Thrombin-Induced Platelet Aggregates Have a Dynamic Structure
Time-Dependent Redistribution of Glycoprotein IIb–IIIa Complexes and Secreted Adhesive Proteins
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The role of glycoprotein (GP) IIb–IIIa complexes and of adhesive proteins in mediating platelet aggregation is now well defined. However, less is known of the changes that occur once aggregation has begun. We report immunogold staining of thin sections of platelets or platelet aggregates, embedded in Lowicryl K4M, after the use of polyclonal antibodies to GP IIb or GP IIIa, fibrinogen (Fg), von Willebrand factor (vWF), and thrombospondin (TSP). Bound immunoglobulin G (IgG) was located by species-specific anti-IgG coupled to 5-nm gold particles and by electron microscopy. Initial experiments with platelet-rich plasma confirmed the feasibility of visualizing adhesive proteins between platelets in aggregates. Experiments then continued, using stirred suspensions of washed platelets incubated with α-thrombin. After 20 seconds, platelets were in contact without detectable release, although giant secretory vesicles containing adhesive proteins were seen. Internal pools of GP IIb–IIIa were progressively externalized within the aggregate. Secreted Fg was readily detected between platelets at 40 seconds. After 3 minutes, when most of the secretion had occurred, Fg had a patchwork-like distribution within the aggregate. After 6 minutes, zones with closely interspaced surface membranes, usually representing pseudopods, were dominant and Fg free. Results for vWF and TSP were similar to those for Fg. Nonetheless, GP IIb–IIIa complexes continued to be located between adjacent surface membranes throughout the aggregate. Thrombin-induced platelet aggregates were isolated, and sodium dodecyl sulfate-soluble extracts were obtained. Western blot experiments showed that, although fibrinopeptide A had been cleaved, degradation of adhesive proteins by platelet proteases had not occurred. These results emphasize that a platelet aggregate is a dynamic structure and suggest that not all surface-contact interactions are mediated by Fg or the other adhesive proteins tested in this study. (Arteriosclerosis and Thrombosis 1991;11:704–718)

Platelet adhesion and aggregation at local sites of vascular injury are essential events that stop blood loss. Recent advances point to a role for membrane glycoproteins and “adhesive” proteins in these phenomena. For example, an essential pathway for platelet adhesion at high shear rates requires the binding of von Willebrand factor (vWF) exposed within the vessel wall to its platelet receptor, membrane glycoprotein (GP) Ib.1,2 Other platelet membrane glycoproteins implicated in platelet adhesion mechanisms include VLA-2 (GP Ia–IIa) and GP IV, purported receptors for collagen,3,4 and VLA-5 (GP Ic–IIa), the purported receptor for fibronectin.5 Platelet attachment is followed by platelet activation and secretion. Released ADP and metabolites, such as thromboxane A2, participate in platelet activation and thrombus formation.6 Another physiologically important platelet agonist is thrombin, generated in part as a result of procoagulant activity expressed at the activated platelet surface. Recent evidence from the pharmacological inhibition of thrombin generation in animal models of hemostasis points to a key role for thrombin in the formation of mural thrombi.7,8 Platelet activation is accompanied by the development of adhesive properties at the platelet surface,
which promote the formation of membrane bridges and platelet aggregation. Overwhelming evidence points to a role for membrane GP IIb-IIIa complexes in mediating platelet aggregation.5,10 These complexes, members of the integrin superfamily of cell surface receptors,11 express binding sites for fibrinogen (Fg) and other adhesive proteins on stimulated platelets. In vitro experiments show that occupancy of GP IIb–IIIa complexes by Fg correlates well with the onset of platelet aggregation.12,13 Recent results show that Fg binds to activated GP IIb through the extreme carboxyl-terminal amino acid sequence of the Fg γ-chain.14,15 In addition, Fg contains the Arg-Gly-Asp (RGD) sequence at two sites on the Fg α-chain. This sequence binds to GP IIb–IIIa complexes via a receptor on GP IIIa14,16 and is thought to be responsible for the fact that other adhesive proteins, such as vWF or fibronectin (Fn), are also able to mediate platelet aggregation.17 GP IIb–IIIa complexes occur at high density on the platelet surface, with monoclonal antibody binding studies revealing over 50,000 copies per platelet (see Reference 9). Studies of the binding of monoclonal antibodies to platelets or immunocytochemical studies of platelet sections have demonstrated the presence of internal pools of GP IIb–IIIa that are externalized during the activation of platelets by thrombin.18–20 The adhesive proteins are also present in two pools: in plasma, where Fg at approximately 3 mg/ml is by far the most abundant, and the platelet α-granule (see Reference 9). Platelet secretion is accompanied by the release of this storage pool of adhesive proteins, part of which become bound to surface receptors and are retained by the platelet.19,21–23 Although reduced in amount in comparison with the plasma pool, secreted adhesive proteins may play an important role in the local environment of a growing thrombus. As well as Fg, vWF, and Fn, α-granules also contain thrombospondin (TSP), a well-characterized glycoprotein present in trace amounts in plasma under normal conditions, but which participates in platelet aggregation after secretion, probably through its interaction with the Fg already expressed on the activated platelet.23–25

