

Anniversary Issue Contribution

Do ADP-activated platelets *in vitro* aggregate as discocytes ?Eberhard Morgenstern¹, Nicole Boeckel¹, Ernst F. Lüscher^{2†}¹Formerly Medical Biology, Saarland University, Homburg, Germany; ²Formerly Theodor-Kocher-Institute, University of Berne, Switzerland

Ernst F. Lüscher had posed some interesting questions in 2000 about the time flow of the first platelet reactions after activation: shape change (SC) or aggregation (1). Regarding in-vivo studies – commencing on the observations of Bizzozero in 1882 (2) – Lüscher deduced from the literature and from own investigations (3, 4) that shape change which precedes aggregation would be an in-vitro artefact. Indeed, a plethora of ultra-structural studies dealt with the examination of aggregated platelets starting with the investigations of Born et al. 1980 (5), and Born and many others used transmission electron microscopy (TEM) to support the view that the initial decrease in light transmission in platelet aggregometry was caused by platelet shape change. Yet, recent morphological findings could attribute aggregates of discoid platelets (designated discocytes) to the initial change in the aggregometer curve (6). More recently, a shear-dependent in-vitro aggregation of discoid platelets was demonstrated using differential interference contrast microscopy or scanning electron microscopy (7–9). These initially unstable aggregates arise under the formation of membrane tethers between platelets and an adhesive surface. Tether formation involves the adhesive function of glycoprotein Ib/V/IX complex on von Willebrand factor. The conversion of aggregates from discoid platelets into stable aggregates consisting of platelets showing shape change requires the release of ADP. Apart from a study investigating aggregation in a genetic defective mouse (10), until now no in-vitro aggregate formation between discoid mammalian platelets could be observed in the TEM. Inspired by the aforementioned implications (1) we investigated whether it is possible to demonstrate aggregation of discoid platelets *in vitro*. To induce aggregation we chose ADP, an agonist of platelets released from damaged cells, particularly erythrocytes, at sites of vascular injury (11). Lüscher initiated these investigations and inspiringly accompanied them up to his sudden death in April 2002.

Results presented in this report indicate that already 1.5 seconds (s) after ADP-stimulation of discoid platelets in-vitro fibrinogen molecules initiated the first contacts between platelets still retaining their discoid shape. The contacts were seen at any site of the platelet surface. One second later, shape change took

place, and focal contacts were formed on the platelet body associated with the constricting contractile gel. The existence of bridging fibrinogen molecules during the aggregation was confirmed by immunolabelling with anti-fibrinogen antibody. Thus, platelet aggregation appears to start with discoid platelet-fibrinogen contacts under the chosen in-vitro conditions, and the two-stage process leads to formation of stable aggregates of platelets with a changed shape.

Materials and methods

Antibody

Rabbit-anti human-fibrinogen IgG Dakopatts (Denmark) was used.

Platelet preparation

Platelet-rich plasma (PRP) was obtained by centrifugation (16 minutes [min], 230 g) from ACD NIH (formula A) anticoagulated venous blood of healthy volunteers. Platelets were stimulated with ADP (20 µg/ml PRP) under stirring at 37°C. After 1.5 or 2.5 s the platelet reaction was stopped by conventional fixation or paraformaldehyde fixation (see below). Platelet aggregates (1 min after ADP stimulation) were prepared for freeze sectioning and immunolabelling (see below).

