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# Growth and Stability of Thrombi in Flowing Citrated Blood: Assessment of Platelet-Surface Interactions with Computer-Assisted Morphometry

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**Key words**

Perfusion chamber – Shear rate – Thrombus growth – Thrombus stability – Computer-assisted morphometry

**Summary**

The differential quantitation of platelet deposition in perfusion studies is a major problem. We report on methods to prepare semithin sections of platelet deposits on collagen coated on glass and plastic cover slips, to study growth and stability of thrombi in three dimensions, and the development of a computer-assisted differential quantitation of platelet-collagen interactions. The interactions were quantified as percentage of the surface covered with platelets (platelet adhesion), thrombus height, thrombus density and thrombus area per unit sectional length, respectively.

Cover slips coated with fibrillar equine collagen in parallel-plate perfusion chambers were exposed to flowing citrated blood at shear rates ranging from 200 to 2,600 $s^{-1}$. Thrombi, partially enmeshed in the collagen meshwork, prevailed on the surface at all shear rates. Maximal platelet adhesion and thrombus density were seen at >5 $\mu g/cm^2$ collagen, while thrombus area and height were maximal at >10 $\mu g/cm^2$. The volume of the thrombi appeared correlated to the number of deposited platelets ($r = 0.92$). En face preparations showed deposits of platelet islands which grew in diameter with time, particularly in the direction of the blood flow, becoming progressively confluent. Sections cut parallel to the direction of the blood stream indicated that this growth pattern was at least partially caused by thrombi bent in the direction of the blood flow. This view is consistent with data from corresponding sections cut perpendicular to the direction of the blood flow showing that the initial thrombus growth at 2 min is isotropic, while anisotropic growth, characterized with decreased growth in height, is observed at 5 and 10 min.

Our three-dimensional analysis suggests that the growth occurs mainly in height, and that blood shear forces may bend the thrombi toward the surface resulting in platelet thrombi preferentially elongated in the direction of the blood flow.

## Introduction

Many approaches have been developed to quantify blood platelet interactions with collagen. One widely used method is the induction of platelet aggregation in stirred platelet-rich plasma by the addition of collagen fibrils (1). The aggregation is monitored by turbidometry (2). Platelet deposition on collagen has also been assessed by means of radio-labeled platelets (3–8), by measuring the decrease in platelet count following incubation of platelet suspension with collagen and subsequent filtration through Sepharose columns (9), by en face microscopy of deposited platelets on collagen coated cover slips (6, 10), by automated microdensitometry of platelets deposited on collagen coated slides (5), and by morphometry of semithin sections of collagen coated gelatin (11) and of α-chymotrypsin digested subendothelium, a surface rich in collagen fibrils (12). Differentiation of platelet deposition in adhesion and thrombi, which is crucial for the proper assessment of platelet function and surface reactivity, is only possible by morphometry in cross-sections (13).

Collagen coated glass has been used in platelet-collagen interaction studies (3, 5–7). It is possible to obtain sections of platelet deposits on glass support for morphometric analysis, but it is a time-consuming process which requires some skill (5).

We describe a new technique for producing semithin and ultrathin sections of platelet deposits on collagen coated glass and plastic cover slips and a computer-assisted morphometric method allowing quantitation and analysis of platelet-collagen interactions in a detail unequaled by other approaches. This paper deals primarily with methodological aspects of assessing three-dimensional thrombus growth and stability in flowing blood.

## Materials and Methods

### *Blood Samples*

Citrated blood. Blood from healthy individuals was collected into 1/10 vol of 108 mM trisodium citrate following venepuncture with a no. 19 Butterfly Infusion Set (Abbott Laboratories, North Chicago, IL). The citrate concentration in plasma was adjusted to 20 mM by adding 108 mM trisodium citrate depending on the hematocrit. All donors denied having taken any medication during the 10 days preceeding the donations. Hematocrits (39–46%) and platelet counts ($1.6–2.8 \times 10^{11}$/l) were all within the normal range. The blood was kept at 22° C for 30–60 min before it was used in perfusion experiments.

Reconstituted blood with radiolabeled $^{51}$Cr-platelets were made from citrated blood (14, 15). Briefly, platelets in platelet-rich plasma prepared by centrifugation of citrated blood (200 × g, 22° C, 10 min) and diluted with 1 vol of 130 mM NaCl, 2 mM KCl, 12 mM $NaHCO_3$ and 20 mM trisodium citrate (pH 5.0), giving a final pH of 6.2, were pelleted (500 × g, 22° C, 10 min) and subsequently resuspended in the same buffer, but at pH 6.0 and in the presence of 2.0 μCi $^{51}CrO_4$/ml. Labeling took place at 22° C for 20 min, and the platelets were washed free of exogenous $^{51}CrO_4$ by three successive centrifugations (500 × g, 22° C, 10 min) with the same buffer at pH 6.0. Platelet-free plasma and three-times-washed erythrocytes were prepared by centrifugations (3,000 × g, 4° C, 30 min and 3,000 × g, 22° C, 2 × 5 min and 1 × 20 min, respectively). Blood was reconstituted with washed $^{51}$Cr-platelets, washed erythrocytes and platelet-free plasma to a platelet count and a hematocrit of $1.2 \times 10^{11}$/l and 40%, respectively. Characterization of washed and radiolabelled pre- and post-perfused $^{51}$Cr-platelets was previously reported (6, 16). The perfusates were stored at 22° C and used in perfusion experiments within 60 min after reconstitution, corresponding to about 2.5 to 3 hrs after venepuncture.

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*Preparation of Collagen Coated Cover Slips*

Cover slips of glass (18 × 18 mm; Menzelgläser, Braunschweig, FRG) and of plastic (22 × 60 mm; Thermanox™, Miles Laboratories, Inc., Naperville, IL) were used. The plastic cover slips were cut to a size of 22 × 18 mm with a specially constructed device in order to match the recess (22 × 18 mm) in the cover slip holders of the perfusion chambers.

