

REVIEW

Integrin VLA-5: modulator and activator of mast cells

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Introduction

An allergic response can be divided into two phases. During the sensitization phase the production of allergen-specific IgE and subsequent binding to the high-affinity receptor for IgE (FcεRI) on mast cells is initiated [1]. During the second phase multivalent allergen triggers the release of various inflammatory mediators of sensitized mast cells [2].

The incidence of allergy has increased rapidly during the last century. The word 'allergy' for 'deviation from the original state' was introduced at the beginning of the 20th century and originally described a variety of harmful immune reactions to vaccination. To differentiate the familial syndrome of asthma and hay fever from these mixed reactions the term 'atopy' for 'strange disease' was used. During time, allergy has lost its original broad meaning and currently the terms atopy and allergy, both IgE-mediated diseases, are used interchangeably [3].

Although several studies strongly indicate a role for environmental factors as well as genetic predisposition in the development of atopic diseases the exact mechanisms remain poorly understood [4]. Most of these factors may be explanatory for the skewing of the immune responses towards atopy. However, they do not clarify why not all individuals that are atopic by skin test – indicating that they are sensitized – show symptoms of an allergic disease. This observation strongly suggests that factors from the mast cell microenvironment can play an important role in whether or not a sensitized mast cell will release its inflammatory content upon contact with allergen. Indeed, the number of factors reported to affect the functional repertoire of mast cells *in vitro* increases rapidly, and at least some of these factors may prove to play an important role in the modulation of mast cell activity *in vivo*.

One example of this is the $\alpha_5\beta_1$ integrin VLA (very late antigen)-5, which was identified as the key receptor for adhesion of mast cells to extracellular matrix-component fibronectin [5]. It was demonstrated that adhesion to fibronectin enhances mast cell degranulation *in vitro* and that VLA-5-blocking antibodies strongly suppress passive cutaneous anaphylaxis [5,6]. These data indicate that VLA-5 can be a potent modulator of mast cell function. Understanding of the mechanism by which VLA-5 modulates mast cell function is of potential interest for the development of future therapy against allergy.

Adhesion via VLA-5

VLA-5 is a member of the integrin family of adhesion receptors. Integrins are heterodimeric membrane receptors that mediate cell–cell and cell–extracellular matrix interactions and play a role in various physiological processes [7]. Currently, eight α - and fourteen β -chain isotypes have been identified. A dimer of a particular α - and β -chain isotype displays affinity for a particular ligand [8]. VLA-5 consists of an α_5 and β_1 chain and binds the arginin-glycine-aspartic acid (RGD) sequence in fibronectin. On resting cells, integrins exist in a low-affinity conformation and in order to induce adhesion, a conformational change to a high-affinity state is required. Various mediators such as interleukins [9], chemokines [10] and growth factors such as SCF [11] have been shown to induce VLA-5-mediated adhesion to fibronectin. The mechanism through which each of these mediators modulates integrin affinity involves intracellular signalling events and is hence referred to as 'inside-out' signalling [12]. In addition, the divalent cations Mn^{2+} and Mg^{2+} have been shown to directly induce a high-affinity state of VLA-5, and this effect can be antagonized by Ca^{2+} [13]. The extracellular domain of the VLA-5 α -chain contains several cation-binding motifs that are highly homologous with the calcium-binding EF-hand motif of calmodulin [14]. Binding of a cation to α_5 'EF-hand-like' domains induces a conformational

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change, which thereby modulates the ligand-binding capacity of the integrin.

Ligand binding and subsequent clustering of integrins triggers 'outside-in' signalling [15], involving tyrosine phosphorylation and influx of calcium. Subsequent recruitment of a range of cytosolic proteins to the cytoplasmic tails of the integrins leads to the formation of complex protein structures, focal adhesion complexes (FACs), at the cell–fibronectin interface. FACs play a role in cytoskeleton reorganization of the cell and have been indicated as hot-spots of cellular signalling [16,17].

Choice of experimental set-up

Enhancement of mast cell degranulation by VLA-5-mediated adhesion to fibronectin was initially reported in RBL-2H3 mast cells [18,19]. These cells have been shown to be invaluable for the investigation of signalling through FcεRI and are still widely used as an *in vitro* model for mast cells. For detailed study of adhesion-mediated modulation of mast cell degranulation, an *in vitro* model system is required in which both adhesion and degranulation can be controlled independently. RBL-2H3 cells are unsuitable for this purpose, because they constitutively adhere to various surfaces without the assembly of specialized adhesion structures at the cell–substrate interface [20]. This major discrepancy with *in vivo* mast cells makes RBL-2H3 cells unsuitable for our investigations.

