Flow dynamics and haemostasis

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Summary. Fluid-dynamic conditions that are compatible with tensile stress on the bonds between platelet glycoprotein Ibα and immobilized von Willebrand factor A1 domain (VWF-A1) led to Ca^{++} release from intracellular stores (type α/β peaks), which preceded stationary platelet adhesion. Raised levels of cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate inhibited these $[Ca^{++}]i$ oscillations and prevented stable adhesion. Once adhesion was established through the integrin $\alpha_{IIb}\beta_3$, new $[Ca^{++}]i$ oscillations (type γ) of greater amplitude and duration, and involving a transmembrane ion flux, developed in association with the recruitment of additional platelets into aggregates. We have defined the distinct roles that the two ADP receptors, $P2Y_1$ and $P2Y_{12}$, play in the early events that follow the initial platelet interaction with immobilized VWF-A1 under high flow conditions. We have examined the consequences of specific pharmacologic inhibition of P2 receptors and our findings demonstrate a differential role of $P2Y_1$ and $P2Y_{12}$, respectively, in platelet adhesion and aggregation onto immobilized VWF under elevated shear stress, and highlight the distinct contribution of signaling pathways dependent on Src family kinases, PLC, and phosphoinositide 3-kinase (PI 3-K) to these processes. Results have been achieved through original experiments under flow, thoroughly characterized by *ad hoc* image analysis techniques and quantitative kinetic analysis.

Key words: platelets, ADP receptors, signaling, von Willebrand factor, adhesion equilibrium constant, image analysis.

Riassunto (*Fluodinamica ed emostasi*). Le condizioni fluidodinamiche in grado di determinare adeguata tensione al legame tra la glicoproteina pistrinica GPIbα e il dominio A1 del fattore di von Willebrand immobilizzato sulla superficie adesiva portano al rilascio di Ca^{++} dai depositi intracellulari (picchi α/β), fenomeno che precede l'adesione piastrinica stabile. Elevati livelli di adenosina monofosfato ciclico (cAMP) e guanosina monofosfato ciclico (cGMP) inibiscono il rilascio di Ca^{++} dai depositi intracellulari e prevengono l'adesione piastrinica stabile. Stabilita l'adesione piastrinica attraverso l'azione dell'integrina $\alpha_{IIb}\beta_3$, si evidenziano nuove oscillazioni di $[Ca^{++}]i$ (type γ), più durature e di maggiore entità, che coinvolgono anche un flusso ionico transmembrana, e che sono associate ad un reclutamento di nuove piastrine nel formare l'aggregato. Abbiamo definito i ruoli distinti dei due recettori piastrinici per l'ADP, il $P2Y_1$ e il $P2Y_{12}$, nelle prime fasi dei fenomeni adesivi che seguono immediatamente l'interazione tra la piastrina e il dominio A1 del VWF immobilizzato sulla superficie di contatto in condizioni di flusso ad alte forze di scorrimento. Abbiamo esaminato le conseguenze dell'inibizione dei due recettori P2 mediante l'uso di inibitori specifici e abbiamo dimostrato un ruolo differente, per quanto riguarda il $P2Y_1$ e il $P2Y_{12}$, nell'adesione e aggregazione piastrinica sul VWF immobilizzato in condizioni di elevato *shear rate*. Abbiamo infine dato rilievo al contributo specifico delle vie di trasmissione del segnale dipendente dalle Src kinasi, PLC e fosfoinositide 3-kinasi (PI 3-K). Questi risultati sono stati ottenuti mediante specifici esperimenti in condizioni di flusso, estensivamente caratterizzati mediante tecniche di analisi di immagine *ad hoc* e analisi cinetiche quantitative.

Parole chiave: piastrine, recettori per ADP, trasmissione del segnale, fattore von Willebrand, costanti di equilibrio di adesione, analisi d'immagine.

INTRODUCTION

Blood circulates with greater velocity at the centerline of a vessel than near the wall, and this difference creates a shearing effect between adjacent layers of fluid that is greatest at the wall. The shear rate (s^{-1}) is directly proportional to the shear stress (N/m^2) and inversely proportional to the viscosity of the fluid ($N/m^2 \cdot s$). Quiescent platelets circulate in the blood-

