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Disintegrins: integrin selective ligands which activate integrin-coupled signaling and modulate leukocyte functions

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Abstract

Extracellular matrix proteins and cell adhesion receptors (integrins) play essential roles in the regulation of cell adhesion and migration. Interactions of integrins with the extracellular matrix proteins lead to phosphorylation of several intracellular proteins such as focal adhesion kinase, activating different signaling pathways responsible for the regulation of a variety of cell functions, including cytoskeleton mobilization. Once leukocytes are guided to sites of infection, inflammation, or antigen presentation, integrins can participate in the initiation, maintenance, or termination of the immune and inflammatory responses. The modulation of neutrophil activation through integrin-mediated pathways is important in the homeostatic control of the resolution of inflammatory states. In addition, during recirculation, T lymphocyte movement through distinct microenvironments is mediated by integrins, which are critical for cell cycle, differentiation and gene expression. Disintegrins are a family of low-molecular weight, cysteine-rich peptides first identified in snake venom, usually containing an RGD (Arg-Gly-Asp) motif, which confers the ability to selectively bind to integrins, inhibiting integrin-related functions in different cell systems. In this review we show that, depending on the cell type and the microenvironment, disintegrins are able to antagonize the effects of integrins or to act agonistically by activating integrin-mediated signaling. Disintegrins have proven useful as tools to improve the understanding of the molecular events regulated by integrin signaling in leukocytes and prototypes in order to design therapies able to interfere with integrin-mediated effects.

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Integrins and signal transduction

Extracellular matrix (ECM) proteins and integrins play essential roles in the regulation of cell adhesion and migration (1). Integrins are important components not only for the structure and architecture of tissues but also for signal transduction leading to regulation of many biological functions in cells. These proteins are connected to intracellular proteins that include diverse signaling molecules recruited to sites of focal adhesion,

and are also linked to the actin cytoskeleton (2). These heterodimeric receptors are composed of an α and a ß subunit, with a long extracellular domain binding to the ECM, a transmembrane domain, and a short cytoplasmic domain that associates with the actin cytoskeleton and adaptor proteins (3). At least 18 distinct α subunits and 8 or more ß subunits of the integrin receptor family have been cloned in mammals, leading to the generation of 24 distinct αß heterodimeric receptors (4). Recently, two types of signaling by integrins have been extensively discussed: transmission of signals into the cell following binding of ligands or counter-receptors to the integrins (outside-in signaling), and regulation of the avidity and conformation of integrins by signals generated by other receptors within the cell (inside-out signaling) (5). Interaction of integrins with the ECM proteins can induce tyrosine phosphorylation of many intracellular proteins.

Integrin clusters in macromolecular complexes, ligand occupancy, and tyrosine phosphorylation are the key events that result in diverse processes such as cell migration and differentiation, tissue remodelling, cell proliferation, angiogenesis, and tumor cell invasion and metastasis (3,6). Focal adhesion kinase (FAK) becomes tyrosine-phosphorylated during integrin-mediated cell adhesion and is believed to play important roles in integrin signal transduction (7,8). FAK, a non-receptor tyrosine kinase, interacts with a pool of intracellular signaling proteins, including c-Src, phosphatidylinositol 3-kinase (PI3-K), Rho GTPase family members, Grb2, and $p130^{CAS}$ (6). *In vitro* studies have suggested that FAK-generated signaling is involved in cell survival (9) and seems to be essential for development. The developmental defect observed in FAK-deficient mice may be associated with impairment of cell migration and enhancement of cell adhesion, as suggested by studies with FAK-/- fibroblasts (10). FAK can be phosphorylated on different tyrosine residues. Phosphorylated Tyr^{397} conforms to the consensus binding for SH2 domains for c-Src (11) and the p85 regulatory subunit of PI3-K (6). It was also shown that the Src interaction with FAK leads to phosphorylation of FAK at several sites including Tyr^{407}, Tyr^{576}, Tyr^{577}, Tyr^{861}, and Tyr^{925}, which then results in the adaptor protein Grb2 binding to FAK at residue Tyr^{925} (12). Phosphorylation at Tyr^{925} may result in Grb2 adaptor protein binding to FAK, which in turn may activate the Ras/Raf/mitogen-activated protein kinase (MAPK) pathway (13).