Although the molecular basis for the above pathways appear well defined, most previously reported studies have been performed on platelets activated in suspension and under "nonaggregating" conditions. As a result, little is understood of the surface contact interactions that occur within a platelet aggregate. For example, do the internal pools of GP IIb–IIIa complexes participate in thrombus formation? How are the secreted adhesive proteins organized within the aggregate? These are important questions related not only to platelet involvement in normal hemostasis but also to the pathological role of platelets in the development of arterial thrombosis and to the pharmacological inhibition of this process. We now report our initial results of the organization of GP IIb–IIIa complexes and adhesive proteins within platelet aggregates formed in vitro by thrombin.

Methods

Blood Sampling and Platelet Isolation

Unless otherwise stated, blood was collected from the antecubital vein of healthy volunteers and anticoagulated with acid-citrate-dextrose, National Institutes of Health formula A [ACD-A]; one part anticoagulant to six parts blood. Platelet-rich plasma (PRP) was prepared by centrifugation at 120g for 10 minutes at room temperature. PRP was immediately acidified to pH 6.5 by the addition of one tenth volume ACD-A; then, 100 mmol/l prostaglandin E,, (Sigma Chemical Co., St. Louis, Mo.) and 25 μg/ml apyrase (Sigma) were added as inhibitors of platelet activation. Platelets were sedimented by centrifugation at 1,200g for 15 minutes and washed three times as described elsewhere.22 The washed platelets were resuspended in a modified Tyrode's buffer consisting of (mmol/l) NaCl 137, KCl 2, NaHCO₃ 12, NaH₂PO₄ 0.3, CaCl₂ 2, MgCl₂ 1, glucose 5.5, N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES) 5 (Sigma), and 0.35% bovine serum albumin (BSA; Sigma), pH 7.4 (HEPES-buffered modified Tyrode's solution; HBMT). The platelets were incubated for 20 minutes at 37°C before use.

Platelet Aggregation

Initial experiments were performed with PRP prepared from blood anticoagulated with 3.8% sodium citrate. ADP (4 μM, Sigma) or human α-thrombin (Fibrindex, 0.1 unit/ml, Ortho Diagnostic Systems, Raritan, N.J.) was added to samples adjusted to 3x10⁸ platelets/ml and incubated at 37°C in a platelet aggregometer (model PAP-4, Biodata Corp., Hatboro, Pa.) with stirring (1,100 rpm). Otherwise, washed platelets (3x10⁸/ml) in HBMT were stimulated with human α-thrombin (0.1 unit/ml) without the addition of exogenous Fg. Immunocytochemical studies of platelet aggregates were performed on samples taken from the aggregometer cuvette with aggregation stopped at different times after the addition of the agonist. A typical experiment is illustrated in Figure 1. As a control of the efficiency of the washing procedure, platelet suspensions were regularly tested for their aggregation response to ADP (4 μM) in the presence or absence of added Fg. Only those preparations that yielded rapid aggregation in the presence of added Fg and a negligible response in its absence were used in this study. Ristocetin-induced platelet aggregation was performed on samples (0.2 ml) of washed platelets mixed with an equal volume of platelet-poor plasma (PPP) prepared by centrifuging blood anticoagulated with 3.8% wt/vol sodium citrate at 12,000g. Ristocetin (H. Lundbeck & Co., Copenhagen, Denmark) was added to 1.5 mg/ml, and the samples were stirred to obtain a full-scale response on the aggregometer recorder.

Sample Preparation for Electron Microscopy

Suspensions (0.5 ml) of platelets were added to 20 ml 1.25% (vol/vol) glutaraldehyde (Fluka AG, Buchs,
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Switzerland) diluted in 0.1 mol/l phosphate buffer, (PBS), pH 7.2.25,26 Fixation was continued for 20 minutes at room temperature, after which the platelets were washed twice with PBS. The samples were preembedded in a low-gelling-temperature agarose and dehydrated through a series of graded alcohols at -20°C before being embedded in Lowicryl K4M (Taab, Aldermaston, U.K.). Photopolymerization of the hydrophilic resin was performed at 4°C for 2 days. Ultrathin sections were obtained using an Ultracut E Ultramicrotome (Reichert, Vienna) and were subsequently mounted on collodion carbon-coated nickel grids. Platelets from a patient with type I Glanzmann's thrombasthenia (J.R.) were examined in early studies performed with this procedure. J.R. is patient 8 in the recent report of George et al.27