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stream whereas the activation of platelets at sites of vascular injury plays a key role in haemostasis. At high shear rates, equivalent to those generated by blood flow in arterioles or stenotic arteries, adhesion requires von Willebrand factor (VWF) endogenously present in the subendothelial matrix or absorbed onto injured tissue components exposed to plasma. The drag that opposes platelet adhesion and aggregation increases with the flow rate, which in turn increases the wall shear stress; consequently, its effects on platelet thrombus formation are more relevant in arteries than in veins and, particularly, in arterioles. The highest shear rate values in the normal circulation may range from 500 to 5000 s^{-1} with a median value of 1700 s^{-1}. The binding of glycoprotein (GP) Ibα to the A1 domain of VWF is the main adhesive interaction capable of tethering platelets to a surface even when the flow velocity is elevated, but cannot mediate irreversible adhesion by itself [1]. Rather, the interaction maintains platelets in close contact with the surface, albeit with continuous translocation in the direction of flow *(rolling)*, until other receptors and ligands mediate a stable attachment after activation. When VWF is bound to collagen, the transition from rolling to stable adhesion occurs in seconds indicating a rapid activation that may be aided by signals originating from the mechanical stimulation of VWF-GP Ibα bonds under tensile stress [2]. At this stage of the process, plasma VWF binds to the surface of adherent and activated platelets, through an interaction that involves engagement of the integrin $\alpha_{IIb}\beta_3$ with the Arg-Gly-Asp sequence in the VWF C1 domain. Membrane-bound VWF is the substrate to which, in a high shear rate environment, flowing platelets attach through their GP Ibα to maintain thrombus growth in a process that repeats itself in successive layers. Like in the initial adhesion to the vessel wall, the binding of other ligands to activated $\alpha_{IIb}\beta_3$ is also required to support stable platelet aggregation [3].

MATERIALS AND METHODS

Blood preparation

Venous blood from medication-free healthy volunteers, who gave their informed consent according to the declaration of Helsinki, was mixed with 1/6 final volume of citric acid/citrate/dextrose, pH 4.5, or a specific α-thrombin inhibitor, either hirudin (Iketon, Milan, Italy) at the final concentration of 400 units/ml or PPACK (Calbiochem, La Jolla, CA) at the final concentration of 50 μM. Fifty ml of ACD-containing blood was centrifuged at 1000 *g* for 50 s at 22-25 °C, and the supernatant platelet-rich plasma (PRP) was collected. The platelets were incubated for 20 min at 37 °C with the fluorescent calcium probe Fluo-3AM (Molecular Probes, Eugene, OR) at the final concentration of 8 μM. Erythrocytes separated from the same blood were washed three times in a divalent cation-free Hepes-Tyrode buffer pH 6.5 and finally resuspended in the same buffer and with the addition of 1.75 mM Probenecid (Sigma, St. Louis, MO), used to prevent leakage of Fluo-3AM from the platelets. An adequate volume of PRP containing 2-8x10^8 FLUO 3-AM loaded platelets/ml was mixed with an aliquot of the erythrocyte suspension (50% hematocrit) and apyrase (grade III; Sigma) at the final concentration of 5 ATPase U/mL. The mixture was centrifuged at 1000 *g* for 15 min, the supernatant was discarded and the cell pellet was suspended in autologous plasma (prepared from the blood collected in hirudin or PPACK by centrifugation at 1650 *g* for 15 min at 22-25 °C) or Hepes-Tyrode buffer pH 7.4 and 1.75 mM Probenecid in a proportion such that the hematocrit was 42-45%. The labeling procedure did not significantly alter the function of platelets as evidenced by the response to agonists and the expression of surface activation markers [2].

Flow experiments

Human plasma VWF was diluted in phosphate-buffered saline, pH 7.4. Different concentrations of protein were used to coat the glass coverslips (24 x 50 mm) that represented the lower surface of a parallel plate flow chamber. A silicon rubber gasket determined the flow path height (125 μm) between the glass coverslip and the upper plate. The chamber was assembled and filled with PBS, pH 7.4. A syringe pump (Harvard Apparatus Inc., Boston, MA) and silicone tubing connections were used to aspirate blood through the chamber at the desired flow rate. The perfusion system was mounted onto an inverted microscopes equipped with epifluorescent illumination (Diaphot-TMD; Nikon Instech, Kanagawa, Japan) and intensified CCD video camera (C-2400-87; Hamamatsu Photonics, Shizuoka, Japan). All these experiments were recorded at a video rate of 25 fps on S-VHS videotape. Recorded images were captured and digitized from videotape. Image analysis was then performed off-line.

Image analysis technique

Image analysis is crucial for the following theoretical discussion. It must provide reliable identification and tracking of cells on long image sequences, rapidly (*i.e.*, without manual operations on individual images) and consistently (no subjective criteria, operator dependent). For that purpose we developed our own algorithms and programs [4], based on the MATLAB Image Tool (The MathWorks, Inc.). The method includes original options for automatic thresholding grey-scale images, and dedicated tracking algorithms, able to account for the physics of the flow experiment. Object identification between subsequent frames is obtained with a time-varying, two dimensional probability density function build around each single identified object [5] and oriented with the flow.

Adhesion data analysis

Thanks to the automatic particle tracking algorithm that we developed, details on each particle feature can be collected over time, providing information on the

establishment and breakage of adhesive bonds between a platelet moving in shear flow and the substrate, as well as the path traveled by the cell on the surface and cytoplasmic Fluo-3Am/Ca^{2+} concentration in the cells, as a function of time.