The cover slips were washed with chromic acid (glass) or ethanol (plastic), rinsed four times with deionized water and dried at 70° C for 1 hr. Subsequently, 3 to 10 cover slips were coated simultaneously with fibrilar equine collagen (Collagen Reagent Horm, 1 mg/ml; Hormon Chemie, Munich, FRG) with the aid of a retouching air brush (Badger, Model No. 100 IL, Badger Air-Brush Co., Franklin Park, IL) at a nitrogen operating pressure of 1 atm (5, 6). 97 and 119 µl collagen dispersion per glass (3.2 $cm^2$) and plastic (4.0 $cm^2$) cover slip, respectively, resulted in a collagen density of about 30 µg/$cm^2$. The collagen solution was applied in multiple runs, and after each run the slides were allowed to dry at 22° C. Some plastic cover slips were sprayed with Horm buffer only. The cover slips were stored at 22° C for about 16 hrs before they were used in perfusion experiments.

*Perfusion Chambers*

Parallel-plate perfusion chambers with central flow slits of 74.0 mm length, 10.0 mm width and heights of 0.6 mm and 0.4 mm and with a collagen coated cover slip positioned in a cover slip holder were used (6). The recess of the cover slip holder was 18 mm wide with a depth of 0.204 mm. The average thickness of the plastic cover slips and of the standard collagen coat (30 µg/$cm^3$) was 0.176 and 0.004 mm, respectively. Thus, the average height of the 0.6 mm high flow slit at the collagen surface was 0.624 mm; 0.624 mm= [0.6 + 0.204 − (0.176 + 0.004)] mm. The average thickness of the glass cover slips was 0.156 mm, resulting in an average height at the collagen surface (30 µg/$cm^2$) of 0.644 mm; 0.644 mm = [0.6 + 0.204 − (0.156 + 0.004)] mm. The corresponding height for collagen coats on plastic cover slips in the chamber with 0.4 mm slit height were 0.424 mm.

*Perfusion*

Perfusions were carried out at 37° C under pulsatile blood flow conditions (17) using an occlusive roller pump with six rollers (Perpex Jubilé, Model 1/10, Werner Meyer AG, Luzern, Switzerland), a polyethylene container for the blood sample, and silastic tubings ($\varnothing_{inner}$ = 3.0 mm; Dow Corning Corp., Midland, MI) to recirculate the blood sample. The perfusion system, including the collagen surface, was preperfused with prewarmed (37° C) phosphate buffered saline (PBS; 58 mM $Na_2HPO_4$, 1 mM $NaH_2PO_4$, 75 mM NaCl), pH 7.4, and left filled with PBS at 37° C for about 5 min. Any air-blood interphase was avoided by the presence of PBS in the system when the 37° C preheated (5 min) blood sample was successively recirculated. The perfusions were terminated by removing the silastic tubing from the blood container, resulting in successively removal of blood from the chamber by the pump. The cover slip was immediately removed from the chamber and briefly rinsed in PBS and then immersed in freshly prepared fixative at 4° C (18).

Average flow rates of 7.6, 24.6 and 49.2 ml/min were maintained for 5 min in the 0.6 mm slit height perfusion chamber with the plastic cover slip resulting in average shear rates at the collagen surface of approximately 200, 650 and 1,300 $s^{-1}$, respectively. The same shear rates in the same perfusion chamber were maintained for 5 min when glass cover slips were used, corresponding to flow rates of 8.0, 26.0 and 52.0 ml/min, respectively. 2,600 $s^{-1}$ shear rate was maintained for 5 min in the 0.4 mm slit height chamber with a plastic cover slip at 45.4 ml/min flow rate.

*En Face Preparations*

En face preparations on cover slips for light microscopic investigation were fixed and stained according to Muggli et al. (5).

*Fixation, Epon Embedding, Sectioning and Staining*

Collagen coated cover slips were fixed in 2.5% glutaraldehyde/0.1 M cacodylate, pH 7.4, at 4° C for 2 hrs.

Postfixation was performed with 2.0% osmiumtetroxide followed by 0.1% uranyl acetate for 30 min each. Dehydration was carried out with graded ethanol concentrations, and the side of the cover slip with the collagen film was subsequently infiltrated with Epon, approximately 1–2 mm thick. Following polymerization of the resin, glass cover slips were dissolved with 38% hydrofluoric acid (Fluka AG, Buchs SG, Switzerland) at 22° C for 30 min, and the preparation was successively washed with distilled water and PBS for three periods of 10 min each, and dried at 70° C for 2 hrs. Plastic cover slips were peeled off from the hardened resin after warming to 70° C for 1 min and subsequent exposure to dry ice for 1 min. The embedded preparations, each with an intact whole collagen surface, were cut parallel to the direction of the blood flow into two halves, and each half into three pieces of equal size perpendicular to the direction of the blood flow, resulting in two upstream pieces, two middle pieces and two downstream pieces. The three sets of the two corresponding pieces were reembedded in Epon (18).

Semithin sections of about 0.8 µm thickness, perpendicular and parallel to the direction of the blood flow, were cut from the upstream pieces. The sections were stained at 70° C with toluidine blue (0.01% toluidine blue in 2% disodiumtetraborate) for 45 s, dried and subsequently stained with basic fuchsin (0.01% basic fuchsin) for 15 s.

Sections for transmission electron microscopy (Philips EM 300 microscope) were stained with uranyl acetate and lead citrate.

