β3 integrins: major therapeutic targets of the near future

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Abstract

Integrins are major cell adhesion receptors which assume two important functions: first they act as anchoring molecules allowing firm cellular attachment to the extracellular matrix and second they work as signaling receptors being able to transduce signals in both directions (outside-in and inside-out) through the plasma membrane. Their biological importance is determined by their involvement in many physiological phenomena. Furthermore, their implication in various diseases and their accessibility to drugs make of them interesting therapeutic targets.

Keywords

integrins, αIIbβ3, αvβ3, signaling, cell adhesion, cardiovascular disease, cancer metastasis

1. Introduction

Cell-cell and cell-extracellular matrix (ECM) interactions are involved in many physiological phenomena such as the development and function of tissues, hemostasis, inflammation, wound healing, elimination of infectious agents, and also in pathophysiological disease states such as osteoporosis, cardiovascular and inflammatory disorders and tumor metastasis. Both types of interactions are mediated by cell surface proteins that are termed cell adhesion molecules (CAMs). The most numerous and most versatile group of CAMs are integrins.

The name "integrin" was first coined by Tamkun *et al*. who described an integral transmembrane protein linking the ECM with the intracellular cytoskeleton (1). Today however, we know that integrins not only serve this architectural function as anchoring molecules, but they also function as signaling receptors and mediate bidirectional transmembrane signaling. Through inside-out signaling (from the cell interior to the outside), the cell is able to upregulate the binding affinity of the integrin for its extracellular ligand. This process, also termed activation, is a characteristic feature of certain integrins (2). In the opposite way, comparable to other receptors, integrins bind extracellular ligands and transmit signals from the cellular environment into the cell (outside-in signaling). These signals direct cell adhesion and regulate other aspects of cell behavior including cell differentiation, migration and growth, and determine cell survival (3).

2. Structure and diversity of integrins

Integrins are calcium-dependent heterodimeric transmembrane glycoproteins composed of one α and one β subunit. Each subunit has a large extracellular domain,

a single membrane-spanning domain, and a short, non-catalytic cytoplasmic tail. The extracellular domains form binding sites for numerous ligands, whereas the cytoplasmic domains anchor the integrin to cytoskeletal and signaling proteins. Figure 1 shows the complete set of mammalian integrins (based on extensive searches of the human and mouse genomic sequences), comprising 18 α and 8 β subunits, known so far to assemble into 24 distinct integrins, varying in their ligand binding specificity, intracellular signaling properties and tissue distribution (4).

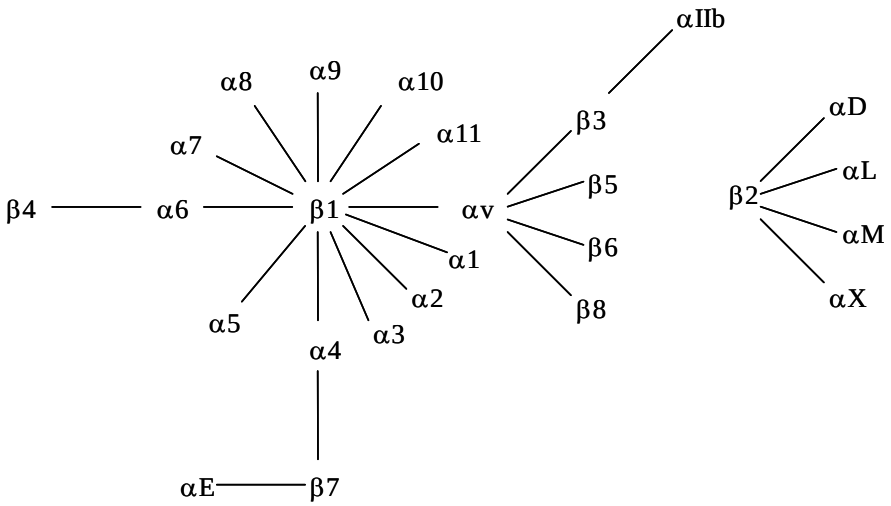

Figure 1: Integrin diversity

As shown in Figure 1, two members of the integrin family share the β3 subunit (5). These two integrins, also termed cytoadhesins, are the platelet-specific αIIbβ3 integrin, also known as the platelet fibrinogen receptor GPIIb-IIIa, and the more widely distributed vitronectin receptor αvβ3.