The platelet stays on the surface for a certain time, then rolls on the surface, stops, starts rolling again, many times before final detachment, and the duration of each step may vary. The actual history of platelet displacements is decisive in understanding and quantifying the strength of adhesion, as well as characterizing the surface density of ligands. After a transient, deposition and reentrainement of platelet equilibrates, reaching an adhesion equilibrium and the average number of surface events remains constant. The length of the initial transinet indicates the minimum interval of observation required to collect statistically significant data. Often, quite a long observation period (approximately from 125 to 1500 frames with a temporal resolution of 25 fps) must be allowed to reliably identify the average surface concentration (equilibrium between the on and off process). On the other hand, the asymptotic value changes according to the experimental conditions such as wall shear stress, platelet count, hematocrit, and surface concentration of active sites. Varying a certain condition, like the wall shear stress (through different flow rates imposed by the pump), often requires adjusting the observation time to have a significant value for the deposition/removal rates. All our experiments were recorded up to steady-state conditions on the surface are achieved. Secondary data, directly determined from observables, can then be derived such as the distribution of lifetimes for all the interacting platelets.

Of specific interest for a kinetic and thermodynamic study is the direct measurement of on- and off-rates. The cumulated number of platelets that tethered and detached in the field of view up to a given time are determined and reported into diagram. The slope of the curve represents the rate of attachment (R_{on}) and detachment (R_{off}) [5].

HOW SHEARING AFFECTS PLATELET ADHESION AND ACTIVATION ONTO VON WILLEBRAND FACTOR SURFACE

Platelet GP Ibα may function as an adhesion receptor and signaling receptor when platelets are adhering onto VWF surface at high shear rate conditions. We identified 2 types of effects, based on the intracellular Ca^{2+} concentration, its variation rate and relation with platelet motion on the surface. One type appears while platelets translocate on the VWF surface and it is characterized by a rapid increase of intracellular Ca^{2+} concentration. This peak was named α when the [Ca^{2+}]i rises above 400 nM or β when lower. In experiments performed with a wall-shear rate of 3000 s^{-1}, approximately 20% of the translocating platelets exhibit at least one α or β Ca^{2+} peak, and 9% established stationary adhesion within the observation period. Approximately 30% of the firmly adherent platelets had a distinct type of Ca^{2+} increase, named γ, which reached levels higher than 2000 nM, and even 3000 nM in many cases, can last several seconds, pulsing the Ca^{2+} concentration *(Figure 1)*. After a Ca^{2+} increase of type γ, platelets were observed to promote the arrest of other platelets translocating in their vicinity, and these in turn showed pronounced cytosolic Ca^{2+} elevations and started to form aggregates. Once established, these aggregates could grow quickly, displaying periodical and synchronous [Ca^{2+}]i pulsations.

Our observations demonstrate that the interaction of platelet GPIbα with VWF leads to 2 distinct types of [Ca^{2+}]i elevations linked to sequential stages of integrin $\alpha_{IIb}\beta_3$ activation. The first Ca^{2+} increase appears to be initiated by a mechanical stimulation, their frequency increasing with wall shear stress above 2 Pa. Within this group, the distinction between type α and β peaks was based solely on Ca^{2+} concentration and the fact that α peaks have a reproducible shape that facilitates the analysis of their relation to platelet motion. Indeed, type α peaks reached a maximum while platelets were transiently arrested but at a predictably short time before detachment from the surface, when the tensile stress on the GPIbα-VWF bonds is larger. These findings support the hypothesis that GPIbα has a mechanoreceptor function, although the proximal events

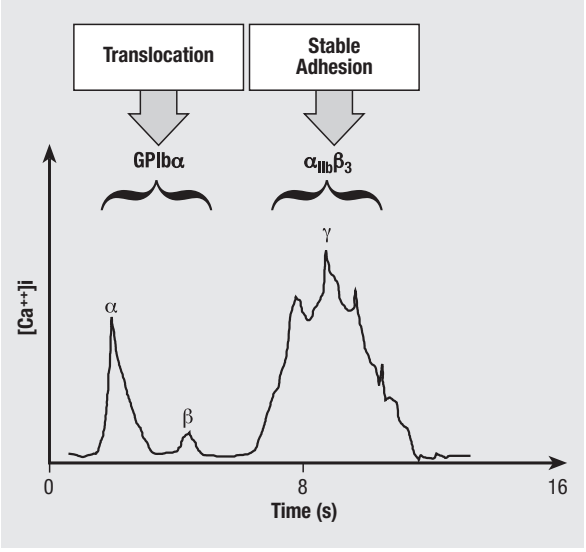

Fig. 1 | *Real-time analysis of [Ca^{2+}]i during platelet translocation and aggregate formation on immobilized VWF. Platelets loaded with Fluo-3 AM were suspended with washed erythrocytes in homologous plasma and perfused over immobilized VWF for 3 minutes at a shear rate of 1500 seconds-1. Platelet appears in the optical field and moved in the direction of flow. The translocation of platelet occurs mostly during a few seconds of relatively rapid movement, coincident with the appearance of transient [Ca^{2+}]i peaks (α/β); a higher and longer lasting increase in [Ca^{2+}]i (γ) develops while the platelet is stationary.*

responsible for transducing forces into a biochemical signal remain to be fully explained. Binding of the GPIbα cytoplasmic tail to the membrane skeleton through filamin-a [6] and to the ζ isoform of 14.3.3, [7], a regulatory molecule in cellular signaling, may be relevant. Neither association is needed for the GPIbα-dependent induction of $\alpha_{IIb}\beta_3$ activation in heterologous cells, but a role in flowing platelets is likely. Notwithstanding these uncertainties, it is clear that type α/β peaks are the consequence of rapid Ca^{2+} release from intracellular stores. Such cytoplasmic Ca^{2+} elevations are likely mediated by inositol-1,4,5-trisphosphate generated with diacylglycerol through the action of phosphatidylinositol-specific phospholipase C.