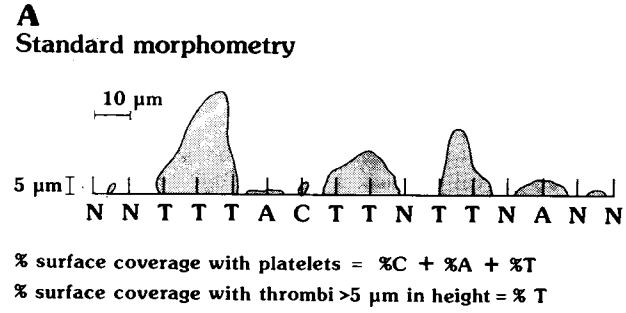


*Fig. 1* Principles for morphometric evaluation of platelet-surface interactions with standard morphometry and with computer-assisted morphometry. A) Standard morphometry (10): Platelet-surface interactions are evaluated at 10 µm intervals at the surface and are classified as naked (N), contact platelets (C), spread platelets <5 µm in height (A) and platelet thrombi >5 µm in height (T). Platelet adhesion and thrombus formation are defined as the percentage of the surface covered with platelets (C + A + T) and with thrombi >5 µm in height (T), respectively. B) Computer-assisted morphometry. All platelet deposits – single adherent platelets, monolayers of platelets and platelet thrombi > 2.5 µm in height – are registered with an electromagnetic pen as outlined in the text. The following parameters are calculated: the percent surface coverage with platelets (platelet adhesion), the number of thrombi/100 µm section length (thrombus density), the thrombus area/µm section length (µm²/µm) and the average thrombus height (µm). The computer program allows classification of thrombus areas and thrombus heights

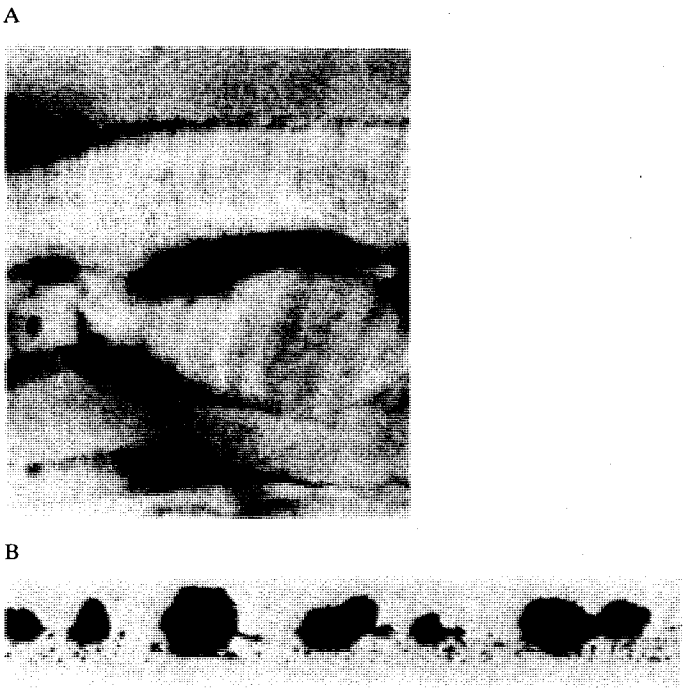


*Fig. 2* A) Electron micrograph of collagen fibrils on plastic cover slips, 122,000×. B) Light micrograph of mural platelet thrombi on the collagen meshwork in sections cut perpendicularly to the direction of the blood flow. Note thrombi partly enmeshed in the collagen meshwork. 5 min perfusion with citrated blood at 650 $s^{-1}$ shear rate, 820×

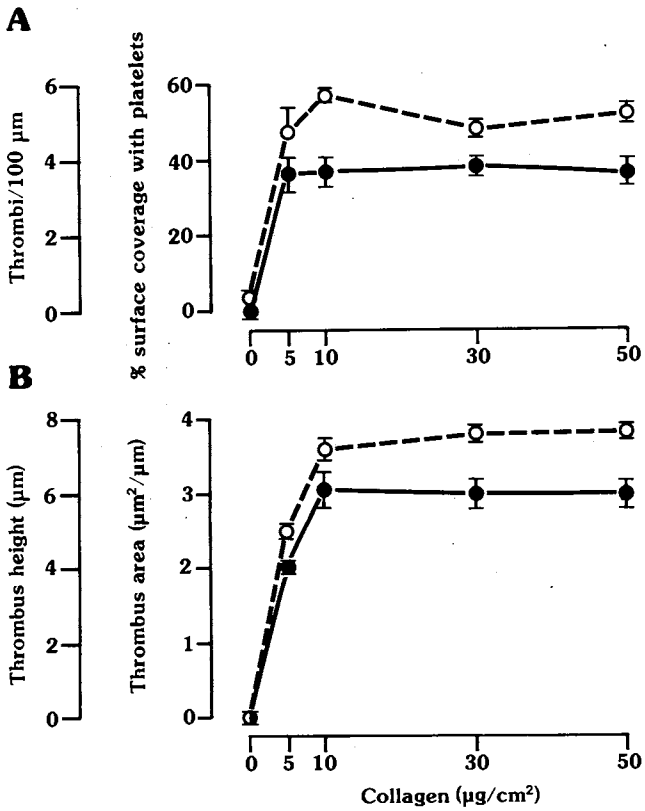


*Fig. 3* Platelet deposition in citrated blood as a function of collagen density after 5 min perfusions at 1,300 $s^{-1}$ shear rate. Sections cut perpendicularly to the direction of the blood flow. Computer-assisted morphometry, mean ± S.E., n = 3. A) Percent surface coverage with platelets (○--○) and number of thrombi/100 µm section length (●—●). B) Thrombus area per unit section length (●—●) and thrombus height (○--○)