Integrin αIIbβ3 acts as a receptor principally for fibrinogen (Fg), but also for fibronectin, von Willebrand factor (vWf) and vitronectin (6) and it is the most abundant platelet receptor. Furthermore, αIIbβ3 is a particularly good model of an integrin whose tightly regulated activation is of great physiological significance (7). Indeed, integrin αIIbβ3 is essential to the control of bleeding since it mediates platelet aggregation at sites of vascular injuries: platelets are stimulated either via adhesion to

exposed subendothelium or via exposure in the circulation or in a growing thrombus to a variety of agonists like ADP, collagen or thrombin. The binding of the agonists to their respective receptors generates a signaling cascade leading to the activation of integrin αIIbβ3 through an inside-out signaling mechanism: αIIbβ3 aquires the ability to bind soluble Fg. Because Fg is a dimer, its binding leads to bridging of adjacent platelets, platelet aggregation and formation of a blood clot which ultimately seals the injured tissue. Since the concentrations of both Fg and platelets are so high in the blood, αIIbβ3 must be maintained in a resting or inactive state under normal conditions to ensure proper blood flow and to avoid formation of arterial thrombi (8) (Figure 2).

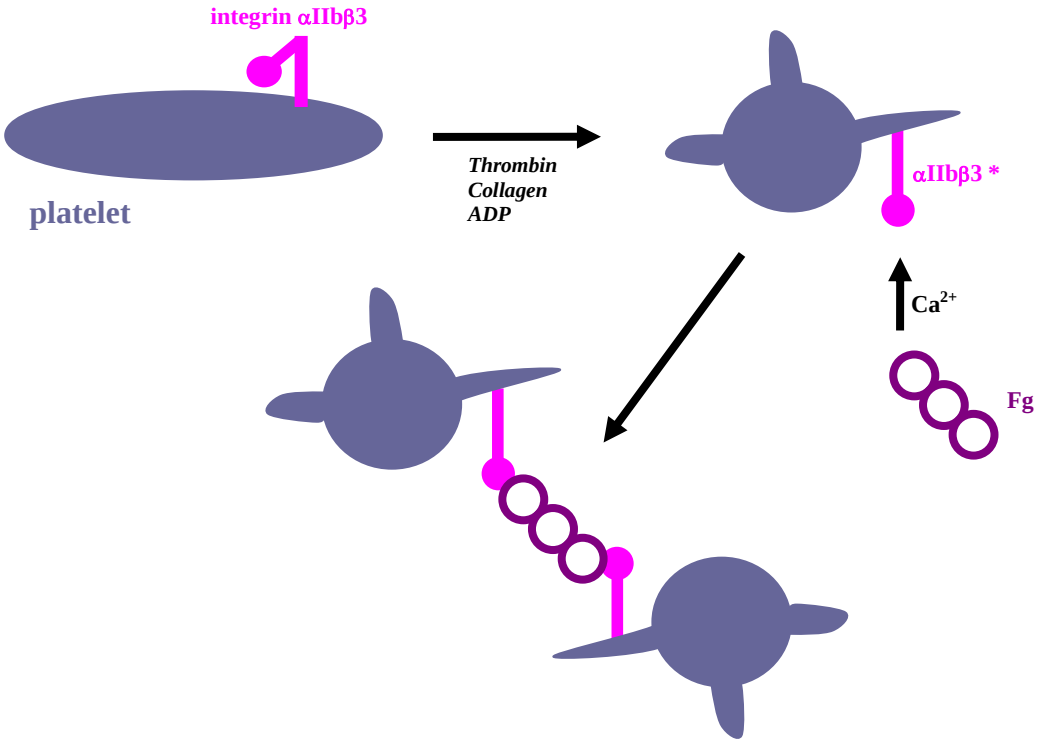

Figure 2: Integrin αIIbβ3 in platelet aggregation

Upon platelet stimulation by agonists, integrin αIIbβ3 switches from the resting state into the activated state (αIIbβ3*) and becomes able to bind soluble macromolecular ligands such as Fg or vWf, leading to platelet aggregation.