The first level of $\alpha_{IIb}\beta_3$ activation induced by platelet interaction with VWF under shear stress leads from transient to stable adhesion and is regulated by the cellular levels of cAMP and cGMP that control type α/β Ca^{2+} signals. However, thrombus formation cannot progress at this stage, possibly because $\alpha_{IIb}\beta_3$ molecules are activated only in the vicinity of stimulated GPIbα and they can bind immobilized VWF but not soluble VWF and fibrinogen as required for aggregation. A second level of $\alpha_{IIb}\beta_3$ activation must be reached for aggregation to occur, and this appears to require signal amplification associated with Ca^{2+} elevations of type γ, induced by ADP released in response to the initial GPIbα stimulation [2].

Then, it was established that secreted adenosine diphosphate (ADP) is necessary for the shear-induced platelet aggregation initiated by the interaction of soluble VWF with GPIbα. ADP binds to different G protein-linked P2 receptors, two of which ($P2Y_1$ and $P2Y_{12}$) are present on platelets [8]. Ligation of $P2Y_1$, linked to Gq, activates phospholipase C (PLC) and mobilizes Ca^{2+} from intracellular stores, leading to the activation of protein kinase C and subsequent platelet aggregation. Ligation of $P2Y_{12}$, linked to Gi, inhibits adenylyl cyclase, lowers cAMP levels, and potentiates ADP-induced platelet aggregation [9]. The function of both $P2Y_1$ and $P2Y_{12}$ is required for platelet aggregation after adhesion to collagen-bound VWF under flow conditions [10]. Distinct roles of two ADP receptors in the activation of platelets interacting with immobilized VWF in a flow field were described. We identified two sequential Ca^{2+} signals associated with the activation of flowing platelets interacting with immobilized VWF *(Figure 1)*. The earlier α/β peaks, linked to the engagement of GP Ibα by VWF-A1, involved Ca^{2+} release from intracellular stores and anticipated stationary adhesion. The results now obtained with selective antagonists of the two platelet ADP receptors modify earlier concepts reported in the literature, demonstrating a previously unrecognized role of $P2Y_1$ in generating the early signals associated with a first level of $\alpha_{IIb}\beta_3$ activation required for stable adhesion to VWF. Such a conclusion rests on the observation that platelets with blocked $P2Y_1$ exhibited the relatively rapid translocation velocity and short transient arrest times sustained by GP Ibα binding to VWF-A1 without $\alpha_{IIb}\beta_3$ engagement, as seen with PGE1-treated platelets. Blocking $P2Y_1$ function decreased by approximately 30% the proportion of platelets that exhibited type α oscillations while translocating on VWF, and reduced by approximately 500 nM the $[Ca^{2+}]i$ of a type α peak. The reduction in activation mirrors the proportion of platelets that show a γ peak after an α peak , indicating that signaling from $P2Y_1$ following GP Ibα-dependent ADP release may be crucial for the increase of $[Ca^{2+}]i$ to the levels needed for $\alpha_{IIb}\beta_3$ activation. This hypothesis is supported by the observation that inhibition of PLCβ, which acts downstream of $P2Y_1$ and generates an effector of Ca^{2+} release from intracellular stores *(Figure 2)* inhibits platelet adhesion to VWF and related Ca^{2+} oscillations as effectively as the $P2Y_1$ antagonist. $PLC_{\gamma}2$, which is also inhibited by U73122,29 may contribute to GP Ib-dependent signaling as previously inferred from studies on platelet adhesion to VWF under static conditions [11]. Thus, as also suggested elsewhere, intracytoplasmic Ca^{2+} levels may be related to the degree of activation of platelets interacting with VWF under high shear stress conditions: less than 200 nM equals resting; 400 nM equals β peak, subactivation; 1000 nM equals α peak induced by GP Ibα signaling, leading to ADP release; 1500 nM equals α peak+$P2Y_1$ signal, first level of $\alpha_{IIb}\beta_3$ activation and stable adhesion; more than 1500 nM equals γ peak, full $\alpha_{IIb}\beta_3$ activation with platelet aggregation modulated by $P2Y_{12}$ function *(Figure 2)*. The contribution of $P2Y_1$ to GP Ibα-initiated $\alpha_{IIb}\beta_3$ activation, therefore, may have different functional relevance in relation to heamodynamic parameters of the blood circulation. Src family kinase-dependent pathway is initially involved in the transduction of the signal originated by VWF-A1 binding to GP Ibα, as shown by the abrogation of all Ca^{2+} peaks caused by the Src inhibitors PP1 and PP2. Similar conclusions have been reached by others using different experimental conditions [12, 13].