Immunogold Staining

All primary antibodies were polyclonal antibodies to purified proteins. Sera containing rabbit antibodies to GP IIb and GP IIIa were the kind gifts of Rodger McEver (Oklahoma University Medical Center, Oklahoma City) and Dominique Fidard (Unité 150 INSERM, Hôpital Lariboisière, Paris, France). Both sets of antibody have been previously characterized and have been shown to be monospecific for antigens carried by the GP IIb–IIIa complex.28,29 A serum containing rabbit antibodies to TSP was generously supplied by Deane Mosher (University of Wisconsin, Madison, Wis.). Commercial rabbit antisera to Fg and vWF were purchased from Dakopatts (Glostrup, Denmark). Each of the above antibodies was tested by Western blotting against platelet or plasma proteins and was confirmed to be monospecific for the antigen in question (see "Results"). For most experiments, immunoglobulin G (IgG) was isolated by chromatography on Protein A–Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden) performed according to standard procedures. Controls were performed with IgG from nonimmune rabbit sera. Antibody concentrations were those shown in preliminary experiments to give maximum labeling of sections of Lowicryl-embedded platelets. Incubations were for 2 hours at room temperature in a moist chamber and were performed by floating the grids (each bearing a single section) onto a drop of antibody-containing solution (primary antibody concentrations are given in the figure legends). Grids were then rinsed by floating five times onto PBS containing 0.5% wt/vol BSA. They were then transferred to a solution containing a 1/70 dilution of affinity-purified IgG of goat antisera to rabbit IgG coupled to 5-nm gold particles (Auroprobe EM GAR G5, Janssen Pharmaceutica, Beerse, Belgium). Incubation was again for 2 hours, after which the sections were rinsed with PBS–BSA, and the sections were "stained" with uranyl acetate and lead citrate before being observed in a Philips Model EM 201 electron microscope (Eindhoven, Holland).

Western Blot Procedure

Washed platelets were prepared as described above except that BSA was omitted from the buffer used in the final washing step and from the HBMT resuspension medium. Samples (0.5 ml) were stimulated, with stirring, at 37°C with 0.1 unit/ml α-throm-
bin in the platelet aggregometer. Here, hirudin (Sigma, 1 unit/ml) was added at different time intervals (see text), and the platelets were immediately sedimented by centrifugation at 12,000g for 0.5 minute in an Eppendorf centrifuge (Eppendorf GmbH, Hamburg, F.R.G.). The pellets were resuspended in 10 mmol/l tris(hydroxymethyl)aminomethane (Tris) HCl, 0.15 mol/l NaCl, 3 mmol/l EDTA, and 5 mmol/l N-ethylmaleimide, pH 7.0 (TNEM buffer); a one fifth volume of 12% wt/vol sodium dodecyl sulfate (SDS) was also added. Supernatants containing the released proteins were diluted with an equal volume of TNEM, and a one fifth volume of 12% SDS was likewise added. Protein concentrations were estimated (see Reference 29), and samples were heated at 100°C for 5 minutes in the additional presence of 0.1 mol/l dithiothreitol. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 7-12% gradient gels according to the Laemmli system as previously detailed by us.29-30 Protein staining was performed with Coomassie Blue R-250.30 Electro-transfer of proteins to nitrocellulose membranes (Bio-Rad, Richmond, Calif.) was effected by use of a semidy transfer apparatus (Bioloyon, Dardilly, France). Saturation of protein binding sites was achieved by incubating the membranes, after transfer, for 1 hour with 0.15 mol/l NaCl, 5% wt/vol skimmed milk (Gloria SA, Paris, France), and 20 mmol/l Tris HCl, pH 8.2 (saturating buffer). Incubation with primary antibodies (those listed above, with the addition of rabbit antibody to fibrinopeptide A from Diagnostica Stago, Paris, France) was performed in saturating buffer but with the skimmed milk diluted to 0.5%. Incubations were overnight at room temperature. After extensive washing, the membranes were incubated for 4 hours with a 1/150 dilution of goat antibody to rabbit IgG absorbed onto gold particles (Auroprobe BL plus GAR IgG, Janssen Pharmaceutica). After several rinses in buffer and then in distilled water, revelation of bound antibody was enhanced by silver staining (IntenSE Blotting, Janssen Pharmaceutica), performed according to the manufacturer's instructions.

**Results**

**Detection of Surface-Bound Adhesive Protein in a Platelet Aggregate**

Initial experiments evaluated the feasibility of detecting adhesive protein between adhering platelets by immunogold staining of sections of platelet aggregates embedded in the resin Lowicryl K4M. Studies were first performed with a rabbit antibody to vWF, and platelets agglutinated with ristocetin in the presence of PPP. The samples were fixed in glutaraldehyde 30 seconds after the addition of ristocetin (when the platelet aggregometer tracing had reached its maximum). Figure 2a shows a regular protein-filled gap between adhering platelets and that immunogold staining was regularly distributed within this space. The large size of the vWF polymers clearly facilitated their detection: Distinct membrane-bound "masses" were revealed to be labeled by the gold beads. Figure 2b illustrates platelets aggregated in citrated PRP with 4 µM ADP. Immunogold staining with anti-Fg antibody resulted in distinct labeling of what appeared to be protein bridges linking adjacent platelets. Similar results were obtained with washed platelets stimulated with ADP in the presence of added exogenous Fg; as soon as platelet aggregates started to form (after 10 seconds), Fg could be detected between the external surfaces of platelets in contact (data not illustrated). Figure 2c shows immunogold staining of platelets activated by thrombin in citrated PRP. Here, samples were fixed well after the onset of platelet aggregation (30 seconds) but before clot formation. Staining was abundant, with the intercellular spaces clearly labeled by the anti-Fg antibody. Fibrin polymerization had already begun, as fibrin strands were revealed attached to some platelets. They were heavily stained. These initial studies confirmed that 1) bound adhesive proteins could be detected in the intercellular space between aggregated platelets and 2) the anti-Fg antibody labeled both Fg bound to platelets stimulated with ADP and fibrin formed when thrombin was added to citrated PRP.