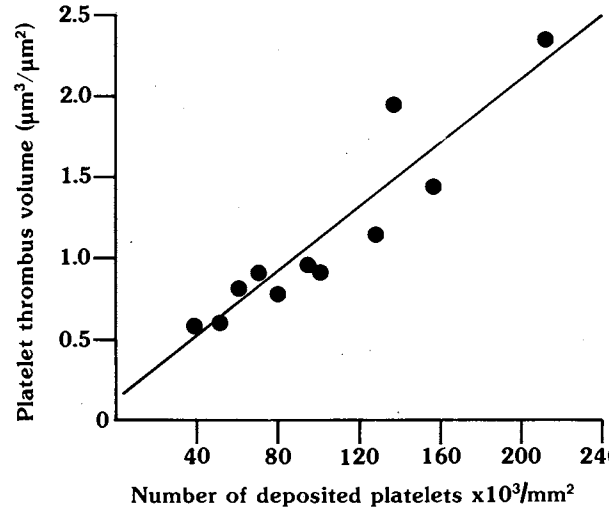


*Fig. 4* Correlation of deposition of $^{51}$Cr-platelets and corresponding thrombus volume per unit surface area on collagen in reconstituted blood with $^{51}$Cr-platelets from 5 donors. Shear rates 200, 650, 1,300 and 2,600 $s^{-1}$ were maintained for 5 min. Linear regression analysis; $p < 0.001$

*Standard Morphometry*

Platelet-collagen adhesion was quantified in semithin sections by standard morphometry as percent surface coverage with platelets at a magnification of 1,000× (10). By moving the surface along an eye-piece micrometer positioned in the ocular of the microscope, platelet adhesion was sampled at about 400 locations, each 10 µm apart (Fig. 1A). Platelet adhesion was quantified on sections cut perpendicular to the direction of the blood flow.

*Computer-Assisted Morphometry*

Computer-assisted morphometry in two dimensions was performed on semithin sections cut perpendicularly to the direction of the blood flow. The microscopic image of the sections was displayed on a colour video monitor (Sony, PVM-2060ME) by a video camera (JVC KY-1900E) fitted to the tube of a microscope (Zeiss) with a final magnification on the screen of 2,700×. All platelet deposits identified – single adherent platelets, monolayers of adherent platelets and platelet masses higher than 2.5 µm (thrombi) – were registered by contouring the objects manually with an electromagnetic pen on a graphic tablet (Fig. 1B). Countours and image were superimposed on the monitor and both were contrasted by a colour effect generator (RGB mixer, EL-Elektronik, Basel, Switzerland). Management of data and data processing were performed with a BIVAS programme (Heinz Meyer, Datalab, CH-3367 Thöringen, Switzerland) and an Apple IIe computer. Data and diagrams were printed on an Epson printer (Epson, Model FX-80).

Platelet adhesion was expressed as percentage of the surface covered with platelets in analogy to standard morphometry (10). The total number of thrombi higher than 2.5 µm was registered and expressed as average number of thrombi per 100 µm surface sectional length, thrombus density. Sectional thrombus areas were calculated by the programme and sectional thrombus heights, the base to peak distance of the thrombi, were registered similarly as in Manual Optical Picture (MOP) morphometry (19). Further characterization of thrombus dimensions was performed by classification of area and height. Thrombus volume per unit surface was estimated by measuring the thrombus profile areas per unit sectional length of the test surface (19). Equivalency of the two parameters, area and volume, can be derived from the principle of Delesse, a fundamental theorem of stereology (20), since there appears not to be a systematic place where thrombi start to grow on the surface.

*Statistical Analysis*

Significance of grouped data was calculated with Student's t-test and p-values <0.05 were considered significant. Linear regression were calcu-

lated with Apple IIe computer utilizing the BIVAS program (Heinz Meyer, Datalab, CH-3367 Thörigen, Switzerland).

## Results

*Standard vs. Computer-Assisted Morphometry*

Comparison of percent surface coverage with platelets evaluated on the same sections with standard morphometry and with computer-assisted morphometry yielded good linear correlation; r = 0.99, n = 10. However, a 12% higher surface coverage with platelets was observed with standard morphometry. Because of the limited resolution of the image on the video monitor, proper differentiation between platelets not spread out (contact platelets) and platelets spread out on the surface is not possible with computer-assisted morphometry.

*Characterization of Collagen Coats*

Transmission electron microscopy of non-perfused collagen coats showed multiple layers of coarse fibrils (Fig. 2A). Light microscopic inspection of collagen coats perfused with citrated blood, and sectioned perpendicular to the direction of the blood flow, showed large thrombi on the surface which appeared firmely anchored to the porous meshwork of collagen fibrils (Fig. 2B). The average thickness of the collagen coat was about 4 μm at a collagen density of 30 μg/cm$^2$.

*Platelet-Collagen Interactions on Plastic and Glass Cover Slips*

No significant differences were noted between platelet interactions with collagen coats on glass and plastic cover slips as measured by standard morphometry, computer-assisted morphometry and by counting of deposited $^{51}$Cr-platelets following perfusions at shear rates of 200, 650 or 1,300 s$^{-1}$ for 5 min.

*Dependence of Platelet-Collagen Interactions on the Amount of Collagen Coated onto the Cover Slips*

Plastic cover slips coated with 0, 5, 10, 30, and 50 μg collagen per cm$^2$ were exposed to citrated blood at 1,300 s$^{-1}$ shear rate for 5 min. The thickness of the coats increased in parallel with the amount of collagen, reaching an average thickness of about 6 μm at 50 μg/cm$^2$. Platelet-collagen interactions were quantified by computer-assisted morphometry on sections cut perpendicular to the direction of the blood flow.

About 50% surface coverage with platelets was observed already at 5 μg/cm$^2$ (Fig. 3A). Increasing the amount of collagen to 50 μg/cm$^2$ had no further effect on the surface coverage.