Electron microscopy

Conventional technique for morphological characterization

Platelet aggregates (1.5 or 2.5 s after ADP stimulation) were prefixed by addition of 40 µl/ml 5% glutaraldehyde (Roth, Karlsruhe, Germany) in 0.1 M cacodylate buffer, pH 7.2, in order to stop all platelet reactions after the chosen time period prior to subsequent centrifugation. Pellets were fixed with the fixation solution for 1 hour (h). Postfixation was performed with osmium tetroxide according to Caulfield, and the specimen were dehydrated in acetone and embedded in araldite. Semi-thin sections prepared with glass knives were stained with methylene blue and examined by light microscopy. Ultra-thin serial sections cut with the Ultracut E ultramicrotome (Reichert-Jung, Vienna, Austria) using a diamond knife were stained (uranyl acetate and lead ci-

Correspondence to:
Prof. Dr. Eberhard Morgenstern
Fakultät 2; Theoretische Medizin
Universität des Saarlandes
Campus Homburg, Geb. 76
D – 66421 Homburg/Saar, Germany
Tel.: +49 6841 16 26252, Fax: +49 6841 16 26227
E-mail: e.morgenstern@mx.uni-saarland.de

[†]Deceased in 2002.

Received May 7, 2007
Accepted May 8, 2007

Prepublished online June 12, 2007
doi:10.1160/TH07-05-0333

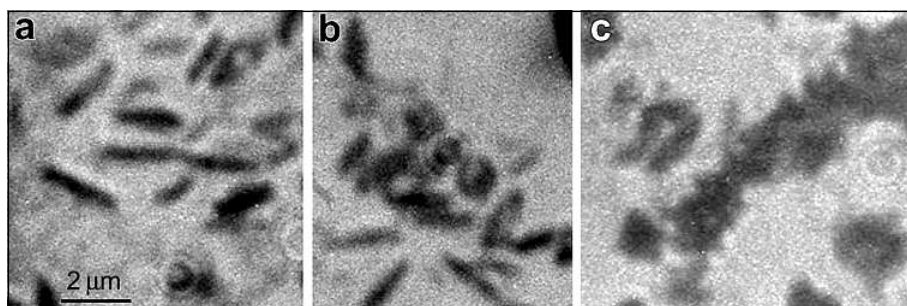


Figure 1: Discoid resting platelets (a), and platelet 1.5 s (b) and 2.5 s (c) after ADP stimulation. Note the differences in platelet interactions before (b) and after shape change (c).

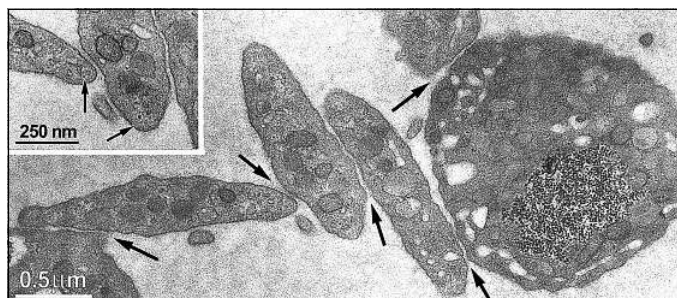


Figure 2: Discoid resting platelets in an aggregate 1.5 s after ADP stimulation. Characteristic contact spaces are indicated by arrows. The enlarged inset demonstrates the marginal bundle of microtubules.