The development of a technique to culture mast cells from murine bone marrow (BMMCs) has opened a new field of possibilities for *in vitro* mast cell research [21]. Through variations in culture conditions maturation into different subtypes can be induced. The phenotypes and responses to various stimuli, such as allergen, of these cells are comparable with the different mast cell populations found *in vivo*. Most importantly, BMMCs do not adhere spontaneously or non-specifically to fibronectin. In our opinion, BMMCs are a more accurate *in vitro* representation of mast cells currently available, and we therefore used these cells for our investigations.

To induce adhesion of BMMCs to fibronectin, we applied a protocol using Mn^{2+} as a stimulus to induce a high-affinity state of VLA-5. A major advantage of Mn^{2+} compared with other known adhesion-inducing stimuli is that this cation does not affect FcεRI signalling directly at the concentrations used.

Our experiments showed that VLA-5-mediated adhesion of BMMCs to fibronectin is achieved in two phases. During the first phase a Mn^{2+} -induced conformational change of the integrin enables ligand binding. During the second phase, which is dependent on kinase and phosphatase activity, the cell–substrate bond is stabilized. These

features reflect the inside-out and outside-in signalling that is characteristic for integrin physiology [12,15].

VLA-5: enhancer and initiator of degranulation

Our data showed that adhesion of IgE-sensitized BMMCs to fibronectin-coated polystyrene beads enhances allergen-induced degranulation. These results correlate with previous studies in which RBL-2H3 cells were used. Besides an enhancement of the response towards allergen, we found that incubation with fibronectin-coated, but not BSA-coated, beads directly triggers degranulation in the absence of an allergic stimulus. Additionally, intradermal injection of *in vitro* Mn^{2+} -stimulated BMMCs into BALB/c mice and evoked local anaphylaxis as determined by measuring leakage of intravenously injected Evans Blue. This *in vivo* response could be inhibited by blocking VLA-5 adhesion with soluble ligand, arginin-glycin-aspartic acid-serine-peptide. Furthermore, we found that in addition to Mn^{2+} , Zn^{2+} , Ni^{2+} and Co^{2+} are able to induce VLA-5 mediated adhesion of BMMCs to fibronectin and subsequently trigger adhesion-induced degranulation *in vitro* and *in vivo* (unpublished data). In the literature there are numerous examples of contact hypersensitivity responses towards manganese, zinc, nickel and cobalt [22–26]; however, the underlying mechanisms are poorly understood. According to our results VLA-5-induced mast cell degranulation could be the underlying mechanism. Overall, our data suggest that the degree of adhesion, determined by the affinity-state of VLA-5, correlates with allergen sensitivity and magnitude of directly triggered degranulation of mast cells.

FcεRI–VLA–5 interaction

FcεRI, the major receptor for mast cell degranulation, consists of an IgE-binding α-chain, a β-chain (FcRβ) that plays a role in membrane stabilization and serves as a signal amplifier, and two γ-chains (FcRγ) that form the connection between the receptor complex and the intracellular signalling events leading to degranulation. Aggregation of FcεRI by binding of multivalent allergen to receptor-bound IgE causes this receptor to associate rapidly with specialized cholesterol-rich membrane microdomains. These microdomains, also called rafts, are also enriched with protein tyrosine kinases (PTKs) of the Src family. In unstimulated cells, rafts are distributed in a patchy manner across the cell membrane and are not associated with FcεRI [27]. Upon association of FcεRI with rafts, the Src PTK Lyn phosphorylates the tyrosine residues of the immunoreceptor tyrosine-based activation motifs (ITAMs) localized in the cytoplasmic tails of FcRβ and -γ. Phospho-ITAMs serve as docking sites for proteins that contain

Src-homology-2 domains. Phosphorylated Fc γ -ITAMs bind and activate cytosolic Syk, a PTK of the ZAP-70 PTK family. Activation of Syk has been shown to be sufficient as well as obligatory for induction of mast cell degranulation [28,29]. A subsequent series of signalling events leads to elevation of the cytosolic calcium concentration and eventually degranulation [30].

