating these processes are likely to play a major role in determining the outcome of these interactions and, to some extent, their expression must be essential in order for cancer spread to occur. Because the normal cell from which melanomas arise, the melanocyte, is derived from the neural crest [4] and cells from this structure migrate extensively during development [5], invading extracellular spaces and interacting with various embryonic cell types [6, 7], is seems possible that the disseminative capacity of melanoma cells simply may be a recapitulation of the characteristics evinced by these embryonic pro-

genitors. For example, highly metastatic murine melanoma cells have been shown to exhibit considerably higher levels of cell surface galactosyltransferase activity than is manifested by their non-metastatic counterparts [8]. Interestingly migrating neural crest cells use this enzyme to recognise and bind to terminal N-acetylglucosamine (GlcNAc) residues on glycoconjugates within the extracellular matrix [9]. Thus in this instance the changes observed in more malignant or progressed tumor types may more closely reflect the embryonic situation than the less progressed tumor.

In the exploration of the development of the role of cell adhesion receptors in malignancy the melanoma system offers some very distinctive advantages. It has long been considered that the accumulation of somatic mutations during the course of tumor evolution, coupled with the strong selection pressures exerted by the host, leads to the emergence of more aggressive clonal subpopulations with a propensity to invade and metastasize [10]. Melanoma exemplifies this process of tumor progression [11, 12] and this characteristic, coupled with the accessibility of neoplastic tissue, has allowed the isolation and analysis of material representing different stages across the whole spectrum of malignant evolution. The finding that normal melanocytes can be grown under tissue culture conditions, as long as agents which activate and down-regulate protein kinase C are included in the medium [13–15], has meant that, unlike many tumor types, reference to the normal counterparts of the transformed cell frequently is possible within *in vitro* systems.

The existence of such distinct stages of melanoma progression has led to the discovery of some fascinating differences between the expression of cell surface molecules at the benign and the malignant stage. Johnson and her colleagues [16–19] for example have conducted some elegant studies to show that the levels of expression of certain cell-cell adhesion molecules of the immunoglobulin superfamily are up-regulated in the more advanced stages of the disease [18, 19].

However, while the system offers real and dramatic benefits for investigation it does pose significant difficulties for anyone attempting to summarise the wealth of information that is becoming available regarding the expression of cell adhesion receptors in even this single tumor type. There is a complexity imposed by changes in receptor expression and function as a consequence of tumor evolution. Furthermore there is the diversity of the cell-matrix and cell-cell interactions that are likely to occur during a process as complicated as tumour spread. Notwithstanding the rather all-encompassing title of this review we have not sought to make our brief the entire field of cellular adhesion receptors. Rather we have opted to concentrate on just two areas of particular significance for our own laboratory. This narrow focus by no means implies any view on a likely hierarchy of relevance. To ignore cell surface galactosyltranferases [8] or gangliosides [20, 21] does not indicate a lack of appreciation for their possible involvement in melanoma metastasis but simply reflects the constraints of space and our own particular bias. Likewise in the areas we have chosen to examine we have been less than exhaustive since excellent reviews, impinging on many of the aspects covered here, have been published elsewhere (e.g. [3, 22–24]). Some of these reviews [22–24] have covered the role of integrins in cell-matrix interactions. Obviously the movement of disseminating cells across basement membranes and through organ parenchyma requires the interaction with extracellular matrix components. Many of these interactions are regulated by members of the integrin superfamily.

The integrin family

The cell surface receptor glycoproteins which constitute the members of this family are trans-membrane, non-covalently associated heterodimers composed of a larger α subunit and a smaller β subunit [25]. While integrin molecules mediate interactions with other cells and components of the complement family [26] they mainly serve to interact with the adhesive proteins of the extracellular matrix (ECM). In many instances the binding of the integrins to the ECM-protein ligand is mediated via recognition of the tripeptide Arg-Gly-Asp (RGD) motif [27]. Originally, the integrin super-