Whereas αIIbβ3 is essential for platelet aggregation and controls platelet function in thrombosis and hemostasis, the vitronectin receptor αvβ3 is more widely expressed and present at the surface of most cell types. This receptor influences cell migration and has an impact on angiogenesis, restenosis, tumor cell invasion and atherosclerosis. Integrin αvβ3 is one of the most promiscuous integrins, as it binds to multiple ligands including vitronectin, fibronectin, Fg, vWf, thrombospondin, and also serves as a receptor for several viruses such as foot-and-mouth disease virus, adenovirus, and human immunodeficiency virus (9).

Very recently, the crystal structure of the extracellular segment of integrin αvβ3 has been elucidated by Xiong *et al*. (10) and, in the crystal, αvβ3 appears as an asymmetric structure forming a 'head' carried on two 'legs' (Figures 3A and 3B). In the resting state, both legs are bent at a defined region, called 'genu' or knee. This reflects an unusual flexibility possibly linked to integrin regulation. The main interface between the α and β subunits has been positioned within the ovoid 'head' of the heterodimer. A further milestone in integrin biology was laid when the crystal structure of the extracellular segment of integrin αvβ3 in complex with a ligand was reported by the same research group (11). Apparently, the binding of the ligand occurs at the major interface between the αv and β3 subunits where the ligand makes extensive contacts with both subunits (Figure 3C).