The average number of thrombi per 100 μm sectional length, was about 3.7 and maximal already at a collagen density of 5 μg/cm$^2$ (Fig. 3A). Thrombus area and heigth were maximal at 10 μg/cm$^2$ collagen, with average values of about 3.0 μm$^2$/μm and 7.5 μm, respectively (Fig. 3B). A significant correlation between average number of deposited platelets and average thrombus volume was established, r = 0.92; p <0.001 (Fig. 4).

*Three-Dimensional Image of Thrombus Growth*

Plastic cover slips coated with 30 μg/cm$^2$ collagen were exposed to citrated blood at 650 s$^{-1}$ shear rate for 2, 5 and 10 min. Platelet-collagen interactions were evaluated in two dimensions with computer-assisted morphometry on sections cut perpendicular to the direction of the blood flow.

Prolongation of the perfusion time increased the average surface coverage with platelets from 19% at 2 min to 61% at 10 min (Fig. 5A). However, the thrombus density remained constant within the range of 3.2 to 3.5 thrombi/100 μm (Fig. 5A).

The average thrombus area and height increased with the perfusion time (Fig. 5B). The relative increase was most pronounced for the thrombus area; 4.5 fold (5 min) and 5.5 fold (10 min) higher average values were observed than at 2 min. The corresponding factors for the thrombus height were about 1.9 (5 min) and 2.2 (10 min). Classification of area (Fig. 6A, B and C) and height (Fig. 6D, E and F) showed a shift from small thrombi with more than 90% being < 1.0 μm$^2$/μm in area and <15 μm in height at 2 min, to larger thrombi with more than 90% being <3 μm$^2$/μm and <20 μm in height at 10 min. Sections cut parallel to the direction of the blood flow showed that most of the thrombi were bent in the direction of the blood flow at 2 and 5 min (Fig. 7A and B), but much less so at 10 min (Fig. 7C). The nesting of platelets into the collagen meshwork was more pronounced with longer perfusion times. The two-dimensional quantitation and the qualitative analysis in the third dimension (parallel to the direction of the blood flow) indicate isotropic thrombus growth at 2 min and anisotropic growth at 5 and 10 min.

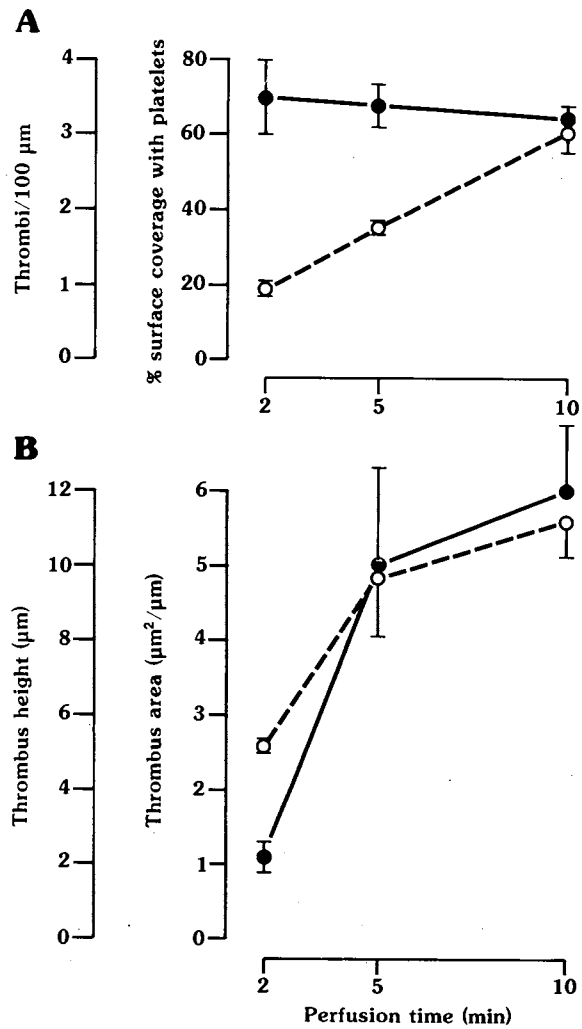


*Fig. 5* Time course of platelet-collagen interactions in citrated blood at 650 s$^{-1}$ shear rate. Sections cut perpendicularly to the direction of the blood flow. Computer-assisted morphometry, mean ± S.E., n = 4. A) Percent surface coverage with platelets (O--O) and number of thrombi/100 μm section length (●—●). B) Thrombus area per unit section length (●—●) and thrombus height (O--O)

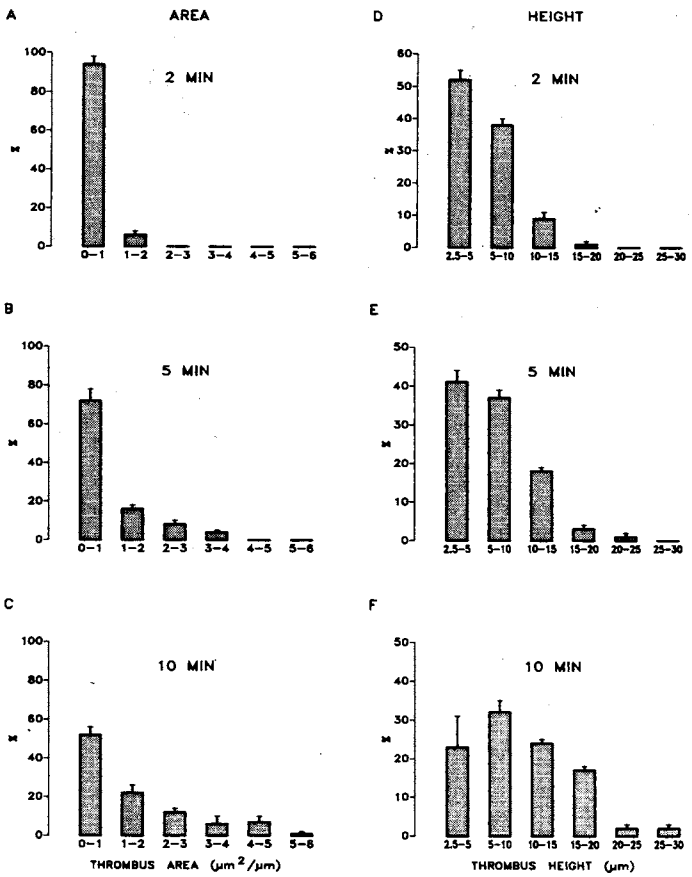


*Fig. 6* Classification in relative percentage of thrombus area at 650 $s^{-1}$ shear rate in citrated blood at 2 min (A), 5 min (B) and 10 min (C), and of thrombus height at 2 min (D), 5 min (E) and 10 min (F). Computer-assisted morphometry