In contrast to the early role of $P2Y_1$ in promoting stable platelet adhesion to immobilized VWF, which is an absolute requirement for subsequent aggregation, we found that $P2Y_{12}$ is not involved in generating any of the Ca^{2+} transients initially associated with platelet activation.

On the other hand, we found that $P2Y_{12}$ function is critical for the full development of platelet aggregates on immobilized VWF, in agreement with its reported role in shear-induced platelet aggregation initiated by soluble VWF binding to GP Ibα and in thrombus formation on collagen-bound VWF. Others have reported that $P2Y_{12}$ is involved in maintaining elevated Ca^{2+} levels in aggregating platelets [14] a function that is likely to be important for thrombus growth, as we confirm here, but appears to be distinct from earlier signaling events that initiate aggregation.

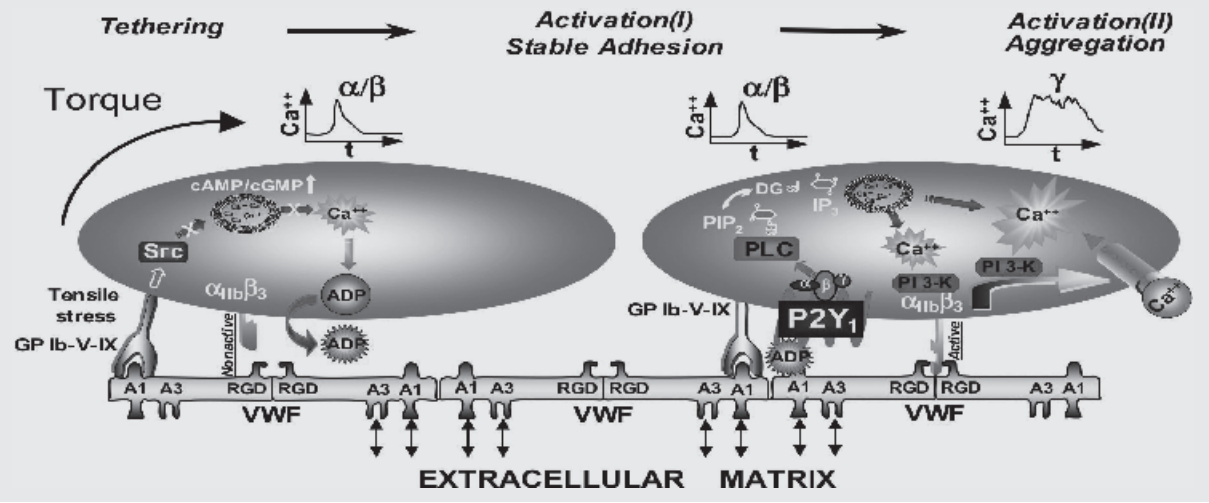

Fig. 2 | *Schematic representation of the sequential signaling events induced by the interaction of platelets with immobilized VWF under high shear stress. On the left, a platelet is shown during the initial tethering to the A1 domain of immobilized VWF mediated by the GP Ib-IX-V complex. An α/β [Ca^{2+}]i elevation is elicited as a consequence of this interaction and leads to the release of ADP from intracellular storage granules. Src family kinases may be involved at this stage, (13) and cAMP/cGMP levels modulate this and other downstream responses. Subsequent events are shown in the platelet on the right. The released ADP binds to the Gq-coupled $P2Y_1$ receptor, which leads to PLC activation and enhances Ca^{2+} release from internal stores during a/b oscillations. At this stage, a first level of localized $\alpha_{IIb}\beta3$ activation is reached that supports a more prolonged platelet adhesion mediated by the interaction with the RGD sequence in the VWF C1 domain. Initial PI 3-K activation may enhance this response. Subsequently, further PI 3-K activation and possibly the involvement of Src family kinases contribute to a more generalized $\alpha_{IIb}\beta3$ activation that permits soluble ligand binding (exemplified here by fibrinogen and VWF) and supports the formation of platelet-platelet aggregates. This second level of $\alpha_{IIb}\beta3$ activation is concurrent with or subsequent to a type γ [Ca^{2+}]i elevation dependent on a transmembrane ion flux. The second ADP receptor, $P2Y_{12}$, supports the formation of larger platelet aggregates through mechanisms that occur after the measured Ca^{2+} oscillations. The thromboxane A2 pathway inhibited by aspirin appears to have a very limited role in the successive stages of platelet adhesion, activation, and aggregation induced by the interaction with immobilized VWF. IP3 indicates inositol-1,4,5-trisphosphate; Src, Src family tyrosine kinases; PLC, phospholipase C; PKC, protein kinase C; PI 3-K, phosphatidylinositol 3-kinase; PIP2, phosphatidylinositol bisphosphate; DG, diacylglicerol.*

