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Article *in* Annals of Medicine · February 2006

DOI: 10.1080/07853890600969130 · Source: PubMed

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TRENDS IN MOLECULAR MEDICINE

Interfering with leukocyte integrin activation—a novel concept in the development of anti-inflammatory drugs

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Abstract
Inflammation is a crucial response against invading pathogens, in which immune cells, including neutrophils and T cells, are recruited into tissue from the bloodstream to help clear infection. However, a prevailing inflammatory response where the immune cells attack healthy tissue is associated with many diseases, including asthma, rheumatoid arthritis, atherosclerosis and multiple sclerosis. Integrins are key players in the recruitment of immune cells from the bloodstream into tissues, and are thus therapeutic targets for intervention with inflammatory responses. Thus far, mainly extracellularly acting therapeutics (monoclonal antibodies) have been developed against integrins, targeting ligand binding sites in these heterodimeric adhesion receptors. However, since these therapeutics nonselectively block all integrin functions, some side effects are expected and have been observed. Therefore, novel concepts need to be developed in the therapeutic targeting of integrins. Recently, major advances have been made in the understanding of integrin biology. Integrin structures have been solved by X-ray crystallography, revealing unexpected data about the activation mechanism of integrins in cells. Additionally, several intracellular factors in the integrin activation process have been identified, providing potential specific targets for therapeutic intervention. Here, we present key events and players in leukocyte integrin activation, and discuss potential new drug targets in the prevention of inflammatory disease.

Key words: *Activation, inflammation, integrin, leukocyte, phosphorylation, therapy*

Introduction

Inflammation is crucial in the response to tissue injury and host defense against invading microbes. Leukocytes are key players in the inflammatory response, and thus they are potential targets for the discovery of novel therapeutics. Leukocytes circulate throughout the body as nonadherent cells, becoming transiently adherent after activation before transmigration through the endothelium into tissues, as well as when they encounter antigen-presenting cells in lymph nodes. The accumulation of leukocytes in particular target organs or tissues contributes to a wide variety of diseases, including asthma, rheumatoid arthritis, atherosclerosis, multiple sclerosis and Crohn's disease (1).

Leukocyte trafficking and adhesion are mediated mainly by the integrin family of cell surface receptors and their ligands, the intercellular adhesion molecules (ICAMs) (2–4). The adhesive activity of leukocyte integrins must be tightly regulated, since deregulation of integrin function could lead to autoimmune diseases and hypersensitivity reactions. Leukocyte integrins on resting cells are not able to bind their ligands, but when the cell has received an activating stimulus they become adhesive. This normally occurs through 'inside-out' signaling or activation, and it occurs through two modes; affinity modulation, where ligand-binding is altered by conformational changes, and avidity or valency modulation, which modifies integrin diffusion and clustering in the membrane that is mediated by cytoskeletal interactions (5,6). Different modes of activation seem to use different mechanisms at the molecular level.

As leukocyte adhesion is a fundamental process in immune function, it holds considerable promise as a source of novel and potent targets for the treatment

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ISSN 0785-3890 print/ISSN 1365-2060 online
DOI: 10.1080/07853890600969130

Abbreviations

Arf-GAP	ADP-ribosylation factor GTPase-activating protein
ECM	extracellular matrix
GTP	guanosine triphosphate
ICAM	intercellular adhesion molecule
LAD-1	leukocyte adhesion deficiency-I
MMP	matrix metalloprotease
Rac	Ras-related C3 botulinum toxin substrate
RhoA	Ras homolog gene family, member A
TCR	T cell receptor
VCAM-1	vascular cell adhesion molecule-1

of inflammation and autoimmune diseases. Leukocyte integrins are of particular interest in this regard, as they are key molecules in many immunological events. While there has been long-standing interest in integrins as a therapeutic target for regulating immunity, only few therapeutic approaches have completed clinical trials (7). Anti-inflammatory agents frequently have limited efficiency and severe unexpected side effects and, thus, more specific blockers of the inflammatory response are required. The understanding of leukocyte integrin structure and activation mechanisms has grown substantially during the last few years, giving us a novel concept in the search for more selective and potent anti-inflammatory drugs.