family was divided into three sub-families based on the presence of one of three distinct, but highly homologous β chains [25]. These distinct β chains associated with one of eleven α subunits and generally it was assumed that it was these α subunits that conferred ligand specificity on the receptor [25]. Thus β_1 associates with one of at least six α-subunits (α_1–α_6) constituting the so-called very-late activation antigens (VLA) which are distributed ubiquitously [28]; β_2 associates with α_l, α_m or α_x to form the LFA-1, MAC-1 and p 150, 95 integrins respectively whose expression is restricted to leukocytes [25, 26]; the β_3 sub-family is composed of two members; the IIb/IIIa (IIb/β_3) complex expressed on platelets and the vitronectin receptor $\alpha_v\beta_3$ which is found on a wide range of cell types [29]. Since this original classification was delineated [25] a number of additional β subunits have been identified (e.g. [30–35]) while it has been found that some of the α subunits exhibit less fidelity in their association with β chains than was thought previously to be the case [36, 37]. A scheme showing the currently known relationships of the integrin family is presented in Fig. 1.

Ligand specificity of the integrins may be monospecific, (VLA-5 or $\alpha_5\beta_1$ for example appears to only bind fibronectin), or multispecific (VLA-3 or $\alpha_3\beta_1$ binds fibronectin, laminin and collagens type I and IV) for as yet undetermined reasons [25, 38, 39]. The specificity of ligand binding may be affected by cell type [40, 41], possibly as a consequence of local membrane composition [42].

Apart from assumptions based on theoretical considerations that the integrins ought to be of importance in determining melanoma metastasis simply because of their function there are results from experimental approaches which confirm this possibility. Thus Gehlsen *et al.* [43] inhibited the invasive capacity of a human melanoma cell line, as determined by the ability to penetrate the amnion membrane, by including RGD-containing peptides in the assay. In a seminal paper, Humphries and his colleagues [44] reported that the co-injection of RGD peptides with B16 melanoma cells resulted in a marked and significant reduction in the resultant number of pulmonary tumor nodules. These findings have been confirmed and extended by Saiki *et al.* [45] who used synthetic polypeptides of RGD, (RGDRGD), which have extended half lives in the circulation. Cell receptors for laminin include several integrins [40, 46] and one 67 kd laminin binding glycoprotein [47]. This latter receptor appears to function by recognising the Tyr-Ile-Gly-Ser-Arg (YIGSR) sequence on the β_1 chain of laminin [48]. Coinjection of YIGSR peptides or laminin fragments containing the cell-binding region also results in reduction of the number of lung tumor nodules resulting from i.v. injected melanoma cells [49]. There is therefore a body of evidence which suggests that interruption of the function of cell adhesion receptors, particularly those of the integrin family or involved in cell-matrix interactions, has a dramatic effect upon the metastatic dissemination of melanomas.

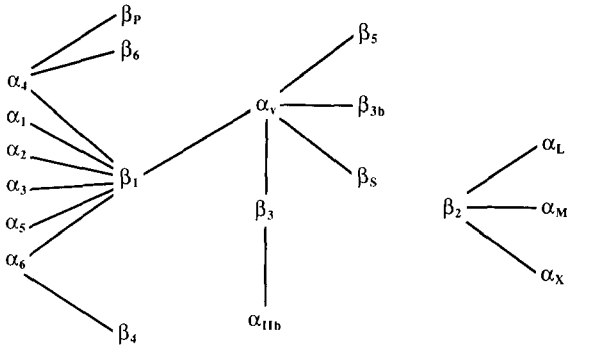

Fig. 1. Subunit associations among the integrins. The α_v subunit has the capacity to associate with multiple β chains.

Integrin function

Cells of melanoma lines have been shown to express a wide range of integrins at their cell surface [50–52] and it would be of considerable interest to determine exactly which of the integrin receptors are the most important in determining transformed and then malignant behavior of this tumor. In other systems for example, it has been found that expression of the VLA-5 ($\alpha_5\beta_1$) integrin has an inverse correlation with tumorigenicity. Thus, when Plantefaber and Hynes [53] transformed rodent fibroblasts with Rous Sarcoma Virus the expression of $\alpha_5\beta_1$ was reduced. Similarly the transfection of Chi-

nese Hamster Ovary cells with cDNA's coding for this fibronectin receptor was associated with suppression of the tumorigenic phenotype [54]. Some clues as to which integrin is most important in cells of the melanocytic lineage have been obtained (see below) but at the present time most effort is still being expended on what might be termed 'taxonomic' classification; simply monitoring whether or not a specific integrin receptor is or is not present in series of unrelated cell lines.