HOW SHEARING AFFECTS PLATELET AGGREGATION MEDIATED BY VON WILLEBRAND FACTOR

A unique feature of the binding of soluble VWF to platelet GP Ibα is the positive regulation by high shear forces. Shear-induced platelet aggregation is initiated by the binding of soluble VWF, through its A1 domain, to platelet GP Ibα. This interaction, which occurs above a threshold shear, triggers platelet signaling events that lead to platelet activation and the modulation of $\alpha_{IIb}\beta_3$ to form a high affinity receptor capable of binding soluble adhesive ligands such as VWF or fibrinogen [3, 15]. Physiologically relevant shear stress alone is capable of inducing this dual-step platelet aggregation without the addition of an exogenous agonist [16, 17]. Although initiated by the VWF/GP Ibα interaction, irreversible platelet aggregation induced by shear requires concomitant binding of VWF to both GP Ibα and $\alpha_{IIb}\beta_3$ complexes [18, 19]. Despite the many advances in our understanding of shear-induced VWF/GP Ibα interactions [20], the underlying mechanisms regulating signaling through GP Ibα remain poorly defined. Binding of soluble VWF to GP Ibα under stationary conditions, can be artificially induced by modulators like ristocetin and botrocetin, which bind to the VWF A1 domain [21]. However, distinct regions of both the ligand and receptor are involved in addition to regions common to both modulators. A panel of anti-VWF, and anti-GP Ibα antibodies have been characterized for their effects on ristocetin- and botrocetin-induced VWF/GP Ibα interactions, in addition to their effect on shear-induced platelet aggregation. Based on these studies, it now appears that ristocetin-, rather than botrocetin-dependent binding of VWF to platelet GP Ibα under stationary conditions more closely simulates the shear-dependent binding of VWF to GP Ibα [22]. Thus, ristocetin-dependent binding of VWF to GP Ibα may provide a useful means of simulating signaling mechanisms relevant to shear-dependent VWF/GP Ibα interactions. In this context, ristocetin-mediated interaction of VWF with platelet GP Ibα evokes a transient Ca^{2+} signal in the absence of extracellular Ca^{2+} [23], indicating activation of the PLC / IP3 pathway leading to elevated cytoplasmic Ca^{2+} levels, mobilized from intracellular stores. An important role of the platelet cytoskeleton in regulating the VWF/GP Ibα interaction has recently been demonstrated [24]. In these studies, pre-treating platelets with inhibitors of actin polymerization enhanced the rate and extent of shear-induced platelet aggregation, and also lowered the shear threshold

required to induce aggregation. Similar treatment also enhanced the rate and extent of platelet aggregation induced by VWF in the presence of ristocetin. These observations raise the intriguing possibility that regulation of the VWF/GP Ibα interaction by the cytoskeleton is key in preventing shear-induced platelet aggregation in the normal circulation.

KINETICS OF BOND FORMATION AND RUPTURE

By perfusing blood through a flow chamber and analyzing surface events using a tailored image processing and particle tracking algorithms, we can quantify deposition and removal rates, and attempt to correlate fluxes with actual shear rate. Statistical reasoning allow formulating single-event observations into mean-field behaviour, suitable for a macroscopic, clinical scale application.

Cell adhesion, mediated by the interaction of dedicated receptor and ligand molecules bound to interacting surfaces, requires suitable, unambiguous criteria for predicting whether a cell-to-cell encounter results in durable adhesion. Rates of adhesion and detachment are generally accepted as key determinants for the purpose, especially in a dynamic flow environment [25]. Critical to the role of the initial bonds platelets-VWF is the balance of their on- and off-binding rates which must be tuned to support platelet tethering and transient arrests without the need to establish stable bonds [26]. While an impressive amount of information has been gathered on the rate of bond dissociation [27, 28], little attention has been given to the frequency of formation of molecular interactions, mainly because variables involved in adhesion rate cannot be easily determined. Progress in this direction has been made [29], however a methodological framework for quantitative investigation of the parameters involved in cell-surface interaction (kinetics of the on- and off- process and characterization of the adhesion equilibrium) with specific attention to blood platelets is lacking.

Adhesion kinetics

According to early formulations (Bell's model [30]) the variation with time of the number of cells bound to the surface, N_b, is given by analogy with a reversible bimolecular chemical reaction between unbound cells *(u)* and surface sites *(s)*:

$$u + s \underset{\text{off}}{\overset{\text{on}}{\rightleftarrows}} b \qquad (1)$$

so that the rate of change of the number of bound cells is given by:

$$\frac{dN_b}{dt} = R_{on} - R_{off} = k_{on}\, N_s\, N_u - k_{off}\, N_b \qquad (2)$$

