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Design and reshaping of an scFv directed against human platelet glycoprotein VI with diagnostic potential

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ABSTRACT

Blood platelets play a key role in physiological hemostasis and in thrombosis. As a consequence, platelet functional analysis is widely used in the diagnosis of hemorrhagic disorders as well as in the evaluation of thrombosis risks and of the efficacy of antithrombotics. Glycoprotein (GP) VI is a platelet-specific collagen-signaling receptor. Clinical studies suggest that increased GPVI expression is associated with a risk of arterial thrombosis. Conversely, GPVI deficiencies have been identified in patients with defective platelet responses to collagen. Currently, there is no standard test available for measuring GPVI expression, essentially because antibodies usually cross-link GPVI upon binding, leading to platelet activation and consecutive changes in GPVI expression. Here, we designed a recombinant monovalent antibody fragment (scFv) derived from an anti-GPVI monoclonal IgG, 3J24, with the characteristics required to analyze GPVI expression. Guided by *in silico* modeling and V-KAPPA chain analysis, a Protein L (PpL) recognition pattern was engineered in the scFv, making possible its purification and detection using PpL conjugates. The PpL affinity-purified scFv is functional. It retains GPVI-binding specificity and allows detection of platelet surface-expressed GPVI without inducing platelet activation. In conclusion, the reshaped scFv may be very useful in the development of diagnostic approaches.

The physiological role of blood platelets is to limit bleeding by forming a clot filling vascular gaps and facilitating healing. On the other hand, inappropriate activation of platelets could lead to arterial thrombosis and ischemic damage of downstream tissues. Several analytical methods are in use to explore platelets. Among them, flow cytometry is a powerful and versatile tool that provides definitive quantitative information regarding the phenotypic status and antigenic properties of platelets (e.g., surface expression of receptors, bound ligands, granules components, or platelet–platelet interactions, interactions with other blood cells or components of the plasma coagulation system), thereby facilitating the diagnosis of inherited or acquired platelet disorders (e.g., Bernard–Soulier syndrome, Glanzmann thrombasthenia, storage pool disease), the pathological activation of platelets (e.g., in the setting of acute coronary syndromes, cerebrovascular ischemia, peripheral vascular disease, cardiopulmonary bypass), and changes in the ability of platelets to activate in response to specific stimuli (e.g., efficacy of antiplatelet therapies). Reliable methods have been developed to characterize the expression of the main platelet receptors such as glycoprotein (GP)Ib[1] (von Willebrand factor receptor) and integrins α2β1 and αIIbβ3 (fibrinogen receptor). Flow cytometry is also used to measure the exposure of platelet activation markers such as CD62P (P-selectin).

One of the most important receptor expressed at the surface of platelets is GPVI, which plays a key role in collagen-induced platelet activation [1]. GPVI is a glycosylated protein of 339 amino acid residues with an apparent M_r of 62 kDa, only expressed on megakaryocytes and platelets as a noncovalent complex with the signaling γ chain common to the Ig receptors (FcRγ) [2,3]. Binding of GPVI-FcRγ to collagen exposed at the site of vascular injury induces clustering of GPVI molecules and initiates a signaling

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[1] *Abbreviations used:* GP, glycoprotein; HRP, horseradish peroxidase; PBS, phosphate-buffered saline; PE, phycoerytrin; PpL, Protein L; PRP, platelet-rich plasma; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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pathway leading to platelet degranulation, activation, aggregation, and procoagulant activity.

Some preclinical studies have provided evidences that enhanced GPVI expression at the platelet surface may be a marker for acute coronary syndrome [4]. In contrast, deficiency or defects of GPVI can lead to profoundly altered platelet responses to collagen. Most often, GPVI deficiencies are revealed by mild bleeding disorders and are acquired in the context of immune dysfunction [5,6]. In rare cases, functional GPVI defects are associated with genetic protein deficiency and dysfunction [7,8]. Furthermore, GPVI has recently been shown to be down-regulated by proteolysis by the coagulation factor Xa which is the target of newly developed anticoagulant drugs [9]. For all these reasons, monitoring of GPVI function and count may be useful in medical practice to understand GPVI-related defects and thrombotic disorders [5,10,11].

Several anti-GPVI monoclonal antibodies have been produced and some of them have great potential for therapeutic antithrombotic applications [12–14]. Others have been used in cytometry for the quantification of surface expressed GPVI or in Western blot to analyze the total cell protein. However, no standardized assays are yet available. Studies conducted in different research laboratories using these various antibodies have contributed to the understanding of GPVI structure, function, and regulation. They allowed the evaluation of normal expression and pathological variations of GPVI: congenital or acquired deficiencies [7,8] or increased expression of GPVI proposed to be associated with a major risk of arterial thrombosis [1]. However, data indicated that the level of GPVI expression varies among healthy individuals. The degree of these variations is a matter of debate, either tightly regulated, varying by only 1.5 or up to 5-fold [10,15,16]. These discrepancies may be due to the use of whole bivalent antibodies that cross-link surface-exposed GPVI and thus may lead to activation signals and modifications in GPVI expression. New sites could be exposed or conversely antibody binding could induce GPVI shedding or internalization [17–19]. This highlights the difficulties in quantifying GPVI expression and the requirement of specific characteristics of antibodies to make them valuable reagents for accurate quantification.