Key messages

- Integrins are key players in leukocyte recruitment from the bloodstream into the site of inflammation, and have therefore been primary targets for therapeutic intervention in the inflammatory process. So far, mainly monoclonal antibodies targeting integrin extracellular domains have been developed.
- Recently, major advances in the understanding of integrin activation have been made. Cytoplasmic factors involved in integrin activation include talin, which induces conformational changes in the integrin intra- and extracellular domains, and seems to play a role in the activation of most integrins. More specific factors for regulation of individual integrins in leukocytes include 14-3-3 proteins binding to phosphorylated $\beta 2$ integrins, paxillin binding to $\alpha 4$ integrins and the Rap1/RAPL/αL system, which seems to work partly through the αL-phosphorylation site Ser1140.
- Especially the specific factors for regulation of individual integrins in leukocytes may provide new, specific targets for therapeutic intervention with inflammatory diseases.

Leukocyte recruitment

The recruitment of leukocytes from blood is crucial in the inflammatory reaction. It occurs through a multistep process, involving tethering and rolling of leukocytes on the blood-vessel wall, firm adhesion and crossing through the endothelial barrier (8) (Figure 1). Activated endothelial cells actively participate in the process by expressing an array of important molecules, such as selectins, chemokines and integrin ligands on their surface. Initial tethering and rolling of leukocytes along the endothelium is mainly mediated by the interaction of selectins with their ligands (9). The next step of leukocyte migration is firm attachment of leukocytes to the endothelium. The most important molecules in this process are leukocyte integrins that bind to their ligands expressed on activated endothelial cells (10,11). The firm cell attachment is followed by migration along the endothelium and invasion between neighboring endothelial cells or directly through the cells (12). Importantly, extensive animal and preclinical studies have indicated that inhibition of leukocyte recruitment can be a highly effective and selective anti-inflammatory therapy (13).

Leukocyte integrins

Integrins mediate the attachment of cells to each other and to the surrounding extracellular matrix, and participate in diverse events such as immunity, hemostasis, development and cancer. Integrins are heterodimeric receptors formed by the noncovalent association of α- and β-subunits. Leukocytes can express at least 12 of the 24 known integrin heterodimers, and the expression pattern depends on the subset and the maturation state of the cell (4). The most relevant integrins for leukocyte migration are members of the $\beta 2$ integrin family, especially $\alpha L\beta 2$ (CD11a/CD18 or LFA-1) and $\alpha M\beta 2$ (CD11b/CD18 or Mac-1), and the two $\alpha 4$ integrins, $\alpha 4\beta 1$ (VLA-4) and $\alpha 4\beta 7$. $\beta 2$ Integrins interact primarily with intercellular adhesion molecules (ICAMs) but they also have other ligands (3). Especially $\alpha M\beta 2$ binds to a wide range of ligands, including the blood coagulation protein factor X (14), fibrinogen (15)

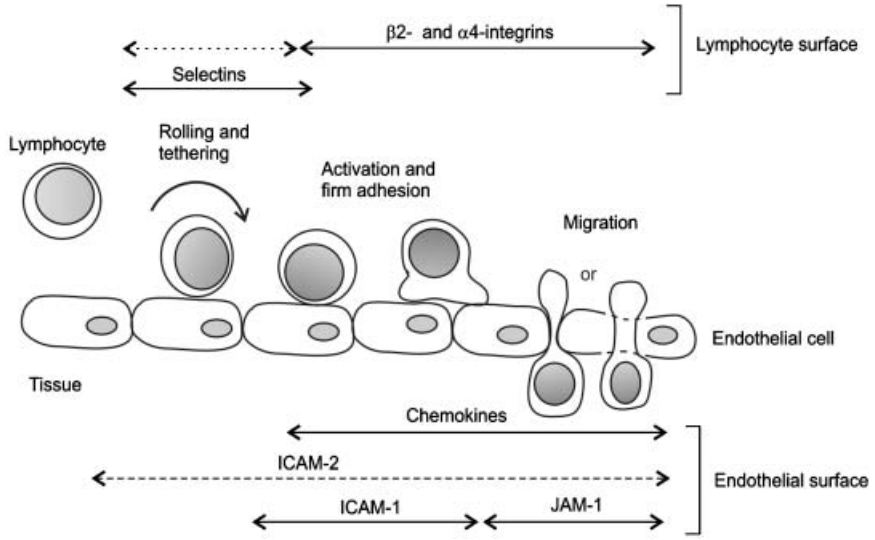

Figure 1. Leukocyte translocation to extravasal tissues. Sugar-binding selectins are the main molecules, which mediate leukocyte tethering and rolling. This event is followed by firm adhesion mediated by leukocyte integrins and their ligands (ICAMs). The migration into tissues may take place between the endothelial cells and possibly through these cells and involve integrins and intracellular signaling.