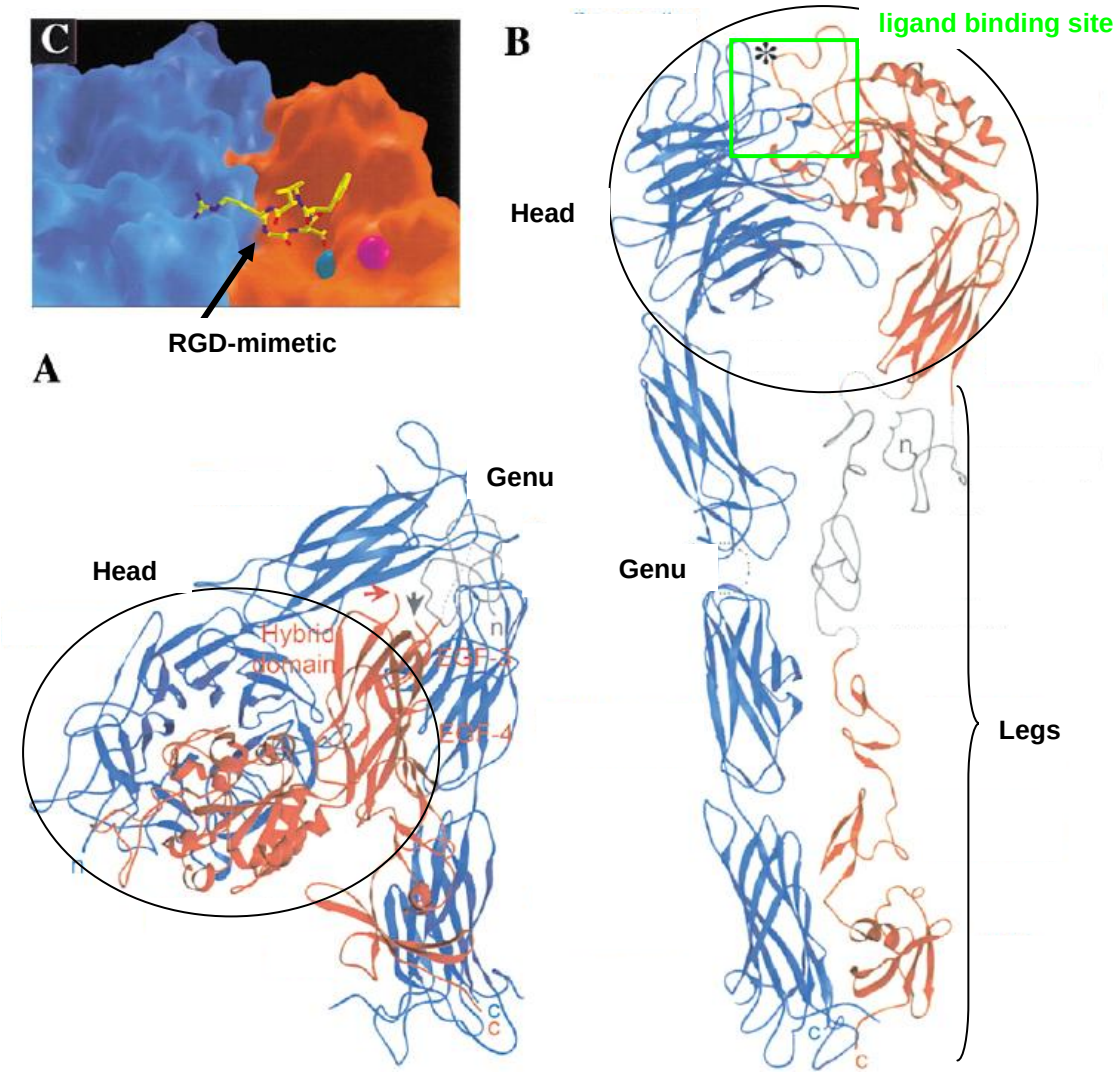

Figure 3a: **Three-dimensional structure of the extracellular segment of integrin αvβ3**

(A) Ribbon drawing of crystallized αvβ3 [shown in blue (αv) and red (β3)]. (B) Model of the straightened extracellular segment of αvβ3. The NH_2-terminal segments of the α and β subunits assemble into an "ovoid" head from which two nearly parallel "tails" or "legs" emerge. In A and B, "n" and "c" indicate NH_2- and COOH-terminus, respectively. (C) Surface representation of the ligand-binding site, with the ligand peptide, an RGD-mimetic, bound to the interface between the αv (blue) and the β3 subunit (red) head domains. (A) and (B) are reprinted with permission from Xiong *et al.* (2001) (10); (C) is reprinted with

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3. Involvement of the β3 cytoplasmic domain in integrin αvβ3 activation and signaling

As mentioned before, the activation of integrins is usually induced by intracellular signaling cascades generated by agonist receptors. As a result, the intracellular constraints that keep the integrin in the low affinity mode are released leading to the propagation of a conformational change from the cytoplasmic part of the receptor to the extracellular ligand binding domain (Figure 4). The integrin aquires the ability to bind soluble macromolecular ligands. Integrin activation can also be induced artificially, for example by deleting the transmembrane or cytoplasmic tails or following the binding of the divalent cation Mn^{2+}. The conformational change within the αvβ3 extracellular domain following small peptide binding or Mn^{2+} treatment has been shown to be dependent on the structural integrity of the β3 cytoplasmic domain (12).

On the other hand, the binding of a macromolecular ligand to the activated integrin also induces conformational changes leading to outside-in signaling events. The cytoplasmic domain of the β subunit has been shown to be a key effector in these events. The results of our work have contributed to prove that structural changes introduced into the β3 subunit cytoplasmic domain severely impair αvβ3-triggered outside-in signals (13).