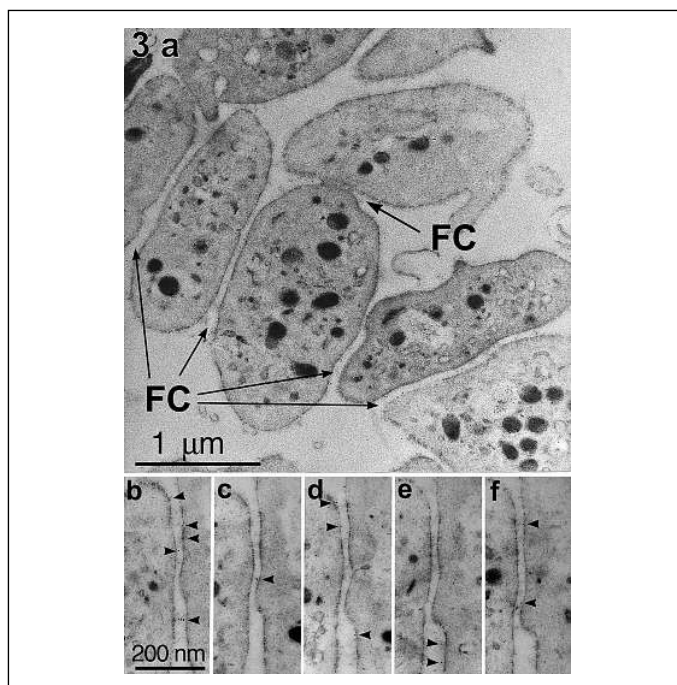


Figure 3: Discoid aggregated platelets from PRP fixed 1.5 s after ADP-stimulation and labeled with anti-fibrinogen. a) An aggregate of discoid platelets from labeled with anti-fibrinogen antibody and gold-conjugated secondary antibody is shown. b-f) In these serial sections of a contact between discoid platelets, gold labeling of fibrinogen is shown to be present within the platelet contacts (arrowheads).

trate) and examined with the EM 109 (C. Zeiss, Oberkochen, Germany).

Paraformaldehyde fixation and cryosubstitution for immunolabelling

Platelet aggregates (1.5 or 2.5 s after ADP stimulation) were prefixed by addition of 30 µl/ml of 3% paraformaldehyde (Serva, Heidelberg, Germany) and 0.1 % glutardialdehyde dissolved in 0.1 M cacodylate buffer, pH 7.2 in order to stop all platelet reactions after the chosen time period prior to subsequent centrifugation. Pellets were fixed with the fixation solution for 30 min at room temperature. After permeation with sucrose using a 2.3 M solution (freeze protection) the samples were cooled with liquid nitrogen and then cryosubstituted with methanol without fixative at -80°C . A low-temperature embedding was carried out using Lowicryl HM20 (Lowi GmbH, Waldkraiburg, Germany) with the cryosubstitution unit CS auto (Reichert-Jung, Vienna, Austria). Serial sections were prepared from these samples.

Immunolabelling

Immunolabelling of serial sections from paraformaldehyde fixed and cryosubstituted samples

For localization of fibrinogen in platelet aggregates, ultra-thin sections were used. After washing in phosphate-buffered saline containing 0.5 M glycine, grids were incubated with the primary anti-fibrinogen antibody for 30–60 min at 20°C , then reacted with goat anti rabbit IgG conjugated to 10 nm gold particles (Aurion, Wageningen, The Netherlands) for 30–60 min at 20°C .

Results and discussion

The aggregation of discoid platelets *in vitro* could not be demonstrated by use of TEM up to now. We discovered two reasons for this phenomenon. Firstly, very short activation times (1.5 s) are indispensable to yield visible aggregates of discoid platelets. Secondly, the aggregates have to be fixed prior to centrifugation in order to avoid additional contact activation. In accordance with these preconditions, the investigation of semi- and ultrathin sections from the initial events is possible, also including immunolabelling with anti-fibrinogen as shown here.

In Figure 1 the sequence of platelet alterations after ADP-stimulation *in vitro* is shown at a glance using light microscopy. The discoid platelets (Fig. 1a) are arranged in aggregates after 1.5 s (Fig. 1b) and 2.5 s after stimulation, they form aggregates consisting of shape-changed platelets (Fig. 1c).

Aggregated platelets 1.5 s after ADP-stimulation

As shown in Figure 2 discoid platelets containing an intact marginal bundle of microtubules form focal aggregation contacts with the width that corresponds with the length of a fibrinogen molecule. Focal aggregation contacts are recognizable on any site along the surfaces of discoid platelets (Fig. 2). Immunolabelling with anti-fibrinogen indicates that in contacts between discoid platelets bridging fibrinogen molecules are present (Fig. 3a-f). Control immunolabelling in the absence of the primary antibody yielded no labels.