and the complement protein iC3b (16). α4 Integrins are expressed predominantly on lymphocytes, monocytes, and eosinophils but only in small amounts on neutrophils (17). The α4 integrin interaction with vascular cell adhesion molecule-1 (VCAM-1) or MAdCAM-1 promotes firm adhesion of lymphocytes to the endothelium in preparation for the transmigration of the lymphocyte. Leukocytes also express trace amounts of αEβ7 integrins and, in common with many other cell types, extracellular matrix (ECM)-binding β1 integrins (α1-α6β1).

The physiological importance of β2 integrins in leukocyte function has been verified by the study of a naturally occurring human disease, leukocyte adhesion deficiency-I (LAD-I) (18). LAD-I is a rare inherited immunodeficiency in which β2 integrin expression is diminished or lost. The lack of β2 integrins results in elevated numbers of circulating neutrophils because the cells fail to migrate across the endothelium. Patients typically have recurrent bacterial or fungal infections, impaired mobilization of leukocytes to infected areas, severe gingivitis, and impaired tissue remodeling and wound healing. Severely affected people often die of infections in early childhood unless bone marrow transplantation is successfully accomplished. A few variant LAD-I syndromes have also been identified, where the expression levels of β2 integrin were normal, but integrins were nonfunctional, indicating a defect in inside-out signaling (19,20).

Also targeted gene deletions ('knockouts') of leukocyte integrin genes in mice show the physiological importance of integrins. β2 Knockout mice display similar phenotypic features as humans with LAD-I, including neutrophilia and defects in the accumulation of neutrophils into inflamed skin (21). α4 Knockout mice are embryonic lethal, because of major developmental defects (22).

Integrin structure and activation

Recent studies have provided important details of integrin structure and conformational changes during activation, thereby facilitating drug development. The integrin polypeptides consist of a large (>700 residue) extracellular domain, a single transmembrane domain and a short cytoplasmic domain (Figure 2). The overall shape of the integrin extracellular domain shows a globular amino-terminal 'headpiece' that binds to the ligand, and

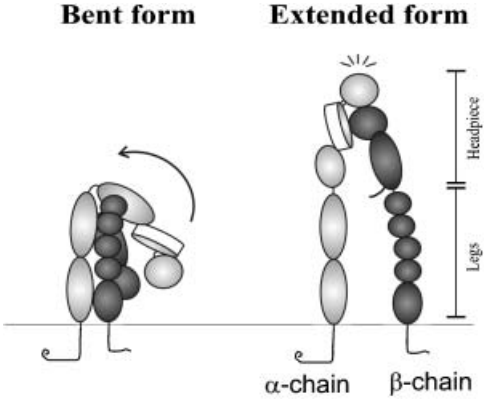

Figure 2. A model for integrin activation. In the resting state the integrins show a bent conformation and upon activation the molecules take an extended structure. The ligand binding site is at the top of the molecular complex.

two long rod-like 'legs' connecting the head to the transmembrane and cytoplasmic domains (23). β2 Integrins, and certain other integrins, contain an additional I-domain in the α chains, which is the major ligand-binding domain (24). The crystal structure of the extracellular domains of $\alpha V\beta 3$ revealed an unexpected bent conformation of the integrin, where the head domain was folded down onto the legs. The bent conformation was proposed to represent a low-affinity state of the integrin, and a switchblade-like opening was proposed to occur upon activation (25,26). In the bent conformation only the closed conformation of the headpiece (I- or I-like domain) is present and the extension reorients the ligand-binding face to the open conformation (25).