To determine a possible role for Fc ϵ RI in adhesion-induced degranulation we generated BMMCs from mice that lack Fc γ due to targeted gene disruption [31]. Our data showed that the absence of Fc γ does not affect adhesion of BMMCs to fibronectin. However, adhesion-induced degranulation *in vitro* was completely abrogated in BMMCs lacking Fc γ , indicating that this signalling unit is utilized by VLA-5. Additionally, detailed analysis using confocal laser scanning microscopy showed that VLA-5 and Fc ϵ RI are colocalized and may be physically associated on the membrane of resting BMMC. Integrin-Fc receptor (FcR) membrane colocalization has been shown previously by cotransfection experiments with the $\alpha_M\beta_2$ integrin CR3 and Fc γ RIIA [32]. Furthermore, Western blot analysis of adhered BMMCs showed accumulation of Fc γ in the cell fraction associated with the fibronectin-coated beads.

This indicates sequestering of Fc γ to the VLA-5 clusters and FACs. We found that adhesion also leads to the recruitment of Syk to this bead-associated cell fraction, possibly due to phosphorylation of Fc γ -ITAMs in the VLA-5 clusters. Adhesion to fibronectin and Fc ϵ RI-stimulation with allergen trigger tyrosine phosphorylation of an identical set of proteins, which is additive when both stimuli are applied. This indicates that VLA-5 clustering triggers the Fc ϵ RI signalling cascade (unpublished data).

Similar data have been demonstrated in bronchial epithelial cells and fibroblasts for β_1 integrins in relation to members of the growth factor receptor (GF-R) family. In these studies adhesion to fibronectin induced coclustering of β_1 integrins and GF-R in FACs, induced growth factor-independent phosphorylation of the GF-R and prevented apoptosis due to growth factor deprivation [33,34].

There appears to be a parallel between integrin-GF-R in, for example, fibroblasts and VLA-5–Fc ϵ RI interaction in BMMCs. In both cases fibronectin serves as a 'pseudo-ligand' and the integrin as a 'pseudo-ligand binding receptor subunit' of the physically associated receptor (GF-R or Fc ϵ RI). Adhesion induces clustering and triggers the signal transduction cascade of the latter. Clustering of

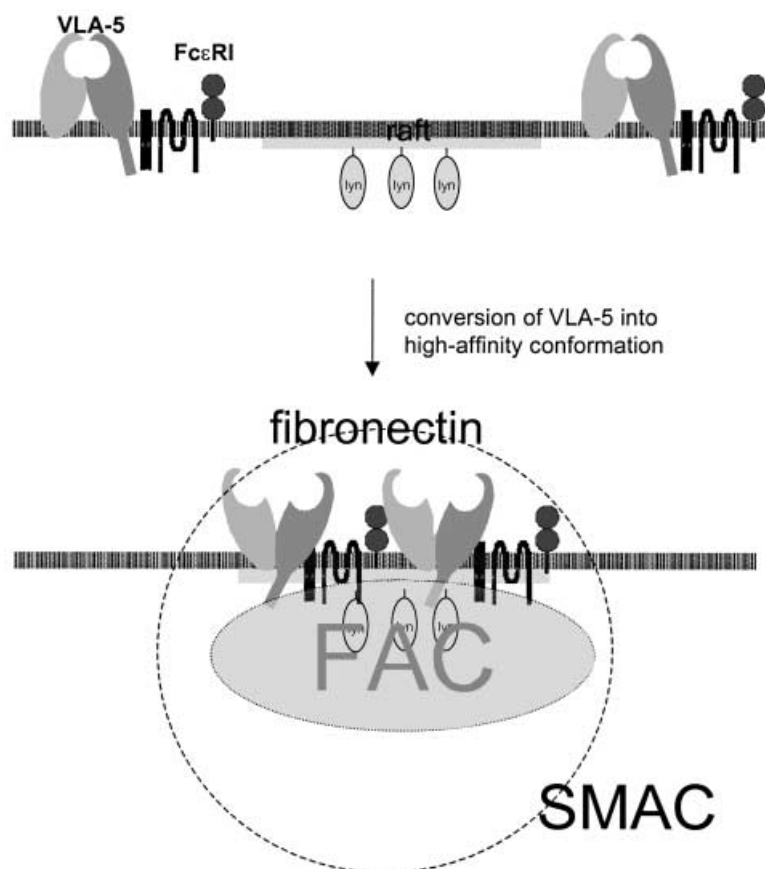


Fig. 1 Adhesion to fibronectin induces the formation of supramolecular activation clusters in mast cells. (a) In resting mast cells, small low-affinity VLA-5–Fc ϵ RI assemblies and rafts are not associated but randomly distributed in a patchy manner on the plasma membrane. (b) Conversion of VLA-5a high affinity conformation induces the accumulation of VLA-5 and Fc ϵ RI at the cell–fibronectin interface. Subsequent association with rafts and focal adhesion complexes (FACs) formation leads to the assembly of supramolecular activation clusters (SMACs).