** Redistribution of Endogenous Fibrinogen During Thrombin-Induced Aggregation of Washed Platelets**

The elevated concentration of Fg in plasma precludes specific ultrastructural studies on the redistribution of secreted α-granule Fg in platelet aggregates formed in PRP. However, as thrombin-induced platelet aggregation does not require added exogenous adhesive protein cofactors,32,33 washed platelets can be used in these studies. Platelets were stimulated with 0.1 unit/ml α-thrombin, the lowest dose that consistently gave a rapid and irreversible aggregation response in the platelet aggregometer (see Figure 1). Figure 3 illustrates the changes in the redistribution of platelet Fg within the first minute of stimulation. Immunogold labeling of ultrathin sections of unstimulated platelets showed abundant labeling of the α-granules (Figure 3a). Labeling was fairly uniform, with the exception of the nucleoid zone. There was no staining of the platelet surface. Granules of the platelets from the patient with type I Glanzmann's thrombasthenia exhibited little or no labeling, confirming the specificity of the anti-Fg antibody used in these studies (data not illustrated). Twenty seconds after addition of thrombin, the platelets showed clear evidence of platelet activation. Pseudopods had formed, and many granules had centralized. Giant secretory vesicles were now evident (Figure 3b). Fg was clearly present within these vesicles and interestingly, immunogold staining was most intense in close proximity to the vesicle membrane. Aggregometer tracings confirmed that at 20 seconds, platelet aggregation had already started (see Figure 1). However, as yet, Fg was rarely observed on the platelet surface. Nonetheless, the
**FIGURE 2.** Photomicrographs showing that adhesive proteins may be detected between aggregated platelets by immunogold staining on sections of Lowicryl K4M-embedded samples. Platelet stimulation was performed in a platelet aggregometer. In panel a, washed platelets diluted with an equal volume of citrated platelet-poor plasma were incubated with 1.5 mg/ml ristocetin. Fixative was added after 30 seconds when platelet agglutination reached maximum. After embedding, thin sections were sequentially incubated with rabbit anti-von Willebrand factor (vWF) immunoglobulin G (IgG) (100 μg/ml) and goat anti-rabbit IgG coupled to 5-nm gold particles. In panels b and c, platelets were stimulated in citrated platelet-rich plasma, and sections were stained for fibrinogen (Fg) with rabbit anti-fibrinogen IgG (100 μg/ml). In panel b, platelets were aggregated by 4 μM ADP, and fixative was added after 1 minute when aggregation had reached maximum. In panel c, platelets were incubated with 0.1 unit/ml α-thrombin for 30 seconds, and fixative was added before clot formation occurred. Here, fibrin strands (FS) can be detected between adjacent platelets. Bar=0.2 μm.

**FIGURE 3.** Photomicrographs showing distribution of endogenous fibrinogen (Fg) on sections of unstimulated platelets (panel a) and thrombin-aggregated washed platelets (panels b–d). Washed platelets (3×10⁸/ml) were stirred with 0.1 unit/ml thrombin for different times. After fixation and embedding, sections were sequentially incubated with rabbit anti-Fg antibody (100 μg/ml) and goat anti-rabbit immunoglobulin G coupled to 10-nm (panel a) or 5-nm (panels b–d) gold particles. Panel a: Unstirred washed platelets were fixed before addition of thrombin. Staining is exclusive to α-granules (G). Panel b: Twenty seconds after addition of thrombin, platelets have lost their discoid form, and zones of their surface membranes are in close contact. However, filamentous structures apparently joining platelets are not labeled. Gold particles are to be seen labeling large secretory vesicles (SV). Panel c: Forty seconds after addition of thrombin, Fg is now detected at the platelet surface, although giant secretory vesicles still predominate. Panel d: After 1 minute, labeling in the intercellular space is more abundant (see arrowheads). Bar=0.5 μm.
sections clearly revealed the presence of platelet-platelet contacts. Immunogold staining was occasionally found on the luminal side of the open canalicular system membranes. After 40 seconds, large aggregates were present (Figure 3c). Despite the fact that much of the Fg was still present in secretory vesicles, immunogold staining was now detected in the intercellular space separating adjoining platelets. After 1 minute, fewer vesicular structures remained, and residual α-granules were rare (Figure 3d). Secretion was clearly well advanced and immunogold staining in the intercellular spaces was more abundant. Nonetheless, the gold beads tended to occur in patches, and not all protein bridge structures between adjacent platelets were labeled. Occasional well-stained zones may represent sites where the secretory vesicles open into the open canalicular system or intercellular space. Exocytosis was clearly continuing, even though the platelets were already attached to each other.