Integrin cytoplasmic domains are normally <50 amino acids in length, with the β-subunit sequences exhibiting greater homology to each other than the α-subunits. The membrane-proximal parts of the integrin intracellular domains have been shown to be α-helical and interact with each other, while the rest of the tails are disordered in the aqueous environment (27,28). A model for integrin activation was proposed, where interaction of the integrin β chain tail with an effector (the so-called head domain from the cytoskeletal protein, talin, see below), would lead to separation of the two cytoplasmic domains and upward movement of the two cytoplasmic domains of the integrin (27,29). This would allow structural changes to be transmitted to the extracellular domains.

However, several stimuli that induce increased ligand-binding do not involve integrin affinity changes, indicating that also other processes are involved (5,30). Affinity-independent mechanisms involve integrin clustering, resulting in increased avidity and providing stronger adhesion at sites of cell-cell contact, and also interactions with and reorganization of the cytoskeleton. Although the integrin α and β cytoplasmic domains are relatively short and do not contain any intrinsic enzymatic activity, they are important both for conformational changes and avidity regulation.

Factors involved in the activation of integrins

Several intracellular proteins take part in the activation of integrins (Figure 3). Some of these proteins are cytoskeletal actin-binding proteins such as talin and filamin, adaptor proteins such as the 14-3-3 proteins and others signaling proteins such as Rap-1 (31). β Chain cytoplasmic tails play important roles in cytoskeletal interactions, while the more divergent α chain cytoplasmic domains in most cases play regulatory roles (32). The binding between integrins and the actin cytoskeleton is crucial for several cell functions; this interaction is especially needed for cell migration and adhesion (33). Since actin filaments cannot directly bind to integrins, the binding takes place via actin-binding proteins, including talin and filamin. Talin is an abundant cytoskeletal protein that binds to β1A, β1D, β2 and β3 integrin tails and weakly to the β7 tails (34–36). The so-called head domain of talin causes integrin activation in many systems; for example, its binding to the β2 chain causes spatial separation of the αL and β2 cytoplasmic domains, thus resulting in integrin activation (37). It has indeed been proposed that the talin-binding is the final common step in integrin activation. In addition to the activating function, talin is critical for localization of the active β2 integrin in cells, ICAM-binding stability and T cell migration (38).

Another actin-binding protein, filamin, has also been shown to bind several integrins. Filamin crosslinks actin, and, in addition, filamin acts as an adaptor protein for numerous signaling proteins that can regulate cytoskeletal dynamics (39). Binding of filamin to β integrin tails has been shown to regulate cell migration and this regulation seems to be integrin-specific, since the β7 tail, which binds strongly to filamin, inhibited cell migration, whereas β1 tails that bind weakly to filamin supported cell migration (40). It has recently been shown that the filamin and talin binding sites in the β tails partially overlap (41). This overlapping causes a competition of binding between talin and filamin, allowing the integrin-filamin interaction to affect the talin-dependent integrin activation. However, the precise functions of filamin in integrin regulation in cells remain elusive.

The 14-3-3 proteins are abundant regulatory proteins which bind to phospho-serine and phospho-threonine containing motifs. They have been shown to bind the β2 integrin specifically through the phosphorylated Thr758, and influence cell adhesion through a mechanism not involving integrin affinity (42) (see below). Also, 14-3-3 proteins have been reported to bind to β1 integrin tails; however, this interaction does not happen via a phosphorylated residue (43).

Rap1 is a small GTPase which has been shown to be involved in integrin regulation in the cases of the $\alpha L\beta 2$, $\alpha M\beta 2$ and $\alpha 4\beta 1$ integrins (44–46). RAPL, a Rap1-binding protein, binds to active Rap1-GTP upon cell stimulation with chemokines or through the T cell receptor (TCR). In the case of $\alpha L\beta 2$, the activated RAPL then forms a complex with the α chain and may in this manner mediate