En face preparations of platelet deposits on collagen coated cover slips showed preferential orientation of the thrombi in the direction of the blood flow. Small and randomly scattered platelet deposits were observed at 2 min (Fig. 8A). After 5 min (Fig. 8B) and 10 min (Fig. 8C) these thrombi had grown and combined with neighbouring thrombi preferentially in the direction of the blood flow.

## Discussion

Differentiation of platelet-surface adhesion and subsequent platelet-platelet cohesion in flowing blood is mandatory for the proper assessment of platelet and surface reactivity, and it requires morphometry of semithin sections (13). This paper describes a computer-assisted morphometric method for the measurement of platelet adhesion and thrombus dimensions on semithin sections of collagen coated glass and plastic cover slip preparations. This new procedure, together with the previously developed parallel-plate perfusion chamber (6), have allowed us to study growth and stability of thrombi in three dimensions in flowing blood. Furthermore, the equine spray-coated collagen coats provide a reproducible thrombogenic surface for experiments where thrombus kinetics are studied.

The computer-assisted morphometric method quantifies a number of platelet surface interaction parameters and yields information which is compatible with that obtained by standard morphometry (10) and MOP morphometry (19). In addition, it makes use of a new parameter: thrombus density (number of thrombi higher than 2.5 μm per unit sectional length). Other advantages include simplified data acquisition and data processing, and collection of more detailed quantitative information about thrombus dimensions than is possible with the available methods. However, the quality of the contrast of the image on the video monitor results in lower adhesion figures than is found with standard morphometry, and does not allow optimal differentia-

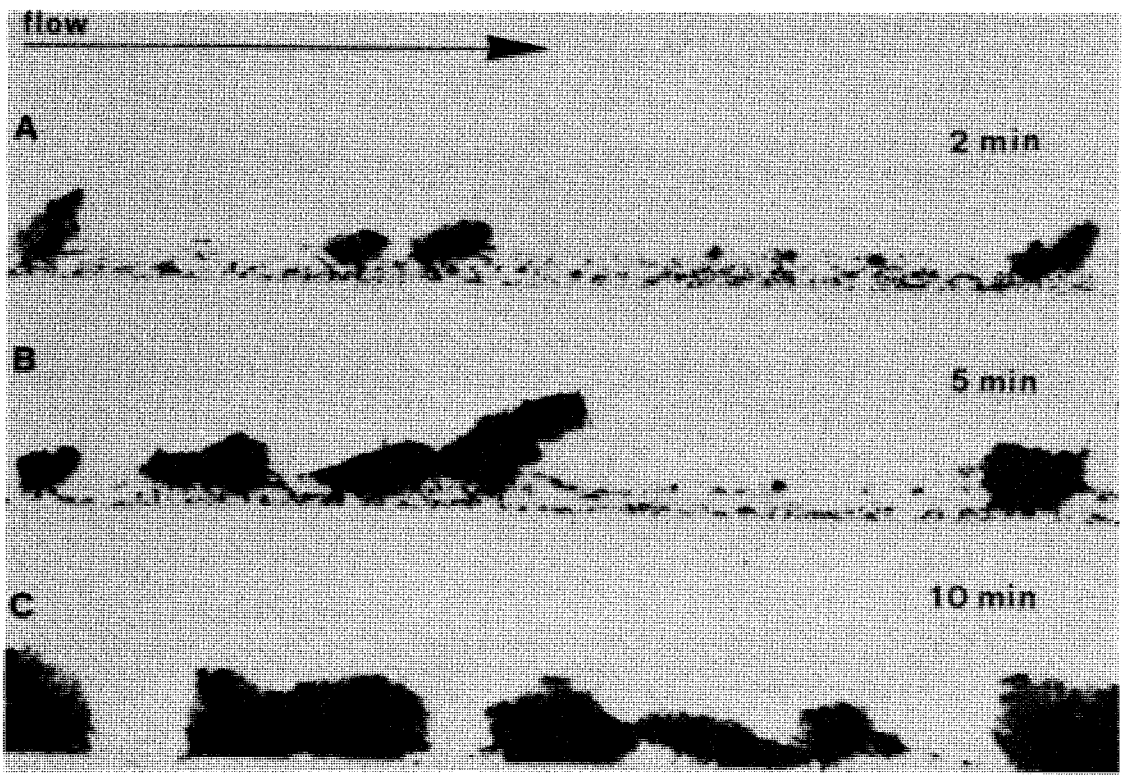


*Fig. 7* Light micrographs of mural platelet thrombi in citrated blood at 650 $s^{-1}$ shear rate and 2 min (A), 5 min (B) and 10 min (C) perfusion time. Sections cut parallel to the direction of the blood flow (arrow), 820×

tion between platelets in contact with the surface and platelets spread out on the surface. Evaluation by standard morphometry is therefore preferred when a detailed analysis of platelet-surface adhesion is required.