Several reports have indicated that antibodies to the β_1 subunit can inhibit melanoma cell binding to a variety of ECM proteins, including fibronectin [50, 51], laminin [40, 55] collagen type 1 [50] and collagen type IV [50, 51]. However, since the β_1 chain associates with one of at least seven different α subunits [28, 36, 37] the nature of the specific integrin involved in binding to some of these ligands is uncertain.

The β_1 sub-family

VLA-1 and VLA-2

When Kramer and Marks [50] chromatographed detergent extracts of the MeWo human melanoma line over collagen type I- or collagen type IV-Sepharose columns they found that EDTA, but not RGD peptides, eluted two heterodimers. These heterodimers consisted of a β_1 subunit associating with either a 200 KD α1 chain or a 150 KD α_2 subunit [50]. It was found that $\alpha_1\beta_1$ bound best to collagen type IV columns whereas $\alpha_2\beta_1$ (VLA-2) bound most effectively to collagen type I columns. Neither of the two receptors bound to fibronectin- or laminin-sepharose columns [50]. By contrast Elices and Hemler [40] found that antibodies to the α_2 chain inhibited the binding of LOX human melanoma cells to laminin substrate. It is clear that the ligand specificity of a single integrin, in this instance $\alpha_2\beta_1$, can vary between two cell lines, LOX and MeWo, representing a single tumor type.

In passing it should be noted that many different integrins expressed on melanoma cells are capable of binding laminin. Thus $\alpha_1\beta_1$ [52], $\alpha_2\beta_1$ [40], $\alpha_3\beta_1$ [40], $\alpha_6\beta_1$ [55] and a novel α sub-unit, which may be specific for melanoma cells only, termed $\alpha_7\beta_1$ [55] have all been shown to be capable of binding laminin. It seems that some of these different integrins may bind to different sites on the laminin molecule [56]. Since laminin plays such an important role in supporting and guiding neural crest cell migration [57] it may be that the variety of integrins capable of recognising this glycoprotein on the surface of melanoma cells is a reflection of their embryonic origin.

VLA-3

The VLA-3 ($\alpha_3\beta_1$) receptor, which has several ligands including fibronectin, collagens type I and IV and laminin [38], is expressed on cells of melanocytic origin. Indeed we have found it to be the major integrin expressed on cultured melanocytes and a panel of melanoma cells (Marshall et al, submitted for publication). In spite of our findings this $\alpha_3\beta_1$ complex was not purified from MeWo cells when detergent extracts were run over laminin-sepharose columns [35] or collagen type I- and collagen type IV-sepharose columns [50]. It was recovered, albeit at a very low level, from LOX cell extracts run over laminin-sepharose columns [40]. A recent report has suggested that expression of $\alpha_3\beta_1$ may vary according to the stage of melanoma progression that the cells are recovered from for analysis. In an examination of clinical material Albelda *et al.* [58] found that 40% of the cells in nevi were positive for α_3 expression but that this percentage had increased to greater than 70% of cells obtained from radial and vertical growth phase samples. Thus the stage of disease from which the cell lines originally were established may have a profound effect upon the spectrum and level of integrin expression at the cell surface.

VLA-4

$\alpha_4\beta_1$ is found mostly on lymphoid and myeloid cells [59] where it may be involved in cell-cell interactions [59–61]. Generally it is not present on most adherent cells, such as epithelial cells, endothelial