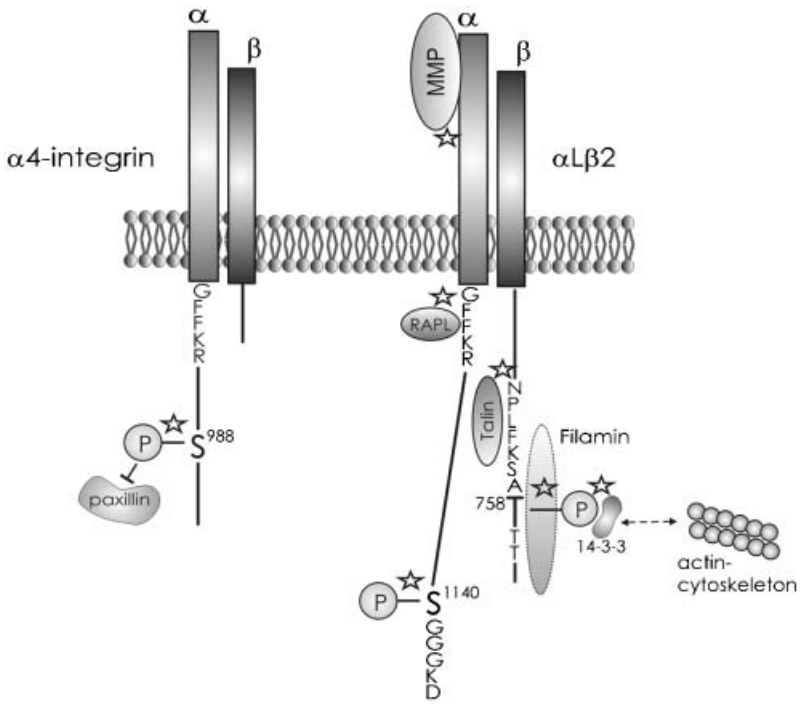

Figure 3. Important sites and molecules involved in integrin activation. Paxillin-binding to the α4 cytoplasmic tail is regulated by phosphorylation of α4, and this interaction regulates cell migration and adhesion. Phosphorylation of Ser-1140 in αL is needed for conformational changes resulting in affinity increase, whereas Thr-758 of β2 phosphorylation is needed for increased avidity. 14-3-3 Proteins bind to Thr-758 phosphorylated integrin. Talin, filamin and RAPL bind also to αLβ2 integrin and are involved in integrin regulation. Metalloproteases (MMP-2 and -9) are associated with the β2 integrin extracellular part. MMPs mediate the proteolysis needed for invasion. Potential targets of the therapeutics are marked by asterisks and discussed in the main text.

Rap-1-induced cell adhesion. Overexpression of RAPL increases the adhesiveness of β2 integrin (44) whereas T or B cells isolated from RAPL knock-down mice are much less adherent to the ICAM-1 (47).

Phosphorylation of integrin cytoplasmic domains

As mentioned above, numerous cytoplasmic proteins bind to the intracellular tails, and thus, spatiotemporal regulation of these interactions is necessary. The phosphorylation of integrin cytoplasmic domains is emerging as a mechanism of regulating integrin functions (48). Both integrin β chains and integrin α chains contain multiple phosphorylatable sites, and have been reported to be phosphorylated in cells (48). Traditionally, the β chain phosphorylations on serine, threonine and tyrosine residues have been much more studied than α chain phosphorylation, mainly due to the fact that the β chain phosphorylations are generally induced by stimuli that induce activation of integrins, such as phorbol esters. This intuitively led researchers to hypothesize that β chain phosphorylations may regulate integrin activation. The α chain phosphorylations were long overlooked as a mechanism of regulating integrins, but lately, they have emerged as novel, surprising regulators of integrin activation, as well as other specific integrin functions.

Phosphorylation of integrin β chains

The β1 and β3 integrins become phosphorylated on tyrosine residues as a consequence of 'outside-in' signaling, whereby ligands bind to integrins, causing integrins to signal into the cell. The β2 integrins, in contrast, are phosphorylated on several serine and threonine residues after cell activation with phorbol esters, TCR ligation or outside-in signaling after ICAM-binding. Thr758 is an important phosphorylation site in the β2 cytoplasmic domain (49,50), and it is included in a region which spans the binding sites of several intracellular molecules, like filamin and 14-3-3-proteins. Recently, 14-3-3 binding to the integrin Thr758-phosphorylated β2 chain has been shown to mediate actin reorganization and cell spreading of T cells, a process that is essential for T cells to bind to target cells (42). This binding takes place after inside-out activation through the T cell receptor, resulting in increased adhesion, but it does