both GF-R and FcRs is obligatory in order to initiate the signal transduction cascade. However, one important difference between both receptor families is that GF-R harbour intrinsic kinase activity and FcR do not. Mere aggregation in VLA-5 clusters is therefore not sufficient for FcR γ phosphorylation, and recruitment of extrinsic kinase activity is needed. In this mechanism there could be a role for rafts, which are enriched with kinases. Previous data have shown a role for rafts in integrin function [15,35] and Fc ϵ RI-signalling [27]. Indeed, we found that disruption of rafts by cholesterol depletion with β -cyclodextrins attenuates adhesion-induced degranulation.

Model

Based on our results we propose the following model (Fig. 1). On the mast cell membrane VLA-5 and Fc ϵ RI are colocalized, and may be physically associated in small assemblies. On resting mast cells (a), VLA-5 is present in a low-affinity conformation, adhesion is absent and these assemblies are randomly distributed on the mast cell membrane. Lipid rafts are distributed on the membrane in a 'patchy' manner and there is no association between the raft and receptor pools. Conversion of VLA-5 into a high-affinity conformation by an external stimulus induces binding to fibronectin and leads to an accumulation of the integrins and Fc ϵ RI at the cell–fibronectin interface. (b) Association with rafts and formed FACs leads to the assembly of supramolecular activation clusters (SMACs

[36–38]). Interaction between Fc ϵ RI and components of FACs has been demonstrated previously [39–41].

The formation of SMACs could account for both adhesion-enhanced and adhesion-induced degranulation. First, stabilization of Fc ϵ RI-aggregates in SMACs could enhance Fc ϵ RI signalling according to the concept of 'kinetic proof-reading', which states that a cascade of intracellular events unfolds as long as the initiating event is maintained [42–44]. Secondly, they could lower the activation threshold of Fc ϵ RI (the minimal amount of allergen required for sufficient Fc ϵ RI and raft aggregation in order to trigger degranulation). Adhesion-induced aggregation, or 'pre-clustering', of Fc ϵ RI and rafts into SMACs, could lower the activation threshold and thus increase the sensitivity for allergen-stimulation. Overall, it is tempting to speculate that degranulation of mast cells depends on the degree of SMAC assembly (Fig. 2) and that several processes may contribute to this process either alone or in combination. In the case of resting IgE-sensitized mast cells, bearing only VLA-5 in a low-affinity conformation, no SMACs are formed (situation A). In these cells degranulation can only be triggered by allergen and these cells therefore have the lowest sensitivity (a high activation threshold). Increase in the number of high-affinity VLA-5 molecules (situation B) and subsequent SMAC assembly lowers the Fc ϵ RI activation threshold and adhesion-enhanced degranulation is observed. At a certain degree of SMAC formation (situation C) the Fc ϵ RI activation threshold is reduced to zero. At this point,