where R are the arrival/removal rates, k the kinetics constants, N_u is the number of unbound receptors on flowing platelets that can interact with the surface, and N_s is the number of sites of deposited substrate available for the platelets to bind. *R'*s can be directly measured from image analysis, but the identification of the intrinsic parameters (*k'*s) requires specifications on the quantities involved in equation (eqn) (2). The instantaneous and average N_b are also experimentally measurable through continuous image analysis; together with R_{off} measurements k_{off} can be easily calculated. Different experiments can reveal the dependence of k_{off} on experimental factors, and specifically the wall shear rate. Variables involved in the adhesion rate *(R_{on})* are not as easily determined. The number of sites on the surface available for the platelets to bind can be expressed as the difference between total number of sites available on the surface, N_s^o, and those already drawn in binding platelets

$$N_s = N_s^o - \alpha\, N_b \qquad N_s^o = K_{VWF}\, C_{VWF} \qquad (3)$$

N_s^o can be related to the concentration of substrate (often von Willebrand factor in our studies), C_{VWF}, used to coat the surface. The complete adsorption curve *(Figure 3a)*, which can be determined by binding assays experiments, at low substrate concentration, before surface saturation, reduces to a simple proportionality through the adsorption equilibrium constant, K_{VWF}. The coefficient α is the number of bonds a single platelet can establish with the surface ligands. It is frequently assumed $\alpha = 1$, the minimum for platelet-surface interaction, although evidences of multiple bonds formation, like rolling on the surface, have been observed. A procedure to estimate α can be devised, to check whether multiple bonds are likely, synergistically opposing tensile stress. N_u, the platelet number close to the surface, is the most critical piece of information. They can be quite different from the blood platelet count, N_p, because of radial segregation of cells in blood [31]. We can assume

$$N_u = \beta\, \delta\, N_P \qquad (4)$$

i.e. the number of platelets in the marginal layer is δ (>1)-times the average platelet count. β is the number of receptors per platelet. Assembling all the above developments in eqn (2), we can express the adhesion rate in term of measurable variables:

$$\begin{aligned} R_{on} &= k_{on} \left(K_{VWF}\, C_{VWF} - \alpha\, N_b\right) \cdot \beta\, \delta\, N_p \\ &= k'_{on} \left(K_{VWF}\, C_{VWF} - \alpha\, N_b\right) N_p \end{aligned} \qquad (5)$$

Platelet count N_p and substrate concentration C_{VWF} are normally known, substrate adhesion

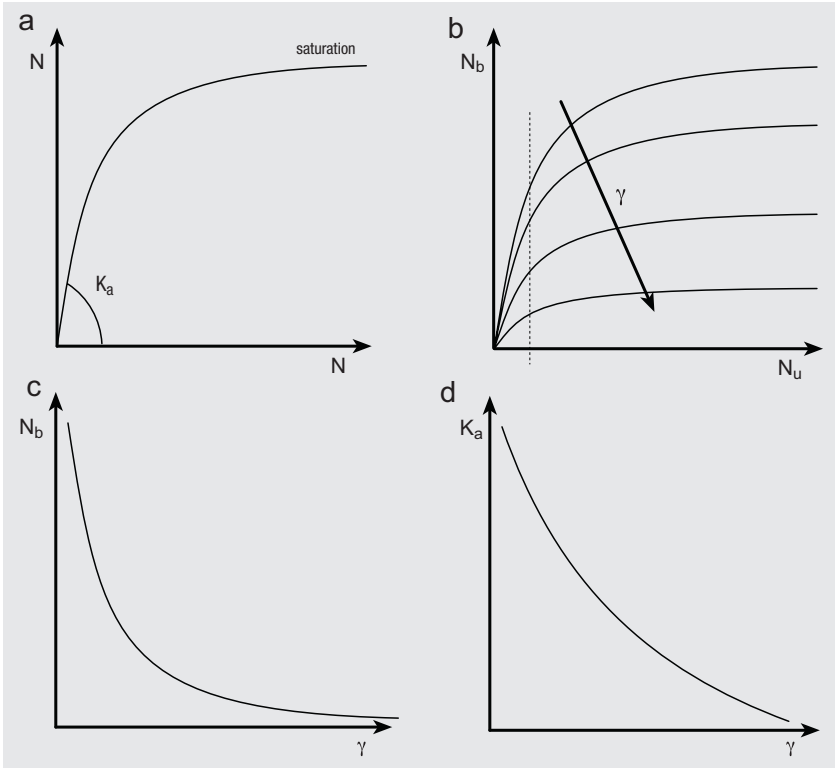

Fig. 3 | *Thermodynamics of adhesion: a) bound versus unbound platelets at equilibrium; b) effect of wall shear rate on the equilibrium curves; non-saturation, constant platelet count conditions are marked by a vertical dotted line; c) effect of wall shear rate on the equilibrium surface concentration of platelets, and d) on the equilibrium constant.*

constant K_{VWF} can be determined through binding assays, while α, β, and δ factors are unknown. According to eqn (5), R_{on} measurements can be used to assess α, the number of bonds truly formed on the surface, from at least two experiments with a different platelet count and/or substrate concentration, being k'_{on} the same. On the contrary, β and δ factors remains undistinguishable from k_{on} within an apparent adhesion kinetic constant, $k''_{on}=\beta\ \delta\ k_{on}$. Unless techniques are identified to independently measure the actual platelet concentration in the marginal layer (*i.e.*, δ) and the number of receptors per platelet (*i.e.*, β), we will not be able to estimate the intrinsic adhesion rate constant k_{on} by measurements of the adhesion rate R_{on}. Uncertainties are mostly in the determination of the platelet concentration at the surface which is heavily dependent on the fluid flow mechanics in specified vessel geometry, and the blood characteristics that can affect it, such as hematocrit and other blood composition variables that can influence its viscosity. We are not aware of any predictive model for radial platelet concentration at the present, so that estimation of pure k_{on} remains an open issue.