not involve talin-binding or affinity changes in the integrin (42). In β7, it has been shown that phosphorylation of these threonines regulates filamin-binding by inhibiting the interaction with the threonine phosphorylated β7 tail, whereas talin-binding was unaffected (41). Downstream effectors of the 14-3-3-β2 complex are still to be elucidated. However, the TTT sequence has been shown to be important for RhoA (Ras homolog gene family member A)-activation in phagocytes, which mediates phagocytosis by iC3b-coated particles binding to αMβ2 integrins (51).

Phosphorylation of integrin α chains

αL, αM and αX have all been shown to be phosphorylated in human leukocytes, mainly on serine residues. The role of αL phosphorylation in integrin-mediated functions in T cells has recently been reported (42). Phosphorylation on Ser1140 is essential for adhesion events which involve a change in integrin conformation/affinity for its ligands. Stimulation of cells with chemokines, ligands or activating antibodies thus cannot activate αL integrins with the phosphorylation site mutated. Notably, T cell stimulation through ligation of the TCR or with phorbol ester, however, results in normal adhesion to ICAM-1. The mechanisms involved in αL regulation by phosphorylation remain to be elucidated; however, it seems like the Rap1/RAPL system may be involved, since a constitutively active form of Rap1 failed to activate the S1140A-mutated integrin in cells.

α4 Integrins are also constitutively phosphorylated (48). Protein kinase A mediates the phosphorylation, and the phosphorylation site mediates regulation of paxillin-binding to the integrin cytoplasmic domain. The phosphorylated integrin does not bind paxillin, and localizes to the leading edge of the migrating cells, thereby mediating lamellipodial stability and cell migration. The molecular mechanism of this phenomenon has been elucidated; the α4-integrin-paxillin complex inhibits Rac (Ras-related C3 botulinum toxin substrate) activation at other places in the cell than at the leading edge, by recruiting an Arf-GAP (ADP-ribosylation factor GTPase-activating protein) that reduces Rac activity (52). Interestingly, integrin adhesiveness is also regulated by the α4-paxillin interaction, since a mutation that blocks paxillin association with the α4 chain also reduces integrin-dependent capture of cells and adhesion-strengthening under shear stress, even if adhesion under static conditions is normal (53).

Therapeutic targeting of leukocyte integrins

Because the leukocyte integrins are involved in numerous leukocyte functions, and several diseases are due to excessive leukocyte activity, it is obvious that therapeutic measures have been taken with the aim to inhibit integrin function. Early on several monoclonal antibodies have been available, which efficiently inhibit human leukocyte adhesion. They can either be directed to a common β chain or to the different α chains.

In several disease models in animals, promising results were obtained and inflammatory reactions were often abrogated. Such diseases include asthma, reperfusion syndromes, arthritis, neuroinflammatory diseases, autoimmune diabetes etc. (54–58). Furthermore, antibodies were used to inhibit rejection of organ transplants (59,60). By 'humanizing' mouse antibodies, whereby most parts of the antibodies were replaced by human Ig-sequences, antibodies were obtained, which retained their antibody activities but had lost most antigenic epitopes. Such antibodies have now been applied in the treatment of numerous human inflammatory diseases. The results from these studies have largely been disappointing (7). However, one should bear in mind that the clinical studies need several years of work and it may, for example, be difficult to find the optimal antibody concentrations to obtain clinically useful responses. An obvious problem exists, however, that by wiping out essential leukocyte functions serious infections and malignant diseases may result.

However, some promising results have been obtained, notably in the treatment of psoriasis, multiple sclerosis, Crohn's disease and ulcerative colitis (13). The results from trials of treatment of psoriasis with anti-αL antibodies are definitely promising (61). Multiple sclerosis has been successfully treated with α4 antibodies, but in a large clinical trial a few cases of progressive multifocal leukoencephalopathy were seen and therefore the treatment was discontinued (62–64). We can, however, anticipate that useful clinical protocols, at least for some inflammatory diseases, will be obtained with time. Due to some of these negative side effects, it would also be useful to develop novel approaches to integrin targeting in order to obtain increased specificity of the therapeutics.