Transmission electron microscopy of nonperfused collagen coats revealed a layered meshwork of fibrils. The coats appeared homogeneous on inspection by light microscopy and no differences in surface reactivity towards platelets were observed for coats on glass and plastic. Light and electron microscopic appearance were similar to Epon-embedded collagen coats detached from carbon coated cover slips following exposure to dry ice/methanol (5), indicating that hydrofluoric acid and shock-freezing of Epon-embedded preparations prewarmed to 70° C did not damage the collagen coats.

The thickness of the coat increased with the amount of spray coated collagen and averaged about 4 μm at 30 μm/cm$^2$, an amount chosen as standard in order to provide maximal surface reactivity. Thrombi prevailed on the surface and only a few singly adhering platelets were encountered. Platelets at the base of the thrombi had apparently migrated into the collagen meshwork, as previously observed with collagen fibrils of α-chymotrypsin digested subendothelium (12). Maximal surface coverage with platelets and thrombus density occurred at 5 μg/cm$^2$ collagen, whereas 10 μg/cm$^2$ was needed for maximal thrombus area and height. The difference in collagen density required for optimal thrombus density and thrombus dimensions is apparently caused by depletion of platelets from the boundary layer by rapidly growing thrombi. The depletion results in to few platelets at the surface to form new thrombi. Evidence for such a mechanism was recently reported (21, 22). The 10 μg/μm$^2$ which triggered optimal thrombus growth is within the range of those recently reported for maximal deposition of $^{111}$In-radiolabelled platelets on purified human type I and type III collagens in a similar perfusion device (8). Furthermore, a close correlation exists between the number of $^{51}$Cr-platelets deposited and thrombus volume, similar to that reported previously for rabbit platelets (4). Such a correlation is expected with thrombogenic surfaces which predominantly trigger platelet thrombi and have only few single adherent platelets. This is in contrast to human artery subendothelium which induces a monolayer of adherent platelets when exposed to aspirin-containing blood at low platelet counts and at shear rates below 800 s$^{-1}$ (14). In these situations, a close correlation between the number of deposited $^{51}$Cr-platelets and the surface coverage of adherent platelets exists (23).

Three-dimensional thrombus growth could be followed by morphological inspection of en face preparations and corresponding semithin sections cut parallel and perpendicular to the direction of the blood flow. However, morphometry was performed in two dimensions only, on the sections cut perpendicular to the direction of the blood flow. The surface coverage with platelets increased steadily throughout the time course, although less steeply than has previously been reported for subendothelium (21). This discrepancy may be explained by the higher thrombogenicity of the collagen surface where rapidly growing thrombi deplete the boundary layer for platelets and thereby lower the rate of platelet adhesion (21, 22). Apparently, the depletion results already at 2 min in to few platelets at the surface for formation of new thrombi, keeping the thrombus density constant. The growth rate of these thrombi exceeded the one reported on subendothelium about 5 to 10 fold (21). The thrombus size, as expressed by area and height increased with time, however, less in height. A similar time-dependent size-distribution for the thrombi was found. The data indicate initial isotropic thrombus growth in the directions perpendicular to the blood flow, turning anisotropic at later stages, i.e. the more or less

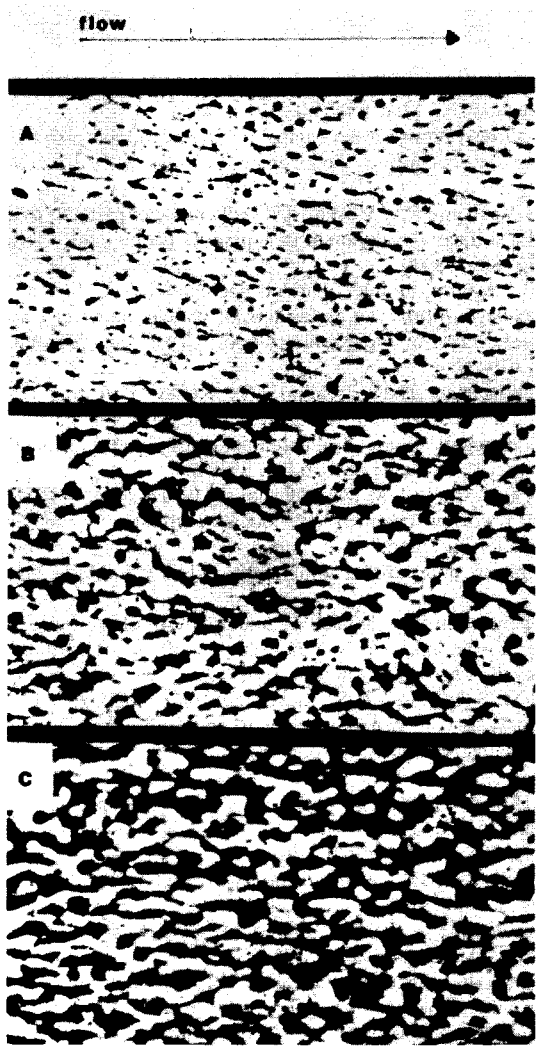


*Fig. 8* Light micrographs of en face preparations with platelet deposits in citrated blood at 2 min (A), 5 min (B) and 10 min (C) perfusion time at 650 s$^{-1}$ shear rate, 200×. Arrow indicates direction of the blood flow

semicircular thrombus profiles at 2 min become slightly flattened at 5 and 10 min when thrombi have grown in size.

En face preparations visualized thrombi preferentially oriented in the direction of the blood flow in the form of long, elongated platelet aggregates. Similar observations were previously reported (5, 24), and it was suggested that growth occurred mainly in the direction of the blood flow. Our data indicate, however, that this need not be the case, because the preferential orientation of platelet aggregates is at least partially caused by thrombi which bend in the direction of the blood flow and eventually attach to the surface, suggesting that the growth occurs at the upstream end of the thrombi and predominantly in height. The stability of these growing thrombi is apparently not strong enough to resist the shear forces which gradually bend them toward the surface. The lack of thrombin generation in the citrated blood may have made the thrombi less stable then those observed in non-anticoagulated blood, as local thrombin generation in and around mural thrombi apears to be essential for the stability and the growth in height (19, 21, 25).