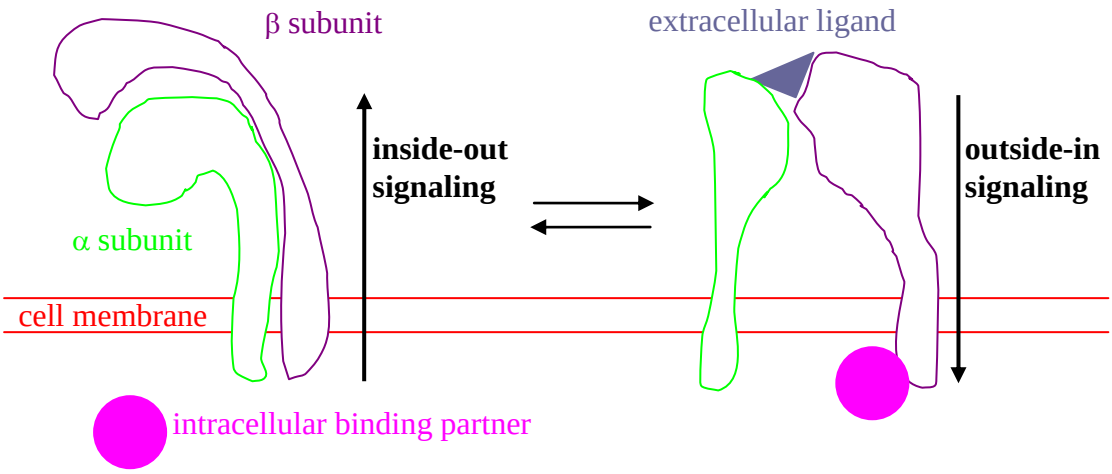


Figure 4: Conformational changes govern inside-out and outside-in signaling

In their inactive state, integrins have a bent conformation and their extracellular domain has a low affinity for ECM ligands. The α and β cytoplasmic domains are closely associated. Subsequent to cell stimulation, the interaction of these cytoplasmic domains with intracellular proteins induces inside-out signaling leading to the straightening and separation of the legs. The integrin is now in an activated state and has a high affinity for extracellular ligands. Following binding of such a ligand, outside-in signals are transduced into the cell.

4. Integrin ligands

As mentioned before, integrins bind to a variety of ligands, including components of the extracellular matrix, cell surface counterreceptors, plasma proteins, as well as bacterial and viral proteins. The minimal target amino acid sequence capable of interacting with an integrin was identified in fibronectin by Pierschbacher *et al*. and corresponds to the tripeptide motif arginine-glycine-aspartic acid (RGD) (14). This RGD motif, which has been found subsequently in a large number of ECM proteins, blood proteins as well as cell surface proteins, is recognized as the "universal cell-

recognition site". The RGD sequence is however not the only motif interacting with integrins, and over the last few years, other cell adhesion motifs have been identified, many of which represent slight variations of the canonical RGD sequence.

The RGD motif is also present in disintegrins, a family of low molecular mass peptides, which have been identified in snake venoms. As a matter of fact, snake venoms contain a complex mixture of pharmacologically active peptides and proteins with various biological activities, such as neurotoxins, cardiotoxins, phospholipases, coagulants and anticoagulants. Predominant in the group of venom proteins that inhibit hemostasis through a non-enzymatic mechanism are the previously mentioned disintegrins. Disintegrins are potent inhibitors of platelet aggregation and exert their biological activity by competing with and preventing the binding of adhesive ligands to integrin αIIbβ3, the platelet fibrinogen receptor. So far, more than 40 peptides and isoforms have been isolated from viper and rattlesnake genera. All disintegrins contain the RGD sequence in a homologous position. Only barbourin, isolated from *Sistrurus miliarius barbouri* (southern pigmy rattlesnake) venom, has lysine-glycine-aspartic acid (KGD) in this region and, as a consequence, has a higher selectivity towards αIIbβ3 than αvβ3 (15).

5. β3 integrins and disease

5.1. Glanzmann's thrombasthenia

As early as in 1918, the Swiss pediatrician E. Glanzmann identified a group of patients with hemorrhagic symptoms and hereditary thrombasthenia (16). At that time, the disease, named Glanzmann's thrombasthenia (GT), was characterized by a list of common diagnostic criteria which included among others: (i) failure of platelets to aggregate in response to common physiological agonists such as ADP, epinephrine,

collagen and thrombin; (ii) prolonged bleeding times; (iii) failure of surface platelet spreading**.** The association of the disease with the platelet αIIbβ3 integrin became apparent when Nurden and Caen (17) and Phillips *et al.* (18) discovered that platelets from some patients with GT were deficient in both αIIb and β3 subunits. Subsequent studies that analyzed the biosynthesis of the receptor showed that both subunits were required to associate and form a surface-expressed complex that functions as a receptor for fibrinogen and other adhesive ligands. Today it is well known that the disease is caused by mutations in the genes encoding αIIb (GPIIb, CD41) or β3 (GPIIIa, CD61) resulting in quantitative or qualitative abnormalities of the platelet membrane αIIbβ3. Most mutations have been found within the extracellular segments of the integrin subunits. One of the mutations causing GT, the β3Ser^{752}->Pro substitution, is however localized within the cytoplasmic tail of the β3 subunit and illustrates well how one single amino acid substitution can strongly impair the functioning of the αIIbβ3 receptor (19). The same mutation introduced into the vitronectin receptor αvβ3 has been shown to prevent αvβ3-mediated outside-in signaling events as well (20).

