Commentary

Role of the β_3 Integrin Subunit in Human Primary Melanoma Progression

Multifunctional Activities Associated with $\alpha_v\beta_3$ Integrin Expression

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Recent research into how cells interact with and function within their different (and sometimes dynamically changing) environments has become a primary focus of cell biology, centering on the examination of cell surface adhesion receptors, most notably the integrins.^{1,2} Integrins form one family of cell adhesion receptors, which also include the immunoglobulin gene superfamily, selectins, cadherins, cartilage-link proteins, and cell mucins (which act as ligands for the selectins). All integrins are heterodimers composed of noncovalently linked α and β subunit transmembrane glycoproteins containing large extracellular domains, short transmembrane domains, and carboxy-terminal cytoplasmic domains of variable length.¹⁻⁵ There are presently 17 α subunits and eight β subunits known, which occur in just over 20 integrins identified so far. However, these numbers may belie the added complexity introduced by the alternately spliced cytoplasmic domains observed in some variants of these subunits.^{1,6} The eight β subunits share approximately 40 to 80% amino acid sequence homology and are similar in size (90 to 110 kd) except for the β_4 chain, which is almost twice as big because of its large intracytoplasmic domain. The β chains contain a fourfold repeat of cysteine-rich segments and a highly conserved cytoplasmic domain with an Asp-X-Ser-X-Ser sequence (where X is any amino acid) associated with cation-dependent ligand binding and with the metal ion-dependent adhesion site motif.^{2,7} This cytoplasmic tail region of the β subunits has been implicated both in cytoskeletal interactions and with signaling complexes. The α subunits, with molecular weights ranging between 120 and 180 kd, tend to be

more heterogeneous than the β subunits. Furthermore, some α units contain light and heavy chains linked by a disulfide bridge in the extracellular domain, whereas other α subunits contain an extra segment of approximately 180 amino acids called the α A-domain⁷ (or I domain)^{1,2} inserted before the last five homologous repeats, which contain a cation-binding domain. This α A-domain contains a sequence homologous to the collagen-binding domains of von Willebrand factor, cartilage matrix protein, and complement proteins. Only recently has functional activity in recombinant versions of this domain permitted the opportunity to study ligand binding; the fragment α L A-domain has been shown to bind the intercellular cell adhesion molecule-2 (ICAM-2) and the fragment α 1/ α 2 A-domain has been shown to bind to laminins.⁷ All α subunits contain a sevenfold repeat of a homologous segment with the last three or four repeats containing the sequence Asp-X-Asp-X-Asp-Gly-X-X-Asp¹ (or related sequence)² motif. This motif is associated with the divalent cation-binding EF-handlike domains^{2,7} and contributes to cation-dependent ligand binding to the integrin receptor. While divalent cations are required for receptor function they can also, depending on the nature of the cation, affect both the integrin's affinity and specificity for ligands.^{1-5,7} Some integrins also require divalent cations for their $\alpha\beta$ subunit association.^{1,8,9} In general, whereas the theoretical number of integrin heterodimers exceeds 100, the 20-plus observed integrins fall into three basic groups based on similar chain structures and/or the ability to recognize similar protein or adhesion motifs. These three groups include integrins which contain the β_1 , β_2 , and β_3 or α_v subunits; three $\alpha\beta$ integrins do not fall within these groups.^{1,2} Many α subunits can associate with just one of the β subunits, although some

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α subunits can associate with more than one β subunit. In particular, the α_v subunit appears to be one of the most promiscuous of the α subunits and can associate with at least five different β subunits, including the β_1 chain (see below). Although originally identified as cell adhesion molecules (both cell-extracellular matrix and cell-cell), integrins have most recently been shown to play significant roles in signal transduction events,^{1-7,10-27} gene expression,^{12,25,28} cell proliferation,^{12,13,15,17,26,27,29} regulation of apoptosis³⁰⁻³³ and anoikis,³² invasion and metastasis,^{2,10-12,21,26,29,33-37} embryogenesis,³⁸⁻⁴¹ tumor progression,²⁹ inflammation and immunity,²⁸ hemostasis,⁴² and angiogenesis.^{26,33,43-48} Recent studies have also identified integrins as points of entry for certain infection agents including hantaviruses, which appear to use the β_3 -containing integrins to gain entry into cells,⁴⁹ and Lyme disease spirochetes, whose attachment to human cells is mediated by the $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins.⁵⁰