Integrin-mediated cell adhesion can activate MAPK including Erk1/2, c-Jun kinase, and p38. Activation of the Erk1/2 pathway by cell adhesion provides a common route leading to transcriptional regulation of certain genes which are critical for cell growth, differentiation and apoptosis (14). Integrin-mediated c-Jun kinase activation is dependent on FAK and is involved in cell cycle progression, cell spreading (15) and cell survival (16). Activation of p38 MAPK by integrins has been reported to regulate collagen gene expression (17) and cell motility. Integrin-mediated cell adhesion also stimulates Rho family members including RhoA, Rac1, and CDC42. After ECM engagement, integrins transduce intracellular signaling cascades that regulate the formation, turnover, and linkage of actin filaments. During early adhesion, cells develop membrane protrusion, whereby integrin-mediated adhesions are associated with the development of tension, activation of Rac1 and CDC42, and actin polymerization. The later adhesion/spreading phase involves RhoA activation and increased contractility (15). In this respect, integrin-mediated activation of Rho family GTPases is critically important in actin rearrangements (18). Cumulative evidence has shown that integrin-mediated activation of RhoA, Rac1, or CDC42 rearranges the actin cytoskeleton network, leading to morphological features of stress fiber formation and focal adhesion turnover, and

the formation of lamellopodia or filopodia, respectively (19). Furthermore, small GTPases influence diverse cellular processes, including cell growth, cell cycle progression and differentiation (18). An important FAK-regulated signaling route is the PI3-K pathway (11). Activated FAK associates with PI3-K through Tyr^{397}, phosphorylating the p85-subunit and activating PI3-K (6,20). PI3-K activation is a common signal transduction event in a remarkable variety of functional responses in different cells. PI3-K-effector proteins are involved in the regulation of integrin-mediated leukocyte migration and immune cell proliferation, differentiation and survival (21). Since tyrosine phosphorylation regulates the catalytic activity of FAK and its association with other signaling molecules, dephosphorylation of tyrosine residues is a potentially important mechanism for the regulation of FAK signaling. Compelling evidence has shown that Shp-2 might regulate FAK tyrosine phosphorylation while phosphatase and tensin homolog deleted on chromosome ten (PTEN) catalyzes FAK dephosphorylation (11). Since different phosphorylation sites function to regulate the catalytic activity and protein-protein interaction, site-specific dephosphorylation of FAK may be an effective mechanism to modulate FAK-dependent integrin signaling.

Disintegrins

A significant development in the study of integrin ligand interactions was the discovery, originally in snake venoms, of potent antagonists of platelet aggregation and integrin cellular adhesive functions, denominated disintegrins (22). The disintegrins are a family of low-molecular weight, cysteine-rich peptide which usually contain the Arg-Gly-Asp (RGD) motif within an amino acid hairpin loop maintained by disulfide bridges in a single-chain (monomeric disintegrins) (23). This tripeptide sequence is the cell attachment site, recognized by integrins, present in many adhesive ECM and cell surface proteins (24). The RGD motif confers to disintegrins the ability to selectively bind to integrins, inhibiting integrin-related functions in different cell systems (23). Despite their close similarities, each disintegrin has a unique RGD-containing loop and distinct biological activities. Most of them are several times more potent as inhibitors of cell adhesion than synthetic linear RGD peptides (23). This higher potency may be due to conformational restraints imposed on the RGD tripeptide and to the arrangement of disulfide bonds, indicating the importance of tertiary structure (23-25). The difference in biological activities and ligand-binding selectivity of disintegrins may reflect differences in the amino acid sequences adjacent to their "RGD loop" (23-25). Although in most peptides the active sequence is the RGD tripeptide, other sequences such as KGD, MVD, MLD, VGD, ECD, or MDG have been identified as integrin-binding motifs in snake venom disintegrins (23).