GT is a rare autosomal recessive disorder with a worldwide distribution. However, since obligate heterozygotes, with 50-60% of the normal number of platelet αIIbβ3 receptors, have no abnormalities of platelet function and no clinically significant bleeding and since consanguineous marriages are common within the geographic regions where the defect occurs, the frequency of GT can be relatively high in these geographic areas. Such populations include the Iraqi-Jews in Israel, the gypsies in France, and some native groups in South-India.

5.2. Integrin αIIbβ3 as a therapeutic target for cardiovascular diseases

There is now solid evidence that the platelet fibrinogen receptor αIIbβ3 plays an important role in several aspects of cardiovascular diseases. Indeed, platelet aggregation, mediated by integrin αIIbβ3, contributes significantly to the formation of arterial thrombi in coronary diseases and embolic stroke, leading to occlusion of arterial vessels, and subsequently to deficient oxygen supply of vital organs such as heart or brain (21). Since integrin αIIbβ3 activation constitutes the final step of platelet stimulation, integrin αIIbβ3 is an attractive target to platelet inhibition (Figure 5). Furthermore, integrins are accessible to and readily inhibited by antibodies, peptides and peptidomimetics, making them excellent drug targets.

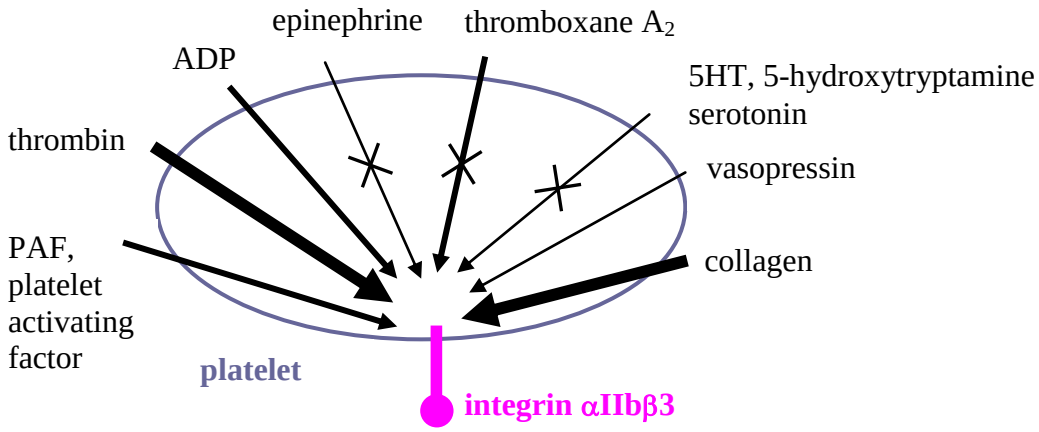
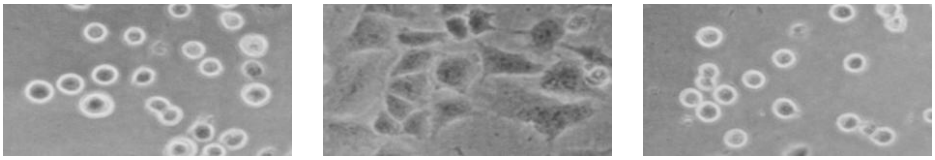


Figure 5: The activation of integrin αIIbβ3 is the final common step in platelet activation

Platelets are stimulated by numerous agonists of varying strength (strength indicated by the thickness of the arrow). After interaction of the agonists with their respective receptors, different signaling cascades are generated, some of which can be blocked by aspirin (barred arrow). Since all the signaling cascades lead to the activation of integrin αIIbβ3, the blockage of this integrin constitutes the best pathway to prevent platelet aggregation and hence thrombus formation.