In this study we used the monoclonal antibody 3J24 directed to the ectodomain of human platelets GPVI [20]. The V-domains of this antibody responsible for the specific antigen-binding activity were cloned and assembled into a synthetic gene encoding a monovalent scFv capable of binding GPVI. Guided by computer modeling and sequence analysis, a variant, here designated as scFv 3J24-P8, was constructed. A single point mutation in V-Kappa FR1 (T 8>P) conferred to the scFv a Protein L (PpL) recognition site, which provides a way for rapid single-step purification or detection using PpL conjugates. The point-mutated scFv 3J24-P8 is fully functional. It retains the ability of the parental antibody to bind monomeric and dimeric shGPVI. Binding of scFv 3J24-P8 to platelets induces neither platelet activation nor aggregation. Therefore scFv 3J24-P8 preserves all the functional properties required in monitoring platelets and assays of GPVI quantification.

Materials and methods

Materials

The anti-GPVI monoclonal antibodies were produced by immunizing Balb/c mice with the DNA encoding a fusion protein corresponding to the extracellular domain of GPVI (residues 1–269) fused at its C terminus via a 3 Ala linker to the human IgG_1 Fc sequence (shGPVI-Fc) using the Rapid Immunization Gene Gun delivery followed by one intravenous injection of 100 μg shGPVI-Fc, 4 days prior to fusion [2]. Hybridomas were screened for secretion of GPVI-specific antibodies by enzyme-linked immunoassay (ELISA) using plate-bound shGPVI-Fc. Selected cell lines were cloned using ClonalCellTM-HY medium D (Stem Cell Technology, Vancouver, British Columbia, Canada). Ascitic fluids were produced, and antibodies were purified by chromatography on Protein A–Sepharose (GE Healthcare, Europe). 3J24 and 9O12 were isotyped as IgG_1 and previously characterized [20,21].

The murine scFv 9O12 directed to the GPVI of human platelets and scFv 9C2 directed against scorpion toxins irrelevant to GPVI have been described elsewhere [13,22].

PpL immobilized on crosslinked beaded agarose resin, peroxidase-conjugated PpL, and biotinylated PpL were from Pierce Biotechnology (Rockford, USA).

The antibodies anti-mouse IgGs and anti-cMyc (9E10) conjugated to horseradish peroxidase (HRP) (Sigma–Aldrich, St. Louis, MO, USA) or FITC (Invitrogen, Cergy Pontoise, France), anti-P-selectin–FITC or phycoerytrin (PE), anti-mouse IgG_1-FITC, and anti-CD41-FITC (Beckman Coulter, Marseille, France) were used according to the manufacturer's instructions.

ScFv 3J24-P8 was coupled to FITC as previously reported and free FITC was removed by extensive dialysis against PBS, pH 7.4 [2].

Dimeric shGPVI-Fc was produced and purified as described [23]. Monomeric recombinant shGPVI-His consisting of GPVI ectodomain fused to a hexahistidine tag at its C-terminal extremity was produced in transfected HEK293 cells and purified using the Probond purification system (Invitrogen).

Blood was collected from healthy volunteers. Platelet-rich plasma (PRP) and washed platelets were obtained according to the previously described procedure [2]. Whole platelet lysates were obtained by solubilization of washed human platelets (10^9 platelets mL^{-1}) in Tris–HCl 20 mM, pH 7.4, containing NaCl 150 mM, EDTA 3 mM, and SDS 2%, according to a described procedure [2]. All chemicals were of standard grade from Sigma–Aldrich or equivalent.

Methods

Construction of single-chain antibody fragment genes

Total RNA was isolated from freshly subcloned hybridoma 3J24. cDNAs encoding the antibody variable domains (IGHV and V-KAPPA) were cloned after RT-PCR using degenerated primers IGH-For (5′-CGG GAT CCT CTA GAC AGT GGA TAR ACM GAT GG-3′) and IGH-Rev (5′-CGG GAT CCT CTA GAG GTS MAR CTG CAG SAG TCW GG-3′) for 3J24 VH amplification or Vκ-For (5′-GGA TAC AGT TGG TGC AGC ATC-3′) and Vκ-Rev (5′-GAY ATT GTG MTS ACM CAR WCT MCA-3′) for 3J24 V-KAPPA and sequenced as previously reported [22,24]. Then, a synthetic gene encoding 3J24 IGHV fused to 3J24 V-KAPPA via a $(G_4S)_3$ peptide linker was designed. Codon usage was adapted to the bias of *Escherichia coli* resulting in a codon adaptation index value of 0.98 (GeneArt, Regensburg, Germany). The scFv gene was cloned into the prokaryotic expression vector pSW1, in frame with the pelB leader sequence at its 5′-end and, downstream, a sequence encoding the c-Myc tag [25].

Plasmid pSW-3J24-P8 encoding the mutated scFv which contains the V-KAPPA point mutation T 8>P was prepared using the Quick Change Kit (Agilent Technologies, Massy, France) and primers 3J24P8For (5′-GCG CTC AGG CTA CTG GGG GTC TGG GTC AGA AC-3′) and 3J24P8Rev (5′-GTT CTG ACC CAG ACC CCC AGT AGC CTG AGC GC-3′).

The constructed vectors pSW-3J24 and pSW-3J24-P8 were sequenced to ensure accuracy before being cloned into *E. coli* TOPP I for expression.

All standard molecular biological procedures were carried out as reported earlier [26].

Bioinformatics

Sequence analysis and data banks search. In order to easily compare V-REGION sequences of IgG 3J24 we used the IMGT unique