Most disintegrins identified thus far are potent inhibitors of platelet aggregation, acting as competitive inhibitors of fibrinogen binding to the $\alpha II_b\beta_3$ integrin on ADP-activated platelets (26,27), and can also inhibit tumor cell adhesion and metastasis, impairing integrin-mediated cell adhesion onto selective ECM proteins (28). Because of these properties, disintegrins have been considered to be potent competitive antagonists of integrin-dependent cell adhesive functions (27).

Monomeric RGD-disintegrins such as eristostatin, echistatin, kistrin, and flavoridin, able to bind to $\alpha II_b\beta_3$, have been reported to be potent inhibitors of platelet aggregation and tumor cell metastasis (29,30). Disintegrins, ligands of $\alpha_5\beta_1$ and $\alpha v\beta_3$, have been shown to block endothelial cell adhesion to matrix proteins (31), to inhibit angiogenesis and to induce apoptosis of endothelial cells (32,33). Alternagin-C (Alt-C), a disintegrin-like protein containing the ECD amino acid

sequence as an integrin-binding motif, recognizes $\alpha_2ß_1$ integrin and inhibits fibroblast adhesion to collagen (34). Contortrostatin, a dimeric RGD-disintegrin, is a potent inhibitor of angiogenesis and breast cancer progression (35,36). Heterodimeric disintegrins such as EC3 and VLO5 carry the VGD motif in the A-subunit and the MLD tripeptide sequence in the B-subunit and are selective and potent inhibitors of $\alpha_4ß_1$ and $\alpha_9ß_1$ integrin-mediated effects (37,38).

Although previously considered to be passive integrin-blocking agents, the disintegrins are capable of interacting with and activating integrin-signaling pathways. Disintegrin-induced activation is usually dependent on the cell type and on the surrounding environment encountered by a given cell. Echistatin, an $\alpha_5ß_1$ and $\alpha vß_3$ ligand, in its soluble form induced detachment of fibronectin-adherent melanoma cells by down-regulating FAK and actin cytoskeleton disassembly (39). In contrast, immobilized echistatin induced platelet adhesion and increase tyrosine phosphorylation (40). Contortrostatin, a homodimeric RGD-disintegrin, induced tyrosine phosphorylation of FAK and CAS in tumor cells, while monomeric disintegrins, echistatin and flavoridin, had no effect. The authors impute the unique effect of contortrostatin to its homodimeric structure (41). Recently, it was shown that Alt-C, an ECD-disintegrin-like protein, strongly activates the PI3-K-Akt pathway and induces endothelial cell proliferation (42).

Leukocytes and integrin signaling

The adhesion process onto biological surfaces driven by cell adhesion molecules is a powerful activator of leukocytes. Integrins have long been recognized as the dominant family of cell adhesion receptors involved in leukocyte interactions with endothelium and ECM (43). Integrin-signaling pathways mediate important functions in leukocytes, including migration, spreading, activation of the respiratory burst, complement binding, cell adhesion, cytokine and chemokine expression, and apoptosis (43, 44). During inflammation, neutrophils roll along the endothelial wall of postcapillary venules, sampling different inflammatory signals. Neutrophil activation is required to generate $ß_2$ integrin bonds with the endothelium that are strong enough to withstand the flow forces and thus achieve arrest from the rolling state. Unlike naive T cells, neutrophils are not only activated by ligation of G-protein-coupled receptors with chemokines and other chemoattractants but also receive signals from the engagement of adhesion molecules including the selectins and $ß_2$ integrins (45,46). The modulation of neutrophil activation through integrin-mediated pathways is important in the homeostatic control of the resolution of inflammatory states. T lymphocytes are the primary cells responsible for maintaining the immune system. There are many intricate mechanisms involved in the regulation of T cells and the integrin family of adhesive surface proteins plays a pivotal role in the control of T lymphocyte activation and function. Adhesion of T lymphocytes to ECM proteins also provides the intrinsic signals needed to direct and coordinate the T cell responses (47,48). During recirculation, T lymphocytes move through distinct microenvironments mediated by integrins, a process that is critical for cell cycle, cell differentiation and gene expression (49). Integrins modulate T cell coactivation by providing a scaffold for signaling and cytoskeletal proteins that are adept at transmitting signals by inside-out signaling or by outside-in signaling. The signaling property of integrins permits rapid responses of lymphocytes to changes in the microenvironment. Therefore, whether the T cell needs to adhere or detach, integrins can quickly accommodate either state of the cell. Once cells are guided to sites of infection, inflammation, or antigen presentation, integrins