Integrin function in normal and pathological processes in terms of ligand and adhesive specificity was initially determined using cell adhesion assays, monoclonal antibodies, and affinity chromatography.^{1,2} It became apparent that individual integrins can often bind to different ligands and that different ligands are recognized by more than one integrin. Integrins bind to extracellular matrix proteins and facilitate cell-substratum adhesion and, in the case of the ligand fibrinogen, can facilitate cell-cell aggregation. Some integrins can also recognize integral membrane proteins of the immunoglobulin superfamily (ICAM-1, ICAM-2, and VCAM-1) and thereby mediate direct cell-cell adhesion (ie, recognize and bind to a counterreceptor on other cells). The first defined integrin recognition site was the sequence Arg-Gly-Asp (RGD),¹⁻⁵ found in fibronectin, vitronectin, and other adhesive proteins. Subsequent binding motifs identified include Lys-Gln-Ala-Gly-Asp-Val (KQAGDV) in fibrinogen, Asp-Gly-Glu-Ala (DGEA) in type I collagen, Glu-Ile-Leu-Asp-Val (EILDV) in an alternatively spliced segment of fibronectin, and Gly-Pro-Arg-Pro (GPRP) in fibrinogen. Whereas the integrins that bind laminin appear to recognize specific parts of the laminin molecule, integrins that bind counterreceptors appear to recognize specific immunoglobulin-like domains.^{1,2}

At present, integrins are described structurally as heterodimeric glycoproteins that contain an extracellular ligand-binding site composed of the *N*-terminal domains of the α and β subunits. This region is connected by two stalks, one from each subunit, to the membrane-spanning segments and ends at the α and β subunit cytoplasmic domains. All current evidence indicates that these cytoplasmic domains interact with cytoskeletal proteins and components. The cytoplasmic tail of some β subunits appears to direct integrin receptors in a ligand-independent manner to focal adhesion sites where the integrins become organized at the ends of actin filaments. These focal contacts also contain the proteins vinculin, talin, and α -actinin, which are thought to mediate interactions between the ligand-integrin structure outside the cell and the actin microfilaments inside the cell.^{1,2,7} The cytoplasmic tail of some α subunits appear to convey ligand-specific signals to the cells in response to the integrin

binding its ligand.⁵¹ In contrast, the $\alpha_6\beta_4$ integrin is unique in that it becomes concentrated in epithelial cells specifically at hemidesmosomes and is thought to interact with the intermediate filaments characteristic of hemidesmosome structure.¹

We now know that individual cells can vary their adhesive properties by selectively expressing different integrins and by modulating their integrin specificity and affinity for ligands through a process known as integrin activation and deactivation. The change in integrins' activation/deactivation state as a conformation change in the receptors' extracellular domains has been detected using both immunohistochemical and biophysical techniques and could relate to the degree of phosphorylation of the β subunit (see below)⁵¹ or result from interactions of the integrins with lipid-derived mediators.¹ Recently, it has been shown that integrin function is also subject to modulation by interaction with other membrane proteins, including other integrins.⁵²⁻⁵⁶ The glycosyl-phosphatidylinositol (GPI)-linked cell surface protein urokinase receptor (uPAR), which can function as an adhesion receptor for vitronectin with distinct sites for binding both vitronectin and urokinase,^{52,53} can interact with the active form of β_1 -containing integrins to form a stable integrin-uPAR-caveolin complex that suppresses the cells' normal β_1 -dependent adhesion to fibronectin. The result is that uPAR alters cells' ability to adhere and interact with different extracellular matrices (and/or different environmental signals) *via* the interactions it establishes through its integrin-uPAR-caveolin connection with the cytoskeleton.^{52,53} Other studies have identified transmembrane adaptors such as the integrin-associated protein (IAP), which is physically and functionally associated with the $\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$ integrins.⁵⁷ IAP not only cooperates with β_3 integrins in binding to thrombospondin, but also appears to activate a heterotrimeric Gi protein-dependent intracellular pathway leading to activation of tyrosine kinase Syk and its association with FAK. As such, IAP seems to work in concert with β_3 integrins to regulate intracellular signaling in response to thrombospondin.^{27,57}