Redistribution of Glycoprotein IIb–IIIa Complexes During Thrombin-Induced Aggregation of Washed Platelets

Figure 4a illustrates the distribution of GP IIb–IIIa complexes in unstimulated platelets as revealed by a rabbit antibody to purified GP IIb. Immunogold staining of the platelet surface was both regular and abundant, and the internal pool was particularly well conserved. Despite the relatively limited conservation of organelle structures using the Lowicryl K4M-embedding technique, anti-GP IIb antibody staining could be clearly localized to the membranes of vacuolar structures, probably belonging to the open canalicular system, and to those of storage organelles. The size of the latter, together with the occasional presence of what appeared to be a nucleoid, strongly suggested that they were α-granules. Identical results to those presented in Figure 4a were obtained using a second rabbit antibody to GP IIb and two different rabbit antibodies to GP IIIa (not illustrated; see “Methods”). When platelets lacking GP IIb–IIIa complexes from the patient with type I Glanzmann’s thrombasthenia were examined, labeling of both the surface and internal membrane systems was drastically reduced, confirming the specificity of the staining (Figure 4b). Stimulation of normal platelets with thrombin resulted in marked changes in the distribution of the GP IIb–IIIa complexes. Results obtained 20 seconds after addition of the thrombin are shown in Figure 4c. Staining was abundant over the entire surface of the platelets, and it should be noted that pseudopods were clearly labeled by the antibody. Labeling of internal membranes now included the peripheral membranes of the giant secretory vesicles. Translocation of the internal pool of GP IIb–IIIa toward the surface was already evident after 1 minute (not illustrated) and after three minutes was well advanced (Figure 4d). This was evidenced by the diminished staining within individual platelets. It is to be noted that the GP IIb–IIIa complexes of surface membranes of adjoining platelets continued to be accessible to the anti-GP IIb antibody and that the staining was regular both over the surface of a single platelet section and within different zones of the aggregate. Interestingly, the high-power insert in Figure 4e shows how the labeling often appeared associated with the short filamentous bridges present between the adherent regions of adjacent platelets.

The Aggregate Is a Dynamic Structure

When immunogold staining with anti-Fg antibody was performed on samples fixed 3 minutes after stimulation, labeling within the aggregate appeared more heterogeneous than after 1 minute (not illustrated). Figure 5 illustrates samples fixed 6 minutes after the addition of thrombin and shows how morphological changes had continued to occur in thrombus structure. In particular, zones enriched in pseudopods were now evident. Immunogold staining showed that practically all GP IIb–IIIa complexes were now located within surface membranes, and little internal staining was seen (Figure 5a). Labeling was fairly uniform across the whole section. In contrast, immunogold staining for Fg had become very heterogeneous, being concentrated in discrete areas within the aggregate (Figure 5b). Contact zones between pseudopods were frequently free of labeling with anti-Fg antibody. No morphological differences could be observed between contact sites labeled by the anti-Fg IgG and those nonreactive with the antibody.

Immunogold Staining for von Willebrand Factor and Thrombospondin

The question arose as to whether other secreted proteins were distributed differently within the aggregate. Immunogold staining with anti-vWf antibody was performed on samples fixed 3 minutes after stimulation. Contact points between adjacent platelets were well stained for GP IIb (small arrowheads). Membranes of central α-granules (G) and giant secretory vesicles (SV) are both labeled. Panel d: After 3 minutes, label is rarely seen within the platelets but is observed on surface membranes throughout the aggregate. Panel e: This higher-magnification view from part of panel d details the labeling pattern observed for GP IIb at points of contact, where gold particles are firmly attached to the radial and repetitive bridge structures. Unless indicated otherwise, bar=0.5 μm.
aggregate and were contributing to the adhesive reactions that linked platelets together. Sections identical to those illustrated in the previously mentioned studies were therefore stained with antibodies to vWF and TSP. As was shown earlier for Fg, both antibodies exclusively stained \( \alpha \)-granules in unstimulated platelets (not illustrated). The anti-vWF recognized distinct structures, first seen by Cramer et al,\(^{33} \) who likened them to Weibel-Palade bodies. In contrast, but like Fg, TSP was distributed throughout the \( \alpha \)-granule, although avoiding the nucleoid. Neither antibody stained surface membranes. Three minutes after stimulation, vWF was primarily located in giant secretory vesicles and in intercellular spaces (Figure 6a). However, staining was weak and was not localized to protein bridges between adjacent platelets. Staining with anti-TSP was much more intense (Figure 6b), possibly related to the increased amount of
FIGURE 6. Photomicrographs showing distribution of secreted platelet von Willebrand factor (vWF) and thrombospondin (TSP) within the aggregate. Sections of thrombin-induced platelet aggregates were stained for vWF (panels a and c) or TSP (panels b and d) using rabbit polyclonal antibodies (immunoglobulin G, 100 μg/ml) to purified proteins. After 3 minutes (panels a and b), both proteins were detected in the intercellular space between aggregated platelets, although staining for TSP was more abundant. After 6 minutes (panels c and d), the distribution within the aggregate was much more heterogeneous. As for fibrinogen, zones enriched in pseudopods (PS) were depleted of both vWF and TSP. Bar=0.5 μm.