can also participate in the initiation, maintenance, or termination of the response (47,48).

Leukocyte functional responses, such as adhesion to the endothelium and ECM, chemotaxis, phagocytosis and formation of immunological synapses, generate dynamic alterations in the actin cytoskeleton network and activation of protein tyrosine kinases, mainly FAK (4,14,43,47). FAK, a focal adhesion-docking protein, recruits many signaling proteins to form a multimolecular complex and alters their activity, including that of PI3-K. Signaling via PI3-K regulates migration and immune cell proliferation, survival and differentiation (21). The role of PI3-K in leukocyte migration has been described in studies showing that neutrophils lacking PI3-K failed to orient toward different stimuli (50). In addition, it has been demonstrated that, in viable gene-target mice lacking the p100 catalytic subunit of PI3-K, T cells show impaired proliferation and interleukin-2 (IL-2) and interferon-γ production in response to anti-CD3 stimulation (51). The triggering of several intracellular signaling pathways linked to FAK and PI3-K activation includes the activation of the Ras/Raf/MAPK pathway, which can control various neutrophil functions, including migration, cell death and survival (52,53). Furthermore, gene expression involved in immune and inflammatory processes is regulated by NF-κB, which regulates the transcription of cytokines, chemokines, stress-response, and anti-apoptotic proteins (54). Integrins themselves have been shown to be involved in these processes by regulating the activity of other integrins and membrane receptors, a process referred to as integrin cross-talk (55).

Leukocytes and disintegrins

Given the crucial role of the integrins in modulating most leukocyte functions, our group has investigated the effects of different disintegrins on leukocyte functions. Studying the effect of RGD-disintegrin on human neutrophils, we recently identified a unique monomeric RGD-disintegrin isolated from *Bothrops jararaca* venom, jarastatin (JT), which is able to bind to $\alpha_M\beta_2$ integrin on neutrophils (56,57). JT inhibited neutrophil migration induced *in vivo* by carrageenan injection in mice, as well as human neutrophil chemotaxis induced *in vitro* by distinct chemoattractants such as IL-8 and fMLP (56). Interestingly, this peptide presents a significant chemotactic activity for human neutrophils and induces homologous and heterologous desensitization to other chemotactic agents such as IL-8 or fMLP (56). The chemotactic effect of JT was inhibited by anti-α_M antibodies, suggesting the involvement of Mac-1 integrin in this effect (57). Neutrophil migration induced by JT *in vitro* is accompanied by profound alterations in the actin network, with an increase in filamentous actin (56). Migration and actin polymerization induced by JT in human neutrophils were completely inhibited by tyrosine kinase inhibitors. Thus, after stimulation with JT, neutrophils show a sharp increase in phosphotyrosine content, with the phosphorylation of FAK and its association with the actin cytoskeleton. Investigating FAK downstream signaling, we also observed that JT activates PI3-K, which modulates its chemotactic effect, and is also capable of activating Erk-2, inducing its translocation to the cell nucleus (57,58). JT-induced MAPK pathway activation seems to be related to the effect of JT in inducing IL-8 synthesis, a potent inducer of migration, exocytosis and respiratory burst (59) and in delaying neutrophil apoptosis (57). These results strongly suggest that this RGD-disintegrin activates integrin-coupled signaling in neutrophils.