A novel concept of therapeutic targeting

The function of leukocyte integrins can be modulated by competitive blockade with antibody, but also by interference with receptor activation and other integrin functions intracellularly. A number of studies show that integrins are activated by several

modes, as discussed above. Since the different activation modes seem to use different mechanisms at the molecular level, interference with the final steps of activation process could give new prospects for therapeutic intervention (Figure 3). Additionally, it would be important to consider factors that are specific only to certain integrins, in order to avoid targeting several or all integrins with the therapeutics.

Integrin β chain cytoplasmic domains are closely related to each other, and thus it may be expected that targeting some of the β chain interactions with activating factors will in fact affect many different integrins, thus not achieving therapeutic specificity. This may be the case for the integrin-talin interaction. Also filamin has been shown to bind to integrin and this binding regulates cell migration (40). This might be a potential therapeutic target affecting leukocyte migration and recruitment; however, also filamin binds to several different integrin β chains, and the molecular mechanisms of filamin regulation of integrins are still not fully understood.

Integrin phosphorylation on the β chain cytoplasmic domains at different residues has recently been shown to be essential for integrin-mediated adhesion and other integrin-dependent functions. This could enable the development of drugs interfering with these phosphorylation reactions directly and specifically. One possibility would be to inhibit the β2 -14-3-3 interaction, which is possible with peptides that block 14-3-3 interactions with its cellular ligands by binding to the phosphopeptide-binding groove in 14-3-3 (65). Since 14-3-3 proteins bind to numerous cellular targets, more specificity could be achieved by developing reagents, which bind the phosphorylated Thr-758 site in β2, thus blocking 14-3-3 binding, or kinases responsible for integrin phosphorylation. Such reagents should be highly specific, since blocking of 14-3-3-target interactions do not interfere with adhesion of β1 integrins to fibronectin, but severely affects β2 integrin-mediated adhesion to ICAM-1 (42).

Integrin α chain cytoplasmic domains and factors that work through the α chains could also be therapeutic targets, since they are quite divergent in structure and presumably mediate integrin functions, which are specific for each integrin. Rap1 has emerged as an important regulator of leukocyte integrin adhesiveness (66), and it is believed to act at least in part through RAPL, which binds to the αL chain of a leukocyte integrin. This interaction gives a potential specific target of anti-inflammatory agents. Similarly, it could be possible to make other α chain-specific reagents. We now know that the Ser-1140 phosphorylation of the integrin αL chain is needed for affinity increases in the αLβ2 integrin after chemokine- and ligand-induced activation (42) and, interestingly, a similar result has also been obtained for the αM phosphorylation site (67). Since the αL and αM phosphorylation sites are only highly specific, elucidating the molecular pathways involved in these activation events may provide exciting new targets for drug development specific for each of these integrins.

Paxillin-binding to the α4 cytoplasmic tail is regulated by phosphorylation of α4 (68), and very recently, a mouse bearing a mutation of the paxillin binding site in α4 has been reported (69). Interestingly, this mouse is phenotypically normal except for a leukocyte migration deficiency into sites of inflammation. Other α4 integrin dependent functions, like hematopoiesis, were retained. This finding also suggests that agents directed against integrin signaling could be developed as useful agents for antiadhesive therapy.

Leukocyte motility is also known to be dependent on the matrix metalloproteases MMP-2 and MMP-9, which have been shown to bind to leukocyte β2 integrin extracellular domain at the cell surface (70,71). MMPs mediate the proteolysis needed for invasion. Recently peptides derived from the MMPs' catalytic domain have been developed which interfere with this integrin-MMP interaction. Such peptides efficiently inhibit leukocyte movement and could turn out useful in clinical medicine (72).

Obviously, we are just in the beginning of an exciting period, when highly specific molecular probes are becoming available and such novel reagents could exhibit the high activity and specificity that is needed for successfully interfering with leukocyte adhesion and function.

Acknowledgements

The original work in the authors' laboratory has been supported by The Academy of Finland, the Sigrid Juselius Foundation, the Finnish Cancer Society, the Magnus Ehrnrooth Foundation and Medicinska understödsföreningen Liv och Hälsa.

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