As a first approach to develop αIIbβ3 inhibitors, monoclonal antibodies to platelets were prepared and the antibodies that inhibited platelet aggregation were selected. Coller *et al*. demonstrated in 1983 that the murine monoclonal antibody m7E3 prevented platelet aggregation by inhibiting fibrinogen binding (22). As illustrated in Figure 6, m7E3 is also capable of inhibiting αIIbβ3-mediated cell adhesion and spreading onto immobilized fibrinogen. The next step in the development of αIIbβ3 inhibitors was the humanization of the effective mouse antibody m7E3 in order to reduce the risk of immune reactions when used in humans. Then followed a long period of clinical testing, which ended by showing that the resulting engineered antibody is an effective supplementary treatment to reduce acute thrombotic events associated with surgery or to prevent restenosis after treatment such as percutaneous transluminal coronary angioplasty to open a blocked vessel. The pharmaceutical abciximab (Reopro, Centocor and Eli Lilly and Co), a Fab chimera that retains the mouse-derived variable portion of m7E3 joined to the constant region of human IgG Fab, is currently in clinical use as an adjunct to coronary intervention.



Negative **Fg** **7E3/Fg**

Figure 6: Chinese hamster ovary (CHO) cells were transfected with the cDNAs encoding human αIIb and β3 integrin subunits. The transfected recombinant human αIIbβ3 enabled the CHO cells to adhere and spread onto the αIIbβ3-specific immobilized ligand Fg (fibroblastoid morphology), whereas the cells did not attach onto bovine serum albumin, used here as a negative control (round

cells). Preincubation of αIIbβ3 expressing CHO cells with monoclonal antibody 7E3 was sufficient to prevent cellular adherence and spreading onto Fg.

Since the tripeptide RGD is present in the binding domain of a large number of integrin ligands as well as in snake venom disintegrins, it was rapidly concluded that small peptides containing the RGD motif or a related sequence could constitute an alternative strategy to antibodies to block αIIbβ3 and hence to prevent platelet aggregation. The fact that the disintegrin barbourin, containing a KGD sequence instead of an RGD, has a high degree of specificity for αIIbβ3 compared with RGD-based peptides, led to the development of the KGD-containing peptide eptifibatide (Integrilin, Millenium) as a clinically useful drug (23).

Another approach has been to mimic the charge and spatial conformation of the RGD sequence via engineered synthetic and semi-synthetic compounds. Examples of parenteral peptidomimetic inhibitors include tirofiban (Aggrastat, Merck Research Laboratories) and lamifiban (Ro 44-9883, F Hoffmann-La Roche, Ltd). Aggrastat, in combination with heparin, is indicated for the treatment of acute coronary syndroms.

The previously described αIIbβ3 integrin inhibitors suffer from the fact that they can only be administered intravenously, which restricts their use to acute situations. There was therefore considerable interest in the development of nonpeptide compounds which can be administered orally for treatment not only of acute, but also chronic disorders. Many such orally active αIIbβ3 antagonists with a variety of structures are now known, and ten of them have already been clinically tested. These agents promised to be potent inhibitors of platelet aggregation, they were specific for αIIbβ3, and displayed excellent bioavailability and pharmacokinetic profiles.

Unfortunately, in the meanwhile, clinical trials of several of the αIIbβ3 antagonists had to be terminated due to adverse effects or because they failed to attain the

anticipated efficacy. Indeed, all of the previously described anti-αIIbβ3 blockers are limited by the degree to which they have bleeding complications as a side-effect.

5.3. Integrin αvβ3 as a therapeutic target for cancer treatment

Cancer metastasis is a complex cascade of events that includes tumor growth and invasion, angiogenesis (the formation of new blood vessels, which is an essential feature for the growth of solid tumors), intravasation of tumor cells into the lymphatic or blood circulation, migration to and arrest in distant organs via interaction of the tumor cells with vascular or lymphatic endothelium, extravasation from circulation and growth to form secondary tumors in the new organ environment (Figure 7). The contribution of integrins to the metastatic process occurs mainly through the regulation of different signaling pathways which dictate the motility, survival and anchorage-independent growth of tumor cells. Moreover, integrins may even have a more complex role in metastasis as they cooperate with serine proteases and metalloproteases to promote tumor cell invasion and angiogenesis. Finally, integrins

mediate cell-cell interactions and favor tumor cell extravasation.