cells or fibroblasts, but it is found on melanomas where it appears to act as the receptor for the IIICS region of fibronectin [62]. Interestingly VLA-4 expression on lymphocytes has been implicated in lymphocyte homing by acting as a Peyer's patch receptor [60]. This normal physiologic process which, as we outline below, appears to have strong similarities to the pathological process of cancer dissemination is regulated by a number of cell surface molecules on both the lymphocytes and the high endothelial venule cells of the lymph nodes [63, 64]. It has been reported that another ligand for VLA-4 is VCAM-1 or INCAM110 which is a member of the immunoglobulin superfamily that can be induced on the surface of endothelial cells by various cytokines [65, 66]. Since it appears that melanoma cells may adhere to endothelial cells via the VCAM-molecule [67–69] and we have found that certain melanoma lines actually release an IL-1α-like molecule which up-regulates VCAM-1 on the endothelial cell surface (Burrows *et al.*, submitted for publication) it is possible that a tumor-driven adhesive interaction may play a role in determining melanoma dissemination in a way which shares many similarities with the phenomenon of lymphocyte trafficking. In this respect it may be of particular interest that there was a difference between the tumorigenic and non-tumorigenic lesions from human patients analysed by Albelda *et al.* [58] in terms of α_4 expression. These authors found that only 2 out of 18 (11%) of the non-tumorigenic lesions expressed α_4, in contrast to the 9 out of 23 samples of tumorigenic origin (39%) which expressed this subunit; a difference which was significant at the $P < 0.005$ level [58]. It certainly will be of interest to determine whether the VLA-4 receptor is the integrin which is responsible for determining extravasation of melanoma cells surviving in the circulation.

VLA-5

Since the only ligand for VLA-5 ($\alpha_5\beta_1$) reported to date is fibronectin [38, 70] this molecule is thought of as being the classical fibronectin receptor. However in some, though not necessarily all, experimental systems it has been found that five other integrins ($\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_v\beta_1$, $\alpha_v\beta_3$ and $\alpha_v\beta_5$) are capable of determining binding to the fibronectin molecule [37, 38, 42, 71]. Antibodies to α_5 inhibited the binding of LOX melanoma cells to fibronectin [40] while VLA-5 was recovered specifically from fibronectin-sepharose columns when detergent extracts of MeWo cells were utilised [55]. Using antiserum raised against a mouse erythroleukemia cell fibronectin receptor Kramer and his co-workers [51] were able to inhibit the binding of the murine B16 melanoma line to fibronectin, laminin, collagen IV and the amnion basement membrane. VLA-5 has, therefore, been found on a variety of melanoma lines. However, as discussed elsewhere in this review, an apparent association has been made between transformation/tumorigenicity and down-regulated expression of $\alpha_5\beta_1$ [53, 54]. At the present time there is little evidence to support the possibility that such a relationship obtains in melanoma. This though probably is just a reflection of the small number of cases or samples that have been examined. Thus Albelda *et al.* [58] found that α_5 was not strongly expressed on a panel of human melanoma lines. However, in what was admittedly a very small sample, they also found that α_5 was more likely to be expressed in the more advanced stages of the disease [58], possibly suggesting that correlations established in tissue culture samples of either human or rodent origin need not be predictive for the *in vivo* clinical situation.

VLA-6

When present on platelets, the VLA-6 ($\alpha_6\beta_1$) integrin serves as a receptor for laminin [72]. Thus Ramos *et al.* [52] were able to purify an integrin, identified as VLA-6, by passing extracts of a B16 melanoma line over a laminin-sepharose column. Nevus cells appear to express α_6 strongly and the sub-unit consistently was found on all malignant melanomas [58].

Lately it has become apparent that the α_6 subunit does not form an heterodimer with β_1 exclusively. In carcinoma cell lines α_6 has been shown to associate with both β_1 and a β_4 chain [73]. The $\alpha_6\beta_4$

integrin appears to be part of the tumor-associated glycoprotein complex TSP-180 [74]. Therefore it may be of some significance that the monoclonal antibody 135-13C, which was raised against the TSP-180 complex initially, but which has been shown to identify α_6 [75], has been found to bind to a greater extent to the more highly metastatic B16-F10 subline of the B16 melanoma than it does to the less metastatic counterpart, B16-F1 [76].