Besides interacting with α_M in neutrophils, a unique feature of JT, this disintegrin also inhibits $\alpha_5\beta_1$- and $\alpha_v\beta_3$-dependent adhesion of K562 transfected cells to fibronectin and vitronectin, respectively (Coelho AL

and Barja-Fidalgo C, personal communication). Thus, for comparison with JT, another known RGD-disintegrin selective ligand of $\alpha_5\beta_1$ and $\alpha_v\beta_3$, flavoridin (FL) (31), was also tested in neutrophils. FL was found to be a potent inhibitor of neutrophil chemotaxis induced by fMLP. However, despite its homology with JT, FL did not induce neutrophil chemotaxis or alterations in actin cytoskeleton dynamics (58). Interaction of FL with human neutrophils induced a slight but significant increase in FAK phosphorylation but decreased PI3-K association to FAK and Erk-2 nuclear translocation below control levels (58). These effects of FL seem to contribute to the mild pro-apoptotic effect of this disintegrin on neutrophils (Coelho AL and Barja-Fidalgo C, personal communication). Non-RGD-disintegrins were also tested in neutrophils and Alt-C was shown to inhibit neutrophil chemotaxis induced by fMLP and to activate the PI3-K and MAPK pathways. The chemotactic effect was related to the ECD motif present in the disintegrin-like domain, and a synthetic peptide containing the ECD sequence induced the same effects as Alt-C (60).

Since early data had shown that EC3, an MLD/VGD-disintegrin, a ligand of $\alpha_4\beta_1$ and $\alpha_9\beta_1$ integrins, inhibited transendothelial neutrophil migration (38), the effect of this disintegrin on human neutrophil migration, activation and functionality was evaluated. Similarly to JT, EC3 induced neutrophil chemotaxis that was inhibited by anti-$\alpha_9\beta_1$, but not by anti-α_M antibodies. Signaling downstream integrin receptors showed FAK phosphorylation and PI3-K activation but, in contrast to JT, EC3 inhibited Erk-2 activation, inhibiting its translocation to the nucleus. This inhibitory effect of EC3 on the MAPK pathway contributes to the decrease in IL-8 mRNA expression and to the potent pro-apoptotic effect of this disintegrin on neutrophils (57). Interestingly, another heterodimeric MLD/VGD peptide, VLO5, which is also able to bind selectively to $\alpha_4\beta_1$ and $\alpha_9\beta_1$ (38), despite its high homology with EC3, has opposite effects. VLO5 activates the MAPK pathway and is a very potent inhibitor of neutrophil apoptosis (Saldanha-Gama R and Barja-Fidalgo C, unpublished data). The data indicate that RGD- and MLD-disintegrins, triggering integrin-coupled signaling, were able to interfere with neutrophil motility and survival and with chemokine expression.

Recent studies by our group have demonstrated the effects of monomeric RGD-disintegrins, FL, kistrin and echistatin, on the activation and proliferation of human T lymphocytes. We observed that these RGD peptides were capable of activating NF-κB in a PI3K-dependent manner, interfering with CD25 expression and T cell proliferation. These recent results allow us to suggest that $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins could act as co-stimulatory molecules on T cells, modulating the activity of T cell receptors by outside-in signaling (Helal-Neto E, Barja-Fidalgo C and de Freitas MS, personal communication).

A significant development in the study of integrin-ligand interactions was the discovery, originally in snake venoms, of disintegrins, which can exert their primary effect by regulating the cell-signaling mechanism triggered by the interaction with integrins. In this review, we focused on the role of integrin-triggered signaling in leukocyte functions, emphasizing migration, survival/death and proliferation. Our studies have elucidated the mechanisms of action of RGD- and non-RGD-disintegrins in human leukocytes. The therapeutic challenge is to design agents for interventions in disorders involving leukocyte dysfunctions. This will require improved understanding of the molecular events regulated by integrin signaling, which coordinates the adhesion and migration steps. In addition, potential ligands interfering with integrin signaling may lead to functional alterations in the cellular events mediated by this adhesion molecule family.

Disintegrins are tools that can be used to understand the cellular events (adhesion, migration, proliferation, and activation) mediated by adhesion molecules.

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