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The spatial molecular pattern of integrin recognition sites and their immobilization to colloidal nanobeads determine α2β1 integrin-dependent platelet activation

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ABSTRACT

Collagen, a strong platelet activator, is recognized by integrin α2β1 and GPVI. It induces aggregation, if added to suspended platelets, or platelet adhesion if immobilized to a surface. The recombinant non-prolylhydroxylated mini-collagen FC3 triple helix containing one α2β1 integrin binding site is a tool to specifically study how α2β1 integrin activates platelet. Whereas soluble FC3 monomers antagonistically block collagen-induced platelet activation, immobilization of several FC3 molecules to an interface or to colloidal nanobeads determines the agonistic action of FC3. Nanopatterning of FC3 reveals that intermolecular distances below 64 nm between α2β1 integrin binding sites trigger signaling through dot-like clusters of α2β1 integrin, which are visible in high resolution microscopy with dSTORM. Upon signaling, these integrin clusters increase in numbers per platelet, but retain their individual size. Immobilization of several FC3 to 100 nm-sized nanobeads identifies α2β1 integrin-triggered signaling in platelets to occur at a twentyfold slower rate than collagen, which activates platelet in a fast integrative signaling via different platelet receptors. As compared to collagen stimulation, FC3-nanobead-triggered signaling cause a significant stronger activation of the protein kinase BTK, a weak and dispensable activation of PDK1, as well as a distinct phosphorylation pattern of PDB/Akt.

1. Introduction

Hemostasis is mediated by a complex interplay involving thrombocytes and blood clotting factors. Lack or dysfunction of any of these hemostasis-mediating components may manifest in life-threating hemorrhage or thrombosis [1]. During tissue and vessel damage, collagen and other extracellular matrix (ECM) proteins are exposed. Platelets attach to collagen and to immobilized fibrinogen and are thereby strongly activated. They become adhesive, take on a dendritic cell shape, degranulate and thus release substances which activate additional platelets. Moreover, the major platelet integrin αIIbβ3 is fully activated, binds to the fibrin network, and stabilizes the thrombus. Platelets directly interact with collagen via two receptors, glycoprotein (GP)VI and α2β1 integrin [2,3]. GPVI is a homodimeric immunoglobulin family receptor [4,5]. Integrin α2β1 is a heterodimeric integrin family adhesion receptor [6]. After ligand binding, integrins recruit cytoskeleton molecules and mechanically link the ECM to the cytoskeleton. Neither integrins nor GPVI possess kinase domains, but they recruit signaling molecules

Abbreviations: BCECF-AM, 2′,7′-Bis-(2-carboxyethyl)-5-(and-6)-carboxy-fluorescein-acetoxymethyl ester; BSA, bovine serum albumin; BTK, Bruton's tyrosine kinase; CBL, Casitas B-lineage lymphoma protein; Col-I, type I collagen; mAb, monoclonal antibody; RT, room temperature; PBS, phosphate-buffered saline, pH 7.4; PDK1, phosphatidylinositol-dependent kinase-1; dSTORM, direct Stochastic Optical Reconstruction Microscopy; TIRF, total internal reflection fluorescence; GABA, gamma-aminobutyric acid; LTA, Light Transmission Aggregometry.

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and thereby trigger platelet activation. Several interlinking signaling pathways were reported in platelets [7]. GPVI associates with FcRγ, which bears an immunoreceptor tyrosine activation motif (ITAM) [3–5], and interacts with additional adaptor proteins, such as SLP76, GADs, and Syk, which eventually activate PI3-kinase [8]. Integrins signal via the FAK family members, FAK and Pyk2 [9], and via members of Src family kinases (SFK), thereby also activating PI3-kinase. The resulting increase in phosphatidylinositol-3,4,5-trisphosphate (PIP3) recruits protein kinase B (PKB)/Akt, which activates additional signaling molecules, such as phospholipase γ2 (PLCγ2) [10]. Consequently, the intracellular Ca^{2+} concentration raises, the cytoskeleton rearranges, and degranulation occurs, which increases the number of activated αIIbβ3 molecules on the platelet surface to 140,000 and allows firm attachment to fibrin [11]. This can be pharmaceutically inhibited with the αIIbβ3 inhibitor tirofiban.

The roles of the two collagen receptors, GPVI and α2β1 integrin, in platelet activation have been discussed controversially. They are not redundant, as the genetic ablation of the two receptors individually does not abolish collagen-induced hemostasis completely, but still allows hemostasis due to the presence of the other receptor [12–15]. In one model, GPVI is likely responsible for triggering platelet signaling and, complementarily, α2β1 integrin was reported to mediate mechanical anchorage to collagen [16]. Farndale and his group delineated the binding sites for both receptors with synthetic triple-helical peptides [2,17]. The molecular framework of the collagen triple helix is essential for both receptors to bind collagen. GPVI recognizes at least two, but preferentially more, repetitive collagenous triplet sequences of GPO, with O being hydroxyproline [18]. Moreover, the affinity strongly increases, if several triple helices are laterally bundled as realized in the collagen-related peptide (CRP) [2,19]. In contrast, α2β1 integrin binds to the trimeric sequence GFPGER [20] presented in one triple helical collagen molecule, commonly referred to as monomer. It does so, even if the collagenous sequence is not prolyl-hydroxylated [21,22].