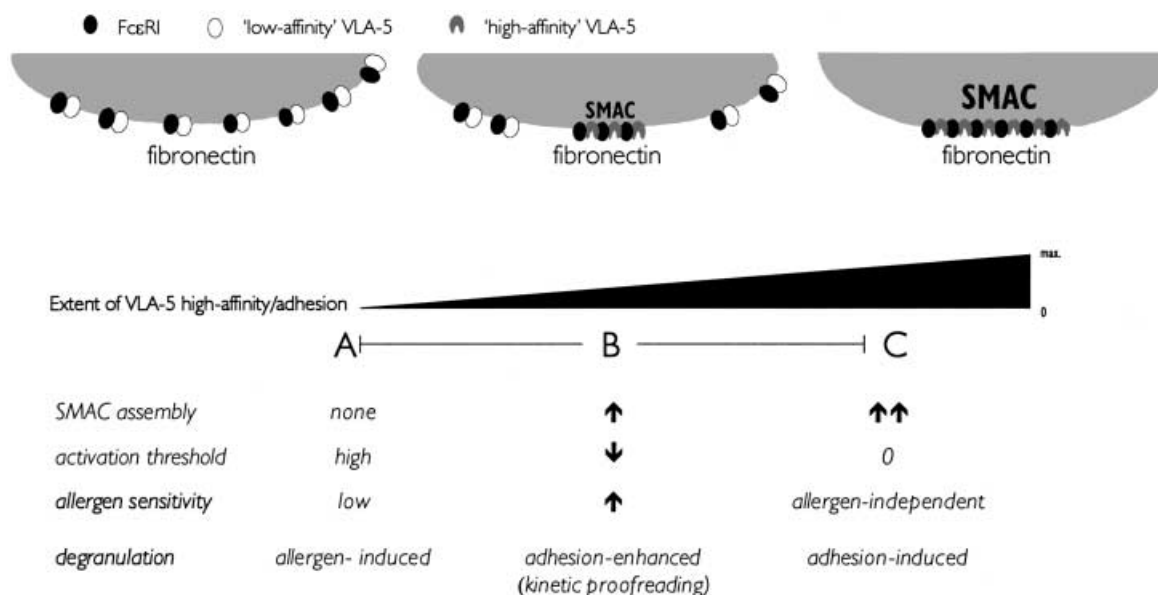


Fig. 2 Regulation of mast cell function by VLA-5. The extent of VLA-5 in high-affinity conformation correlates with supramolecular activation cluster (SMAC) assembly in mast cells. Through SMACs the sensitivity towards allergen and magnitude of degranulation of mast cells are modulated.

allergen is no longer required because through adhesion a sufficient amount of FcεRI is clustered to trigger degranulation by itself. The latter situation is probably the case in our experimental set-up and explanatory for adhesion-induced degranulation of BMMC.

Microenvironmental regulation of mast cell function

Our data suggest that the sensitivity of mast cells for allergen correlates with the affinity status of VLA-5. A range of mediators produced by various cell types can induce activation of integrins. Subtle variations in local conditions of the mast cell microenvironment, for example at sites of inflammation where integrin-activating mediators are abundantly present, could thereby regulate mast cell sensitivity for antigenic stimulation. The observation that skin atopy does not always correlate with allergic disease could be explained by low sensitivity of mast cells in the body as described in Fig. 2, situation A. According to this hypothesis, being allergic or not could be explained by the presence of mast cells in Fig. 2B or A, respectively. Organ restriction of allergy could be due to priming of local mast cells by VLA-5-activating mediators produced by the mast cell surrounding tissue, as in Fig. 2, situation B. At locations of severe tissue inflammation the concentration of inflammatory, and integrin-activating, mediators reaches high levels and situation Fig. 2C could occur. The latter situation could also provide a plausible explanation for hypersensitivity responses to metal, because our data show that cations of these metals can also induce VLA-5 mediated adhesion and subsequent degranulation.

Future directions for anti-allergic therapy

Ra *et al.* and Yasuda *et al.* have shown that the blocking of VLA-5 with specific antibodies inhibits passive cutaneous anaphylaxis responses in mice [5,6]. Blocking of VLA-5 mimics situation 2A (Fig. 2) of our model because SMAC assembly in mast cells is prevented. Decreased mast cell sensitivity and limited allergen concentrations – below the activation threshold – could thereby explain abrogation of *in vivo* activation.

Making mast cells less responsive to allergen by blocking VLA-5 could be a strategy for therapy in allergic responses.

Instead of using antibodies we suggest a different approach to block VLA-5 adhesion. To interfere with VLA-5 function we used peptides CALP1 (for calcium-like peptide 1) and CALP2 that have been previously designed as amino acid sequences complementary to that encoding a primordial EF-hand and were shown to interact with EF-hands of various proteins [45–48]. We found that both

CALP1 and CALP2 are able to abrogate adhesion of BMDCs to fibronectin. Additionally, we showed that the targets for these peptides were located intracellularly, possibly calmodulin and membrane calcium-channels, as well as extracellularly, the VLA-5 cation-binding domains that are 'EF-hand-like' structures [49].

The ability of CALP1 and CALP2 to block adhesion to fibronectin and thereby modulate the activity of mast cells suggests that peptide calcium-mimetic peptides may be possible lead compounds for anti-allergic drugs.

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