Kramer *et al.* [55] have reported that a novel integrin receptor for laminin exists on MeWo cells. This integrin is composed of a β_1 subunit associated with an α chain of 130 kd molecular weight under non-reducing conditions. Since α_1 is 200 kd and α_2-α_6 are 140–160 kd under similar non-reducing conditions [28] this makes it the smallest α subunit yet reported. These authors found that this novel subunit, tentatively labeled α_7, was present on an additional three human and one murine melanoma lines but not on several other cell types or tissues raising the possibility that this heterodimer is melanoma-specific [55].

The β_3 sub-family

Vitronectin receptor (VnR)

The β_3 sub-family of integrins currently is comprised of the IIb/β_3 (IIb/IIIa) complex expressed exclusively on platelets [29] and the $\alpha_v\beta_3$ vitronectin receptor expressed on a variety of cell types including melanomas [58]. The $\alpha_v\beta_3$ VnR has been isolated from M21 human melanoma cells where it recognised vitronectin, fibrinogen and von Willebrand's factor as ligands [77]. The VnR has been found to have several other ligands, including thrombospondin [78], osteopontin [79], fibronectin [42] and laminin [80]. It may be that this range of ligands is a reflection of the heterogeneity of composition of the vitronectin receptor since the α_v chain has been found not only in association with the β_3 sub-unit but also in association with β_1 [36, 37], β_5 [71], β_s [31] and β_{3b} [34]. While all reports in the literature on the VnR of melanoma cells have only documented the presence of $\alpha_v\beta_3$ we have found evidence recently that heterogeneity of VnR composition may be particularly prevalent in melanoma lines. Thus in a panel of ten human melanoma lines we have found one line where the VnR consists of an $\alpha_v\beta_1$ association and one line where there is a possible $\alpha_v\beta_5$ association (Marshall *et al.*, submitted for publication).

Whether variations in VnR composition do affect melanoma behavior it certainly is true to say that there is a small, but interesting, set of reports which appear to indicate that variations in absolute levels of the VnR could correlate with the degree of progression/malignancy in the melanoma system. Thus McGregor *et al.* [81] found that an antibody raised against the platelet IIb/IIIa complex, presumably recognising the β_3 subunit on melanoma cells since a commercially available anti-β_3 (IIIa) antibody showed a similar pattern of reactivity, preferentially stained sections of melanoma but not nevi or normal melanocytes in the skin. Thus LYP18 detected IIb/IIIa-like molecules on 16 out of 21 melanoma cases studied but detected no reactivity in the 9 nevi and 2 normal skin samples examined [81]. It seems quite likely that these changes relate to a melanoma specific characteristic since 72 out of 75 other tumors studied did not bind LYP18 [81]. The investigations of Albelda *et al.* [58] corroborated and extended these observations since these authors noted that in an examination of 42 clinical cases, ranging from nevi to metastatic disease, β_3 was expressed at a high level only in vertical growth phase and metastatic samples. Interestingly, the same authors reported that α_v was present at a high level in all samples [58]. This would appear to suggest that in the nevus and radial growth phase samples α_v must not be associating with β_3 chains; a possibility that accords well with our demonstration that the VnR composition of melanoma cell lines may be particularly variable.

Though nowhere near as strong as the data derived from the analysis of clinical samples, there are supportive indications from experimental tumor systems that also suggest that VnR may be of particular significance in melanoma development. Thus we have found that, in our series of human melanoma lines, there is an apparent association between levels of VnR expression and tumorigenic capacity in athymic mice. The three least tumori-

genic lines in our panel expressed levels of VnR that were comparable to those expressed by normal melanocytes, while the lines which gave the largest and fastest growing tumors in athymic mice tended to express the highest levels of VnR (Marshall *et al.*, unpublished observations). Using the same monoclonal antibody LYP18, that was used by McGregor *et al.* [81] in the study discussed above, Boukerche and co-workers [82] found that the growth of a human melanoma implanted subcutaneously in athymic mice was inhibited by prior treatment of the injected cells with the antibody. This seems to suggest that the VnR is in some way necessary for the expression of the full growth potential of these melanoma cells [82]. Perhaps, since the interaction between neoplastic cells and the stromal matrix plays such an important role in regulating tumor growth and differentiation *in vivo* [83], it may be that the disruption of this interaction, or the failure to form such an interaction in the case of cells with low levels of VnR, is the underlying cause of this growth inhibition. It may not be only in the area of growth regulation that the VnR plays an important part in modulating melanoma behavior. Recently, it was reported that the more metastatic line of two derived from the MeWo melanoma possessed higher levels of VnR than did the less metastatic line and that this integrin appeared to be involved in mediating the attachment of MeWo cells to lymph node sections [84]. It was suggested from these observations that this receptor could therefore play a role in lymphatic metastasis of melanoma [84]. Perhaps then the reported association of β_3 expression with increasing malignancy of melanomas [58] is an indication of the involvement of this molecule in the direct disseminative capacity of cells in these lesions.