Adhesion thermodynamics

Thermodynamics aims at characterizing and predicting the equilibrium in a process. The adhesion-desorption of cells on a surface is said to be macroscopically in an equilibrium state when the rate of surface arrival is equal to the rate of departure from the surface:

$$R_{on} = R_{off} \qquad \text{or} \qquad \frac{dN_b}{dt} = 0 \tag{6}$$

Under such circumstances we can build up the expression of the equilibrium surface concentration of cells by developing the expressions of the rates:

$$R_{on} = k_{on} N_s N_u = k_{off} N_b = R_{off} \tag{7}$$

Combining eqns and we obtain the equilibrium concentration of bound cells:

$$N_b = \frac{K_a N_u N_s^o}{1+\alpha K_a N_u} \qquad K_a \equiv \frac{k_{on}}{k_{off}} \tag{8}$$

definining the adhesion equilibrium constant K_a. eqn has the typical Langmuir adsorption form, as shown in *Figure 3a*.

Figure 3a is similar to results observed in binding assay techniques, in static conditions, that provide the adhesion equilibrium constant (K_{VWF}) used above. Flow experiments give a unique opportunity to investigate the strength of the adhesive bond. Increasing the flow rate, and then the wall shear stress, the amount of platelets bound to the surface as a function of unbound platelet concentrations is expected to decrease, as schematically shown in *Figure 3b*. Experiments at different platelet count can be planned to identify

the whole equilibrium curve, but it is more practical to perform experiments always at the same approximately platelet count. The effect of the wall shear stress on the equilibrium constant can be assessed anyway. For that purpose, non-saturation conditions (see *Figure 3b*) are preferred since K_a variations can be directly measured. In such a case, the number of binding cells is expected to vary with wall shear rate, as shown in *Figure 3c* and consequently the equilibrium constant similarly, as in *Figure 3d*. Note that a lower average concentration of platelets on the surface or a smaller equilibrium constant does not imply lower on- and off-rates. On the contrary they are expected to increase, with the wall shear rate, while the equilibrium surface concentration decreases anyway. The function $K_a(\gamma)$, once experimentally measured, can be used to determine the dependence on wall shear rate of the individual kinetics constants, according to

$$K_a(\gamma) = \frac{k_{on}^0}{k_{off}^0} e^{(B_{on} - B_{off})\gamma} \qquad (9)$$

where *B*'s are the compliance factors explaining the sensitivity of each individual constant to the shear rate. Unfortunately, the assumption of pure removal (neglect of arrival rate) is often used to determine k_{off} so that the observed exponential dependence of the kinetic constants on the applied force (*i.e.*, γ), can be misleading.

CONCLUSIONS

Our results support the definition of a mechanism that links shear-induced stimulation of GPIbα to 2 sequential and distinct stages of platelet activation characterized by specific cytosolic Ca^{++} elevations. These findings provide the basis for a detailed definition of the signaling pathways initiated by the VWF-GPIbα interaction under flow conditions that may regulate platelet participation in hemostasis and thrombosis. The functional importance of these signals in relation to those generated by other thrombogenic substrates, such as collagen, remains to be established. In this regard, the nature of the vascular lesion evoking a platelet response and fluid dynamic conditions may be important in determining which pathway of platelet activation will be followed. For example, injured endothelial cells release VWF that, while bound to their surface, may initiate platelet adhesion and activation in the absence of subendothelial denudation. Clarification of this issue is one of the goals of future studies.

A method of adhesion data analysis, combining tailored image processing techniques, statistical analysis, kinetic and thermodynamics modelling has been proposed. The study is motivated by and has been applied to the initial interaction of platelets with surface-immobilized von Willebrand factor in flow experiments. Results indicate the key role of a reliable automatic image processing procedure to provide information critical in explaining how biomechanical properties of tether bonds ultimately control the process of platelet adhesion. Non-conventional information, such as deposition and removal rates could be obtained in a number of experimental conditions, supporting kinetics and thermodynamics speculations. The effort to develop an appropriate interpretation of adhesion data is a valuable contribution to help elucidating the adhesive properties of platelets that determine their participation in atherogenesis and ultimately the formation of occlusive thrombi.

By understanding the multifaceted mechanism involved in platelet interactions with vascular surfaces, new approaches can be tailored to selectively inhibit the pathways most relevant to the pathological aspects of atherothrombosis.

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