TSP in platelets (see Reference 9). Here, there was abundant staining of the intercellular space between platelets. Six minutes after the addition of thrombin, immunogold staining for vWF (Figure 6c) and TSP (Figure 6d) was much more heterogeneous. As for Fg, staining was now confined to discrete zones within the aggregate.

Western Blot Analysis of Adhesive Proteins Within the Aggregate

The next question was whether the time-dependent changes in the location of adhesive proteins within the aggregate was due to their degradation and/or to their possible elimination from the aggregate. Thrombin was added to stirred, washed platelet suspensions, and samples of the treated platelets (or aggregates) and of the proteins released into the supernatant were analyzed by SDS-PAGE followed by Western blotting with monospecific antisera. Figure 7 first shows the results obtained with anti-Fg sera. In the absence of thrombin, virtually all the Fg sedimented with the platelets (confirming the absence of residual plasma from the suspension). It should be noted that the anti-Fg antisera used in this
Fibrinogen Fibrinopept. A vWF TSP

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**FIGURE 7.** Evaluation of the integrity of adhesive proteins within an aggregate by Western blotting. Thrombin-induced platelet aggregation was performed as before. Hirudin was added at different time points and platelets were sedimented to allow separation into pellet (p) and supernatant fractions (sn) (see “Methods”). Samples (50 μg) of SDS-soluble reduced platelet proteins or sn fractions (50 μl) were subjected to electrophoresis on 7-12% polyacrylamide slab gels before transfer onto nitrocellulose membrane. Membranes were incubated with monospecific antibodies to fibrinogen (diluted 1:200), fibrinopeptide (fibrinopept.) A (diluted 1:100), von Willebrand factor (vWF) (diluted 1:100), or thrombospondin (TSP) (diluted 1:200) before visualization of bound rabbit IgG with a 1:150 dilution of goat antibody to rabbit IgG coupled to gold particles followed by silver enhancement. Molecular weight measurements were made relative to the migration of standard molecular weight markers. IgG, immunoglobulin G; SDS, sodium dodecyl sulfate; unstim./unst., unstimulated; min., minute(s).

**Discussion**

Although much progress has been made in the understanding of the molecular pathways responsible for platelet aggregation (see the introductory section), most reports have concentrated on events that accompany the activation of unstirred platelet suspensions. The objective of the present study was to investigate those changes that occur within platelet aggregates. To do so, we performed immunogold staining of thin sections of platelets fixed at different times after stimulation and embedding in Lowicryl K4M resin. Similar methods have been used previously to investigate the distribution of membrane glycoproteins or adhesive proteins within platelets. An alternative approach has been to perform immunogold staining on frozen sections. In our experience, preliminary studies have shown that the morphology of platelet aggregates was better preserved in resin-embedded samples, although we acknowledge that membrane structure is not well conserved. Another noticeable disadvantage of the Lowicryl K4M–based procedure is that reactivity with monoclonal antibodies is often lost. It was for this reason that polyclonal antibodies were used in our studies.

Initial studies showed staining of both the plasma membrane and internal membrane pools of GP IIb–
IIIa complexes in unstimulated platelets. Although the antibodies used were prepared against SDS-denatured proteins, the fact that results for antibodies to GP IIb and to GP IIIa were identical, together with the previous reports that more than 90% of GP IIb and GP IIIa in platelets are present in the complexed form (see Reference 10), suggest that it is GP IIb–IIIa complexes that are being recognized. Such a conclusion is also in agreement with the recent report of Cramer et al., who observed staining of both membranes of the open canalicular system and of α-granules when sections of unstimulated human platelets were incubated with a polyclonal antibody directed against purified nondenatured GP IIb–IIIa. Studies with antibodies monospecific for each adhesive protein confirmed previous immunocytochemical studies showing that Fg, vWF, TSP40 are primarily localized to the α-granules of unstimulated platelets. Heterogeneity in their distribution within the α-granule, with an absence of staining of the nucleoid, was also confirmed.

A prerequisite of our approach was that the immunocytochemical procedures would be sufficiently sensitive to allow us to detect bound adhesive protein linking adjoining platelets within an aggregate. Studies with the use of a rabbit antibody to vWF showed regular labeling within the largely protein-filled space between platelets agglutinated by ristocetin, a process mediated by the binding of vWF to GP Ib. Such results are consistent with those obtained when ferritin-coupled antibody was used in electron microscopy to examine ristocetin-induced binding of vWF to platelets. Quite large structures were visualized, probably due to the size of the bound vWF multimers (>10^5 daltons). Next, we examined the presence of Fg between platelets aggregated by ADP. A consensus from early ultrastructural studies on platelets was that the gap between adjoining surface membranes of platelets aggregated by ADP was of the order of 50 nm and that protein bridges crossed this space in the aggregate (reviewed in Reference 42). Such bridges could be seen in our sections (Figure 2b), although conditions for their visualization were not optimal (lack of tannic acid staining, for example). These structures were clearly although lightly labeled with anti-Fg antibody. The size of the Fg molecule, 45×9 nm (see Reference 43), and its multivalency for the GP IIb–IIIa receptor (see Reference 13) would permit it to bridge adjacent platelets. Similar results were also obtained when washed platelets were stimulated with ADP in the presence of purified Fg, even at early time points. When platelets in PRP were stimulated with thrombin, staining was more abundant. However, this was due, at least in part, to the rapid formation of fibrin polymers, clearly revealed at the platelet–platelet interfaces 30 seconds after platelet stimulation.