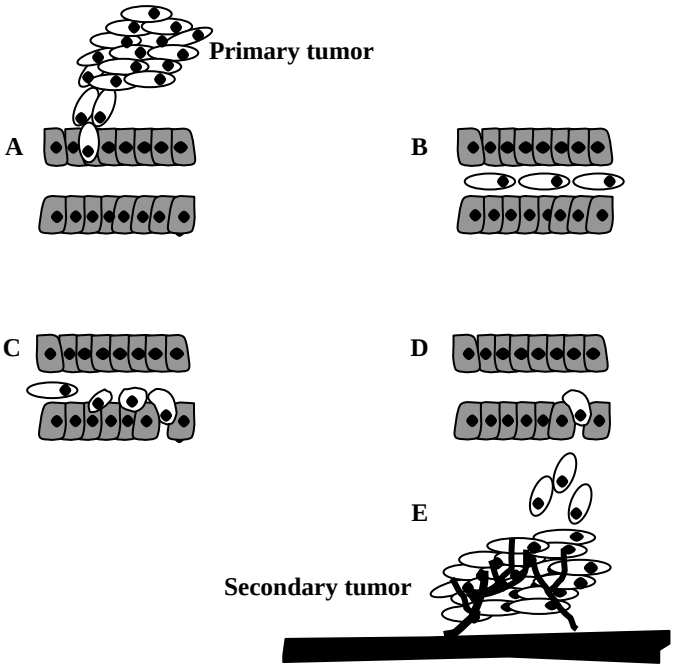


Figure 7: Metastatic cascade

A. **Release of cancer cells from a primary tumor, passage through the endothelial cell layer and entry into the blood or lymphatic system**
B. **Migration in the blood or lymphatic system**
C. **Arrest at the proximity of target organs**
D. **Extravasation**
E. **Development of metastatic colonies and tumor-induced angiogenesis**

This involvement of the integrins in metastatis is greatly mediated by changes in levels of integrin expression and integrin affinity for ECM substrates. Numerous studies have shown marked differences in surface expression and distribution of integrins in malignant tumors compared with pre-neoplastic tumors of the same type (24). For example, the integrin αvβ3 is strongly expressed at the invasive front of malignant melanoma cells and angiogenic blood vessels, but only weakly on pre-neoplastic melanomas and quiescent blood vessels. Furthermore, inducing the

expression of the αv or β3 integrin subunit in a melanoma cell line increases the metastatic potential. On the other hand, increasing the affinity of integrins for their ligand is another mechanism by which cells can alter their adhesive profile to assume a more migratory phenotype. For instance, αvβ3 affinity has been reported to be modulated in a number of cells, and selectively blocking high-affinity αvβ3 has been shown to impair the directed migration of endothelial cells (25).

Therapeutics that are designed to modify cellular invasive and migratory activities might be useful in treating cancer-cell metastasis and furthermore angiogenesis and inflammatory disease. Over the past several years, research has led to the development of integrin and protease inhibitors that are now being tested in clinical trials.

First results of human cancer trials with Vitaxin (Medimmune/IXSYS), a humanized monoclonal antibody to integrin αvβ3 have been described (26): 8 of the 14 treated, late stage cancer patients showed a disease stabilization and/or some objective tumor reduction with no evidence of toxicity in many of these patients. Vitaxin is now in phase II clinical trials against leiomyosarcoma, a soft cell cancer. Antibodies with improved binding characteristics compared to Vitaxin were obtained by antibody engineering and are being tested.

6. Conclusion

Considerable effort has already been put into the elucidation of the functioning of integrins. The structural information which has been provided recently has contributed to a far better understanding of these important receptors. The gain of further insights will surely contribute to the development of more effcient therapeutic

agents targeting integrins in human diseases as diverse as thrombosis, inflammation, atherosclerosis, osteoporosis, cancer and infectious diseases.

Acknowledgments

I thank Dr Nelly Kieffer for critical reading of this manuscript and for helpful suggestions. I would also like to thank the journal "Science" as well as Dr Armin Arnaout for their kind permission to reproduce the crystal structure of integrin αvβ3 in this review.

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