From this brief overview of the presence of integrin receptors on melanoma cells it can be seen that, at the present time, the situation is complex. Certainly a wide range of integrin molecules have been detected on melanomas and, though the numbers of cases analysed is small, there appear to be marked changes in at least two of these molecules, $\alpha_4\beta_1$ and the VnR, associated with tumor progression. Since modulation of metastatic activity has been achieved by co-injection of molecules known to interact with the integrin receptors [44, 45] it seems likely that these alterations in integrin expression have a functional role in determining metastatic capacity. Further efforts at elucidating the role of these structures in the malignant behavior of melanomas will not only involve examination of more substantial numbers of clinical cases but will also focus on the use of receptor-deficient mutants and selective cDNA transfection into appropriate recipient cells [85]. Clearly the presence or absence of specific receptors may have a major impact on cell behavior but cannot constitute the whole story. Rather it is the regulation of adhesion receptor function that is likely to be of most significance in determining cellular responses. For example, it has been shown that VLA binding activity of T cells is augmented to a significant extent following cell activation without any change in the level of expression of the VLA molecules [86]. One attractive hypothesis for explaining how avidity of binding can be regulated is via a phosphorylation event. Treatment of cells with the protein kinase C-activating tumor promoters often has profound effects upon the adhesive characteristics of such cells [87] and it has been found that these treatments may phosphorylate at least some β subunits [31]. Of particular interest is the recent finding that differential phosphorylation of integrins may occur in cells with different transformed phenotypes [88]. Given that alterations in membrane-bound protein kinase C have been found to be induced differentially in variant melanoma lines of diverse metastatic behavior [89] it seems possible that the role of integrin phosphorylation in determining cell migration and adhesion is likely to be of major significance and to have a profound effect on melanoma metastasis. This avenue of exploration promises to be a fertile one in future years.

CD44 expression on melanomas

As Springer [90] has pointed out in a recent review, the cells of the immune system have, during the course of patrolling the body for invading organisms, to circulate as non-adherent cells in the blood and the lymph and migrate as adherent cells

through the tissue. Moreover they must be able to cross endothelial and basement membrane barriers in order to aggregate at the sites of infection [90, 91]. These processes, with their requirement for rapid transitions between the non-adherent and the adherent state, are very similar in many respects to those occurring in the metastatic dissemination of solid tumors [2, 63, 64]. Certainly the characterization of the molecules involved in these immune system interactions is at a much more advanced state than is true for solid tumors and the interested reader is referred to the excellent review by Springer [90] for further details.