The major part of our studies were performed on platelets washed free of plasma proteins and stimulated with human α-thrombin under conditions that led to rapid secretion. Morphological analysis confirmed that platelet stimulation was accompanied in the first 20 seconds by pseudopod formation, granule centralization and, through granule fusion, the appearance of giant secretory vesicles. The latter structures represent one of the first stages of the secretion process. These vesicles were readily stained with antibodies to Fg and to GP IIb or GP IIIa. Interestingly, immunogold staining for Fg was now often concentrated in the area next to the α-granule membrane (see Figure 3b). Two recent reports have suggested that part of the endogenous pool of Fg is expressed on the platelet surface already bound to membrane receptors. This conclusion was based on observations that the surface expression was not inhibited by agents, for example, RGD-containing peptides or monoclonal antibodies to GP IIb–IIIa complexes, that are effective inhibitors of the binding of plasma Fg to activated GP IIb–IIIa complexes. Our findings increase the speculation that at least some of the internal pools of GP IIb–IIIa complexes are activated before their exposure on the platelet surface. Studies of unstirred platelet suspensions have shown that internal pools of GP IIb–IIIa complexes externalize after addition of thrombin. This process may accompany secretion, with α-granule membranes (or those of giant secretory vesicles) fusing with plasma membranes or those of the open canalicular system. Our study confirms that membrane movements and/or translocation of internal pools of GP IIb–IIIa complexes continue long after platelet aggregation has begun and the initial platelet–platelet contacts have been formed. Escolar et al., who showed that surface activation and spreading of platelets on formvar grids was accompanied by a progressive depletion of the open canalicular system. It is tempting to speculate that this also occurs in thrombin-induced aggregates when the “surface” would be provided by adjacent platelets. Whatever the mechanism, one consequence of this process of externalization is an increased number of GP IIb–IIIa complexes available to participate in surface contact interactions within the aggregate.

Immunocytochemical studies on platelets stimulated under nonaggregating conditions suggest that secreted Fg is expressed as discrete masses on the platelet surface. Our studies and those recently published by Suzuki et al. show that the distribution of Fg is heterogeneous within the aggregate. This may be expected if Fg is being released from α-granules or giant secretory vesicles into channels of the open canalicular system. Such a process would lead to zones of high concentration awaiting diffusion of free Fg and/or Fg bound to membrane receptors. What was unexpected in our study was the fact that this heterogeneity became greater as the aggregate aged. After 6 minutes, the morphology of the aggregate had considerably changed, and its appearance suggested that the platelets had formed many long pseudopods. Although GP IIb–IIIa complexes continued to be located between all adjacent surface membranes, many of the apparently “pseudopod-
rich zones were free of Fg. In view of this, we examined the distribution of two other secreted proteins, vWF and TSP, within the aggregate. Even in the 1–3-minute period, staining for vWF in the intercellular spaces was weak compared with that seen for Fg, this despite the antibody having successfully located vWF bound to platelets incubated with ristocetin (see Figure 2). This may reflect the relatively small amount of vWF in platelets (see Reference 9). In contrast, staining for TSP was abundant. In both cases, however, staining was very patchy after 6 minutes, with zones showing little or no immunogold staining for either protein. Preliminary double-staining procedures with goat anti-Fg, and rabbit anti-vWF or rabbit anti-TSP located with species-specific anti-IgG antibodies conjugated to differently sized gold beads suggest that individual secreted adhesive proteins colocalize within the aggregate (E. Heilmann, unpublished observations).

We obtained no evidence for the widespread degradation of secreted adhesive proteins by platelet proteases during the time course of our experiments. Both Fg and vWF are potential substrates for calpain, a Ca\(^{2+}\)-activated platelet protease.\(^{46,47}\) Largely considered as a cytosolic constituent, the presence or absence of calpain from the stimulated platelet surface is controversial.\(^{46,49}\) Nonetheless, surface-bound enzyme was seemingly not active within the aggregate under our experimental conditions. Similarly, we found no evidence for the presence of cross-linked fibrin polymers even 6 minutes after addition of thrombin to washed platelets. This was despite extensive cleaving of fibrinopeptide A from the platelet Fg by thrombin. Such a finding agrees with the immunocytochemical studies of Sixma et al.,\(^{50}\) who showed that factor XIII (subunit a) was distributed throughout the cytoplasm of human platelets.