It is now clear that altered, modulated, or regulated integrin interactions can change the way cells interact with their environment with dramatic and far-reaching consequences for both normal and pathological conditions. This is apparent from studies that have shown that perturbation of certain integrins, either by ligation or by treatment with certain anti-integrin antibodies, can result in the generation of signals that cause an increase in intracellular pH^{26,58,59} and Ca^{2+} levels,^{26,60} changes in inositol lipid synthesis,^{26,61} tyrosine phosphorylation of pp125^{FAK},^{11,12,26} and activation of p34/cdc2^{13,26} cyclin A,^{26,62} protein kinase C,^{26,63} mitogen-activated protein kinase (MAPK),^{15,26} phosphatidylinositol 3-kinase,^{17,18,26} p21 Ras,^{20,26} and NF- κ B.^{20,21,26,64} Furthermore, recent studies have shown that the $\alpha_v\beta_3$ integrin on melanoma cells can bind and localize proteolytically active MMP-2 on the cell surface, facilitating cell-mediated collagen degradation and directed cellular invasion.⁶⁵ Integrins have also been shown to act synergistically with growth

factor receptors to modulate cellular functions including cell growth⁶⁶ and migration.⁶⁷

One of the most diversely functional integrins studied to date is the $\alpha_v\beta_3$ integrin. This integrin is the original, classic vitronectin receptor which recognizes the RGD binding motif found in vitronectin.^{1,2,3} The α_v subunit is now known to associate with at least five out of the eight different β subunits, making it one of the most promiscuous subunits studied so far. When associated with the β_3 subunit, the $\alpha_v\beta_3$ integrin can bind to vitronectin and colocalize to focal adhesion sites with the proteins vinculin, talin, and α -actinin at the end of actin filaments. When associated with the β_5 subunit, this homologous integrin can bind vitronectin but remains randomly distributed over the surface of the cells and does not localize to focal adhesion sites.⁶⁸ Recent work has shown that the cytoplasmic tail of the β_3 chain undergoes tyrosine phosphorylation upon adhesion of the $\alpha_v\beta_3$ integrin to vitronectin, antibody perturbation of the integrin, or incubation with a manganese-containing buffer. This phosphorylation, which requires the presence of the cytoplasmic tail of the α_v subunit, does not occur in the β_5 subunit of the $\alpha_v\beta_5$ integrin under similar conditions.⁵¹

The ability of the $\alpha_v\beta_3$ integrin to cluster on the cell surface and colocalize with cytoskeletal elements in response to specific stimuli can result in polymerization of the actin cytoskeleton and lead to changes in cell morphology and migratory ability. This permits the integrin to act as a physical bridge between external stimuli and the cells' cytoskeleton, and as a transducer of messages from outside the cell to its internal signal processing pathways. Ultimately, changes in adhesiveness of cells for their environment, through integrins like the $\alpha_v\beta_3$ integrin, can lead to gross changes in cellular morphology via the actin cytoskeleton, which is directly involved in cell migration. In this respect, high expression of the $\alpha_v\beta_3$ integrin has been associated with different motile cells *in vivo* including neural crest cells, vascular endothelial cells, and malignant melanoma cells.^{1,2,26} Recent work has identified the NPXY motif within the β_3 subunit (comprising residues 744–747) as essential for cell morphological and migratory responses (cell attachment, spreading, and migration on a immobilized ligand) mediated by the $\alpha_v\beta_3$ integrin both *in vitro* and *in vivo*.⁶⁹ This work showed that the hamster cell line CS-1 could be transfected with and express the wild-type $\alpha_v\beta_3$ integrin, leading to a change in the cells' migratory response to vitronectin and the acquisition of an ability to form spontaneous pulmonary metastases in a chick embryo grown on a chorioallantoic membrane. Mutations in the NPXY region of transfected $\alpha_v\beta_3$ integrin abrogated the cells' metastatic ability and migratory response to vitronectin but did not disrupt the integrin's ability to bind to vitronectin.⁶⁹ Other work has shown that ligation of different integrins, including $\alpha_v\beta_3$ on human melanoma cells, can lead to altered protein expression associated with increased invasive ability *in vitro*.⁷⁰ More specifically, as an extension of the work by Werb and colleagues⁷¹ that demonstrated that the $\alpha_5\beta_1$ integrin could transduce extracellular signals in rabbit synovial fibroblasts resulting in a change in the cells' expression and extracellular