Under physiological conditions, collagens are not monomeric but characterized by a highly ordered supramolecular structure, *e.g.* type I-collagen monomers align laterally in a staggered manner with a periodicity of D = 67 nm [23,24]. Thus, the binding sites for receptors are physically linked within a fibril and form an array of characteristic geometry and distances [25,26]. To study the role of this supramolecular array of α2β1 integrin binding sites in cellular signaling, a collagen molecule is required, which does not assemble into higher aggregates spontaneously, but remains monomeric unless linked by chemical means. Previously, we generated recombinant mini-collagens FC0 and FC3 [21], which lacks or contains, respectively, one α2β1 integrin recognition site [20]. These mini-collagens consist of three identical $(GPP)_{10}$ sequences fused to a foldon trimerization domain. Brought in close proximity by the foldon domains, the three $(GPP)_{10}$ sequences fold into a collagen triple helix. In FC3, the α2β1 integrin binding sequences are inserted into the middle of the host triple helix and are thus presented in a collagenous conformation. Without forming supramolecular aggregates, monomeric mini-collagens stay in solution, which together with the lack of hydroxyproline residues make their binding to GPVI negligibly weak [22]. Using mini-collagen FC3, we here show how monomeric mini-collagen molecules must be arrayed supramolecularly to elicit agonistic effects through α2β1 integrin. Thus, we developed FC3-coated nanobeads as ideal tools to analyze the signals transduced by α2β1 integrin during collagen-induced platelet activation.

2. Experimental section

2.1. Materials

Platelets were isolated from blood taken from healthy volunteers according to [27] with 6 μg/ml of prostaglandin E_1 and, in the last resuspension, with 2 mM $CaCl_2$ and 1 mM $MgCl_2$. The study was conducted in accordance with the Declaration of Helsinki and approved by local ethics committee. Recombinant mini-collagens FC3 and FC0 were produced as described previously [21]. Phosphate-buffered saline, pH 7.4 (PBS) was purchased from Gibco Life Technologies.

2.2. Non-patterned and nanopatterned mini-collagen biofunctionalized surfaces

For non-patterned adhesive surfaces, microtiter plates were coated with FC3, FC0 (1.7–5 μg/ml in PBS) or collagen-I (Col-I) (1 μg/ml in 0.1 M acetic acid) at 4 °C overnight. Wells were washed with PBS and blocked with 0.5% BSA in PBS at RT for 1 h. For morphometric analysis of platelet spreading, six channel μ-slides (ibidi) were coated with FC3 or FC0 (10 μg/ml), or collagen-I (10 μg/ml), and afterwards blocked with 0.05% BSA in PBS. For evenly spaced binding sites, glass surfaces were nanopatterned with hexagonally arranged gold nanoparticles according to Arnold et al. [28] and functionalized with 0.25 mM of linker 2-(2-[2-(1-mercaptoundecyl-11-oxy)-ethoxy]-ethoxy)-ethoxy-nitrilotriacetic acid (NTA-thiol) and 0.25 mM $NiCl_2$. Then, the gold nanoparticles were biofunctionalized with His-tagged FC3 or FC0 (0.05 μg/ml) for 2 h. Non-bound mini-collagen was washed off. The attachment time for platelets was 2 h.

2.3. Biofunctionalization of colloidal beads with mini-collagen

Latex-Beads (Sigma) in three different sizes (50, 100 and 460 nm) were sonicated for 30 min at 4 °C. For consistent coating density, the bead concentration was adjusted to the total surface area of the beads. Beads were coated with a concentration range of FC3 or FC0 from 0.5 to 120 μg/ml in PBS by gently mixing (550 rpm) for 12 h and afterwards blocked with 0.1% BSA for 1 h, both at 4 °C.

3. Results

3.1. Mini-collagen FC3 coated surface caused platelet activation and adhesion

Platelets attached to a surface coated with FC3 or FC0 in a concentration-dependent manner, until saturation was reached. The saturation signal was significantly higher on surfaces coated with FC3 than with FC0 (Fig. 1A). Kinetically, platelets attached to collagen-I, FC3 and FC0 with a similar rate (Fig. 1B). Moreover, in the impedance-based measurement, FC0 coated surfaces showed a lower saturation signal of attached platelets than surfaces coated with collagen-I or FC3, because the latter two allowed not only attachment but also spreading of platelets (Fig. 1C). In fluorescence micrographs and SEM images (Fig. 1C and D), platelet seeded on collagen-I exhibited a dendritic shape with spiky membrane extensions. On immobilized FC3, they appeared roundish and spread prominently. In contrast to those adherent and spread platelets, platelets only attached but did not spread on FC0-coated surfaces, thus failing to increase impedance values substantially (Fig. 1B). Only adhesion and spreading of platelets are caused by intracellular signaling processes.