The evidence for a role for Fg or other adhesive proteins in platelet aggregation is overwhelming (see the introductory section). Studies of patients with Glanzmann's thrombasthenia underline the essential role played by GP IIb–IIIa complexes for an absence or a severe deficiency of GP IIb-IIIa complexes from the platelets of these patients is accompanied by a total absence of platelet aggregation as induced by all physiological agonists.\(^{77}\) Our studies reveal two situations where platelet–platelet contact is seen without immunogold staining of adhesive proteins: 1) within the first 20 seconds of thrombin-induced platelet aggregation and 2) within regions of the aggregate enriched in pseudopods 6 minutes after addition of thrombin. Several explanations could account for these findings (which could involve different interactions). The first is that they are mediated by adhesive proteins known to be secreted from platelets but that were not studied by us. Two such proteins are fibronectin\(^{19,40}\) and vitronectin.\(^{51}\) However, a role for these proteins in the early stages of thrombin-induced platelet aggregation would seem unlikely, as morphological studies revealed that little secretion had occurred, and that more abundant \(\alpha\)-granule proteins, such as Fg, which binds readily to GP IIb-IIIa complexes, were not detected. A second possibility is that secreted Fg or other adhesive proteins mediate these events but are present at concentrations too low to be detected by immunogold staining as performed by us and by Suzuki et al.\(^{36,37}\) This eventuality is difficult to exclude. Kinetic studies show that at least 20,000 molecules of Fg can be bound by platelets activated with ADP or thrombin and that binding reaches saturation within 30 minutes of the addition of the agonist.\(^{12,13}\) Yet, as illustrated in Figure 1, platelet aggregation begins within 10 seconds of the addition of the stimulus. The question arises, therefore, as to how many GP IIb–IIIa complexes are occupied at this time point. It is possible that the binding of relatively few Fg molecules (or those of other adhesive proteins) is required to initiate platelet aggregation and that the high density of GP IIb–IIIa receptors facilitates the platelet–platelet interaction rather than the initial Fg binding. Nonetheless, both Suzuki et al.\(^{36}\) and ourselves (E. Heilmann, unpublished data) have shown that Fg can be clearly detected between platelets aggregated by ADP. Indeed, our own results show this to be so even for samples fixed as soon as 10 seconds after addition of the stimulus.

Another possibility, therefore, is that some platelet contact interactions do not require adhesive proteins. Indirect evidence for this is obtained from platelet pathology. For example, thrombin-induced aggregation of platelets from patients with congenital afibrinogenemia, severely deficient in both the plasma and platelet pools of Fg, proceeds normally.\(^{21,32}\) Although it has been proposed that vWF may substitute for Fg in these patients,\(^{52}\) in the case of washed platelets, this would have to come from the \(\alpha\)-granule pool, and, as we have shown, platelet–platelet binding occurs before the bulk of the secretion has occurred. Furthermore, in the gray platelet syndrome, characterized by deficiencies of all \(\alpha\)-granule proteins, the aggregation of washed platelets by thrombin is not absent although it can be delayed.\(^{53}\) Perhaps, therefore, membrane glycoproteins participate in the surface contact interactions of platelets by binding to each other. A possible candidate is PECAM-1, a recently characterized glycoprotein that is common to platelets and endothelial cells and that is localized within tight junctions of endothelial cells.\(^{54}\) It should be noted that binding with both anti-GP IIb and anti-GP IIIa antibodies identified filament-like structures that extended between adjoining surfaces (see Figure 4e). The reported size of the complexes, upwards of 20–30 nm,\(^{45}\) is sufficient to permit a direct interaction across a 50-nm space, and their involvement in such contact interactions would be compatible with the widespread nature of the platelet aggregation defect in Glanzmann’s thrombasthenia (see above). Nonetheless, gold particles distant from the phospholipid bilayer could represent second-antibody binding to rabbit IgG attached to an
outer part of GP IIB-IIIa. Further studies will be required to evaluate the possibility of direct interactions between membrane glycoproteins within the aggregate.

In conclusion, we have provided evidence that a platelet aggregate is a dynamic structure. Such observations may be of significance with regard to the treatment of thrombotic disease. Monoclonal antibodies to GP IIB-IIIa complexes or peptide sequences inhibiting the interaction of adhesive proteins with their receptors provide an important new approach to preventing thrombus formation in vivo (reviewed in Reference 56). However, these drugs may be less effective in facilitating the dissociation of preformed thrombi. The fact that the secretion of adhesive proteins and the externalization of membrane glycoproteins continue within the aggregate suggests a role for these components in the consolidation of the thrombus. Another possibility is that bound ligands may interact within the plane of the plasma membrane. Although there is evidence that surface-bound adhesive proteins and occupied GP IIB-IIIa receptors can form clusters on single platelets, it is difficult to see how this could be an ongoing event if they were cross-linking two adjacent platelets when motile forces would presumably just as likely be opposing as complementary. Further elucidation of the nature of the interactions within the platelet aggregate may lead to the development of agents better able to effect thrombus dissolution.

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