levels of collagenase and stromelysin, work with the human melanoma cell line A375M showed that ligation of the $\alpha_v\beta_3$ integrin with vitronectin (either matrix bound or soluble) or an activating antibody to the α_v subunit could increase the expression and extracellular levels of the matrix-metalloproteinase-2 enzyme (MMP-2, gelatinase A) coincident with an increase in the cells' ability to invade *in vitro*.⁷⁰ As a whole, these results suggest a role for the $\alpha_v\beta_3$ integrin in the pathological progression of melanoma that is facilitated by specific $\alpha_v\beta_3$ integrin-mediated interactions of cells with their extracellular environment. These interactions can then generate physical (possibly mechanical)^{72–74} and biochemical signaling events that contribute to a change in cell behavior resulting in an aggressive, pathological phenotype.

Significant in light of these observations is the evidence presented by Hsu and colleagues in this issue⁷⁵ that the $\alpha_v\beta_3$ integrin plays a direct role in the progression of human primary cutaneous melanoma from the nontumorigenic, nonmetastatic radial growth phase to the tumorigenic, metastatically competent vertical growth phase. An important aspect of this work was the approach that Hsu and colleagues used to design and perform these studies. First, information about the long-term clinical and histopathological observations of cutaneous melanoma formed the basis for defining the disease and its progression in patients and provided the touchstone for the results obtained in the laboratory. Second, using these *in vivo* observations, Hsu and colleagues isolated and propagated *in vitro* cells derived from different stages of the disease as a model for primary cutaneous melanoma progression as defined *in vivo*. Care was taken to correlate the biological properties of the different isolated cell types in culture with observed biological properties and stages *in vivo*. Significant in the planning and execution of these experiments was the authors' progression from the reductionist *in vitro* cell culture model of the disease stages to the more complex three-dimensional skin reconstruct model which recreates the physiological milieu of the *in vivo* environment. Unlike other traditional invasion assays, this model accounts for both tumor cell-derived mechanisms and microenvironmental factors from stromal cells during the invasive process. Therefore, differences between the control and β_3 transfected cells could be observed and the invasion by transfected melanoma cells deep into the dermis without entering apoptosis could be clearly delineated from the control cells, which did not invade but remained in the epidermis and entered apoptosis. Ultimately, the experiments returned to the *in vivo* SCID mouse model where tumorigenicity of early stage melanoma cells was shown to increase after forced expression of the β_3 subunit.

Central to this experimental approach was the clinical observation that the onset of expression of the β_3 integrin was one of the most specific markers for identifying the transition of primary human cutaneous melanoma from the nontumorigenic, nonmetastatic radial growth phase to the tumorigenic, metastatically competent vertical growth phase. Hsu et al demonstrated that forced expression of the β_3 integrin subunit (via adenovirus gene

transfer) results in the expression of a functional $\alpha_v\beta_3$ integrin and in a malignant phenotype that corresponds to the observed *in vivo* progression of cells from radial growth phase to vertical growth phase. This study not only contributes to the growing body of work that clearly identifies the importance of integrin analysis in the diagnosis and prognostic evaluation of diseases, but also is the first to extend clinical observations about a key prognostic marker for cutaneous melanoma progression to the demonstration of a specific functional role for the β_3 -containing integrin $\alpha_v\beta_3$ *in vitro* and *in vivo*, which directly correlates with the progression of the disease as clinically defined. In conclusion, these results clearly provide the rational basis for research that is just now beginning to identify integrins as unique biological targets for tumor therapy,^{34,76} as potential targets for inhibiting or blocking the metastatic cascade,⁷⁷ and as useful tools for targeted chemotherapy strategies in the treatment of cancer based on the selective expression of integrin receptors in the tumor vasculature.⁷⁸

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