The molecules that are expressed on lymphocytes and which regulate the attachment of these cells to the specialized 'high' endothelial venule (HEV) cells have been termed 'recirculation' or 'homing' receptors [90] and appear to interact with specific ligands, termed 'addressins', on the high endothelial cells of different lymphoid organs [63, 64]. Certainly VLA-4 ($\alpha_4\beta_1$), as discussed above, is a Peyer's patch receptor [60, 92] that is more widely expressed than on lymphocytes alone, suggesting that the putative 'homing' receptors need not be restricted to cells of the lymphoid lineage. Another molecule which was identified as a 'homing' receptor but which is widespread in its distribution is CD44. Originally described by Dalchau *et al.* [93], using the monoclonal antibody F.10-44-2, CD44 was found on T cells, granulocytes, cortical thymocytes and brain tissue [93, 94]. Since that time, this ubiquitously distributed structure has been found to be an integral membrane glycoprotein that exists in a variety of forms, the most prevalent of which is 85–95 kd in size, that arise as a consequence of post-translational modifications of a 37 kd core protein [95–97]. Monoclonal antibodies against the CD44 molecule, the Hermes antigen [98], phagocytic glycoprotein Pgp-1 antigen [99] and the extracellular matrix receptor ECM-III [100] all recognise the same molecule [101] indicating that it is likely to be involved in a wide range of cellular functions. The possible pleiotropic capacity of CD44 was underlined when cloning of CD44 cDNA revealed that the N-terminal domain of the molecule exhibited significant homology with the tandem repeat domains of the cartilage link and proteoglycan core proteins [95, 96, 102]. Intrigued by the observation that two human melanoma lines expressed mRNA for CD44 [96] we wondered whether this molecule might play a role in determining the invasive and metastatic behavior of such cells via much the same sort of mechanism that has been proposed for $\alpha_4\beta_1$ [67–69]. Accordingly we have examined the expression of the CD44 molecule on a panel of human melanoma cell lines and a series of strains of normal melanocytes isolated from neonatal foreskins. We have found that cells of the melanocytic lineage do indeed express the CD44 molecule at their cell surface and that, in both transformed and non-transformed cells, this is the 85–95 kd species (Birch *et al.* submitted for publication). Moreover the amount of CD44 expressed on melanoma cell lines invariably was greater than the amount expressed on normal melanocytes; suggesting that a corollary of neoplastic transformation in cells of this lineage is an up-regulation of CD44 (Birch *et al.* submitted for publication). In an effort to explore further the involvement of the CD44 molecule in the modulation of melanoma behavior we have used iterative sorting on the fluorescence-activated cell sorter [85] to isolate a series of stable CD44-sufficient and CD44-deficient variants from both a human and a murine melanoma line. In both systems we have found that, while there is no difference between the clones in terms of their tumorigenic capacity as assessed by the formation of tumors in the s.c. flank region of either athymic (for human cells) or syngeneic (for murine cells) mice, there are dramatic differences in terms of the clones' abilities to form pulmonary tumor nodules subsequent to i.v. injection into recipient mice. These differences are such that the high-expressing clones produced much greater numbers of lung tumor nodules than did the low-expressing clones (Birch *et al.*, submitted for publication). Because of the problems associated with the existence of cellular heterogeneity for the metastatic phenotype we have tested a total of 12 clonal lines, 6 of which were high CD44-expressors and 6 of which were low CD44-expressors. All clones from one group behaved as

did all other clones within that group appearing to rule out the possibility of a spurious correlation between the cell surface molecule and the *in vivo* behavior. Certainly, our results are in accord with the basic premise that a molecule involved in regulating lymphocyte recirculation may also be involved in affecting the disseminative capacity of melanoma cells.