The appearance and distance of contacts between discoid platelets resembles the fibrinogen contacts in later phases of platelet aggregation. It may be advantageous that at this time platelets are able to form contacts distributed over their entire accessible surface. Apparently, at this early stage, $\alpha_{IIb}\beta_3$ integrins are capable of finding fibrinogen molecules that bridge the contact spaces between the discocytes as demonstrated by immunolabelling with anti-fibrinogen. The width of the contact spaces resembles to that of later phases. However, at this very early phase fibrinogen receptors may still be associated with the sub-membranous cytoskeletal lattice of the discoid platelets and are not yet associated with the contractile cytoskeleton formed after a remodelling of the actin filaments (12–15).

Platelet aggregates 2.5 s after ADP-stimulation

As shown in Figure 4, 2.5 s after activation by ADP, aggregated platelets have changed their shape and centralized their organelles. Fibrinogen-mediated contacts were located on their bodies. The immunocytochemical analysis of such contacts has already been demonstrated elsewhere (12). Not until this time point after stimulation, so-called tight contacts located between the fibrinogen contacts were present (16, 17). Thus, already 2.5 s after ADP-stimulation platelets have changed their shape and centralized their organelles indicating an active contractile cytoskeleton (13, 18–20). The affinity of fibrinogen for the $\alpha_{IIb}\beta_3$ -integrin is increased after its association with the contractile cytoskeleton, and such a binding is required for the internalization of soluble molecules to form a stable aggregate (13, 21) or of fibrin fibres to retract a clot (22).

In conclusion, we found that the first phase of aggregation by discoid platelets *in vitro* takes a very short time. During this time period the affinity for fibrinogen binding to its receptor

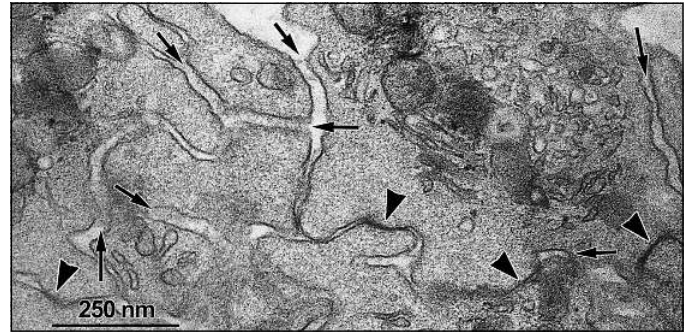


Figure 4: Platelets in an aggregate from PRP fixed 2.5 s after ADP stimulation. These platelets have changed their shape and centralized their organelles. Besides of fibrinogen-mediated contacts (arrows) tight contacts are present as well (arrowheads). These contacts are located between the fibrinogen contacts.

$\alpha_{IIb}\beta_3$ -integrin would be low as for other two-stage aggregation processes (9). Nevertheless, stimulated discoid platelets apparently recognize each other in a fibrinogen-mediated manner. Thus, it is conceivable that the discocytes are able to form fibrinogen-mediated contacts more easily using their accessible surface and to aggregate more rapidly than platelets after their shape change. The implications of the fragile aggregates at the beginning of the haemostasis process were discussed many years ago by Lüscher and Weber considering earlier studies (4). The therapeutic efficacy of inhibitors of the $\alpha_{IIb}\beta_3$ -integrin points to such a first recognition mechanism (23). Recent observations *in vivo* were comparable with the findings in this study (24): Flow cytometric and electron microscopic examination of blood drawn from the coronary sinus demonstrated the rapid formation of aggregates consisting of discoid platelets immediately after rotational atherectomy. As in our *in-vitro* study, ADP is certainly a platelet stimulator mainly available after rotational atherectomy.

Acknowledgements

The authors would like to thank Anne Vecerdea (Homburg, Germany) for her excellent help in manuscript preparation and Dr. Ludwig Edelmann (Homburg, Germany) for experienced support in cryotechniques.

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