With the demonstration that CD44 functions as the principal cell surface receptor for hyaluronate [102, 104] a possible basis for the differences between high- and low-expressing clones was suggested. Hyaluronate, which is a glycosaminoglycan composed of N-acetylglycosamine-glucuronate repeats, is found widely thoughout the body and often is located in the extracellular matrix [105, 106]. Many tumors of diverse origin have been found to exhibit considerable accumulations of hyaluronate [107] and it is well known that transformed cells can exhibit high levels of hyaluronate binding activity [108]. We found that the clones expressing high levels of CD44 attached to hyaluronate substrata *in vitro* to a considerably greater extent, as would be expected, than did the low-expressing clones. Since particularly high levels of hyaluronate have been found in the lung [109] it is tempting to speculate that the increased number of pulmonary nodules resulting from the i.v. injection of high CD44 expressing clones is a reflection of increased arrest in the lung microcirculation. Currently we are using radiolabeled cells of the various clones injected into animals to assess whether this is indeed the underlying basis of the observed phenomenon. If enhanced arrest is observed it need not be solely a consequence of binding of cells to the hyaluronate present in the ECM. It has long been known that hyaluronate is involved in cell-cell interactions and aggregation [110–112]. However, it was interesting to note that the transfection of mouse L cells with CD44 primate cDNA increased the occurrence of self-aggregation [97]. Whether this aggregation was because the transfected L cells already expressed a ligand for the CD44 receptor or whether the molecule was binding in an homophilic fashion was not known [97]. Similarly, we do not know, as yet, whether in our clones aggregation is via an homophilic receptor-receptor interaction or via a receptor-ligand-receptor interaction but what we do know is that the high-CD44 clones form aggregates and clumps to a significantly greater extent than do the low-CD44 clones. It has been shown that larger embolic clumps, and the capacity to form such aggregates, can lead to increased experimental metastatic capacity [113] so that quite possibly melanomas expressing CD44 are trapped more readily in capillary beds. It is clear that individual cell motility and the regulation of this process must play a considerable part in determining invasive and metastatic events [114–116]. The hyaluronate receptor has been shown to be linked to the actin filaments of the cell [117] and descriptions of the proposed role of Pgp-1 in cell motility have appeared [118]. When we assayed our clones for migratory activity, using the wound assay in a confluent monolayer of cells, we found that not only did the high CD44 expressing clones manifest a greater migratory capacity than did the low CD44 expressing clones but that anti-CD44 antibodies inhibited this capacity for increased cell movement. These results suggest that in melanoma cells the CD44 molecule is playing a role in cell motility and, possibly, that the increased malignancy associated with higher expression of the hyaluronate receptor may be due in some measure to this altered cellular capacity.

In summary, it is apparent that the up-regulation of the CD44 molecule, the hyaluronate receptor, on melanoma cells can have a marked effect upon the behavior of such cells. This effect may be mediated through changes in adhesive characteristics, aggregation ability or migratory capacity. Certainly it has been suggested that accumulation of hyaluronate at the interface between the connective tissue and the tumor mass can have a substantial impact on the invasiveness of cancer [107]. Possibly the existence of high levels of the hyaluronate receptor on cell populations could allow the exploitation of this phenomenon and lead to increased malignant behavior. It will be of interest to determine whether the data we have derived working with tissue culture cells have any relevance to the clinical situation. Examination of melanomas, at

different stages of progression for hyaluronate deposition and levels of hyaluronate receptor (CD44) expression currently is underway.

Summary and conclusions

The changes in cellular behavior, and the molecules regulating such changes, associated with tumor progression from the benign to the malignant state are studied readily in melanoma development where the various stages are well defined [11, 12]. It is highly likely that at least some alterations will involve cell surface molecules capable of determining cell-cell and cell-matrix interactions. Indeed, it has already been shown that specific cell-cell adhesive molecules, such as ICAM-1, are changed during the course of melanoma progression [17, 18]. In this article we have focused on two groups of receptor molecules, one the integrin family and the other the hyaluronate receptor (CD44), which are involved in cell-matrix interactions and examined the evidence that expression of these glycoproteins is altered during the course of progression and discussed their possible involvement in the metastatic process.

The integrins have been documented to be widely expressed on melanoma cells and the evidence is strong that these structures have a major role in determining tumor spread. Relatively little is known about what changes do occur during the course of malignant progression but at least two of the integrins, VLA-4 ($\alpha_4\beta_1$) and the β_3 chain in the vitronectin receptor, show alterations in level of expression which correlate with more aggressive behavior.

Originally we started to examine CD44 expression in melanomas based on the rather naive assumption that, since the process of tumor dissemination is analogous in many ways to that of lymphocyte migration, this molecule might play a part in controlling cell arrest. The demonstration that CD44 is the principal cell receptor for hyaluronate has suggested that this membrane glycoprotein probably plays a wider role in the cellular processes involved in metastatic dissemination than by simply affecting cell-cell adhesion. We have found that considerably more CD44 is expressed on melanoma cells than on normal melanocytes. Moreover selection of various lines, differing in levels of CD44 expressed, from a single parental tumor is associated with profound changes in the *in vivo* behavior of these cells such that greater expression of CD44 correlates with enhanced aggression. This has not been a universal observation in all experimental systems [119] and so it will be important to assess the expression of the hyaluronate receptor in clinical cases of malignant melanoma. Efforts to address this question currently are underway in our laboratory.

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