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## References


1 Spaet T H, Lejnieks I. A technique for estimation of platelet-collagen adhesion. Proc Soc Exp Med 1969; 132: 1038–41.

2 Born G V R. Observations on the change in shape of blood platelets brought about by adenosine diphosphate. J Physiol 1970; 209: 487–511.

3 Cazenave J P, Packham M A, Mustard J F. Adherence of platelets to a collagen coated surface: development of a quantitative method. J Lab Clin Med 1973; 82: 978–90.

4 Tschopp T B. Aspirin inhibits platelet aggregation on, but not adhesion to, collagen fibrils: an assessment of platelet adhesion and deposited platelet mass by morphometry and $^{51}$Cr-labeling. Thromb Res 1977; 11: 619–32.

5 Muggli R, Baumgartner H R, Tschopp T B, Keller H. Automated microdensitometry and protein assays as a measure for platelet adhesion and aggregation on collagen coated slides under controlled flow conditions. J Lab Clin Med 1980; 95: 195–207.

6 Sakariassen K S, Aarts P A M M, de Groot Ph G, Houdijk W P M, Sixma J J. A perfusion chamber developed to investigate platelet interaction in flowing blood with human vessel wall cells, their extracellular matrix, and purified components. J Lab Clin Med 1983; 102: 522–35.

7 Adams G A, Feuerstein I A. Platelet accumulation on collagen: drugs which inhibit arachidonic acid metabolism and affect intracellular cyclic AMP levels. Thromb Haemostas 1984; 52: 45–9.

8 Houdijk W P M, Sakariassen K S, Nievelstein P F E M, Sixma J J. Role of factor VIII-von Willebrand factor and fibronectin in the interaction of platelets in flowing blood with monomeric and fibrillar human collagen types I and III. J Clin Invest 1985; 75: 531–40.

9 Legrand Y J, Fauvel F, Kartalis G, Wautier J L, Caen J P. Specific and quantitative method for estimation of platelet adhesion to fibrillar collagen. J Lab Clin Med 1979; 94: 438–46.

10 Baumgartner H R, Muggli R. Adhesion and aggregation: morphologically demonstration and quantitation in vivo and in vitro. In: Platelets in Biology and Pathology. Gordon J L (ed). Elsevier/North-Holland Biomedical Press, Amsterdam 1976; p 23.

11 Turitto V T, Muggli R, Baumgartner H R. Platelet adhesion and thrombus formation on subendothelium, epon, gelatin and collagen under arterial flow conditions. Am Soc Artif Intern Organs 1979; 2: 28–34.

12 Baumgartner H R. Platelet interaction with collagen fibrils in flowing blood. I. Reaction of human platelets with α-chymotrypsin-digested subendothelium. Thromb Haemostas 1977; 37: 1–14.

13 Turitto V T, Baumgartner H R. Platelet-surface interaction. In: Hemostasis and Thrombosis, Basic Principles and Clinical Practice. Colman R W, Hirsh J, Marder V, Salzman E W (eds). J B Lippincott Company, Philadelphia, PA 1982; p 364.

14 Sakariassen K S, Bolhuis P A, Sixma J J. Human blood platelet adhesion to artery subendothelium is mediated by factor VIII-von Willebrand factor bound to the subendothelium. Nature 1979; 279: 636–8.

15 Sakariassen K S, Fressinaud E, Girma J P, Baumgartner H R, Meyer D. Mediation of platelet adhesion to fibrillar collagen in flowing blood by a proteolytic fragment of human von Willebrand factor. Blood 1986; 67: 1515–8.

16 Sakariassen K S, Bolhuis P A, Sixma J J. Platelet adherence to subendothelium of human arteries in pulsatile and steady flow. Thromb Res 1980; 19: 547–59.

17 Baumgartner H R. The role of blood flow in platelet adhesion, fibrin deposition and formation of mural thrombi. Microvasc Res 1973; 5: 167–79.

18 Sakariassen K S, Muggli R, Baumgartner H R. Measurements of platelet interaction with components of the vessel wall in flowing blood. In: Methods in Enzymology. Hawiger J J (ed). Academic Press Inc., New York 1988; 169: 37.

19 Baumgartner H R, Turitto V T, Weiss H J. Effect of shear rate on platelet interaction with subendothelium in citrated and native blood. II. Relationships among platelet adhesion, thrombus dimensions, and fibrin formation. J Lab Clin Med 1980; 95: 208–21.

20 Weibel E R. Volume density measurement. In: Academic Press Inc., London 1979; p 26. Weibel E R (ed). Stereological Methods, Volume 1.

21 Baumgartner H R, Sakariassen K S. Factors controlling thrombus formation on arterial lesions. Ann New York Acad Sci, New York 1985; 454: 162–77.

22 Sakariassen K S, Baumgartner H R. Axial dependence of platelet deposition on collagen in flowing blood: Upstream thrombus growth impairs downstream platelet adhesion. Arteriosclerosis 1989; (in press).

23 Bolhuis P A, Sakariassen K S, Sander H J, Bouma B N, Sixma J J. Binding of factor VIII-von Willebrand factor to human subendothelium precedes increased platelet adhesion and enhances platelet spreading. J Lab Clin Med 1981; 97: 568–76.

24 Adams G A, Brown S J, McIntyre L V, Eskin S G, Martin R R. Kinetics of platelet adhesion and thrombus growth. Blood 1983; 62: 69–74.

25 Weiss H J, Turitto V T, Vicic W J, Baumgartner H R. Fibrin formation, fibrinopeptide A release, and platelet thrombus dimensions on subendothelium exposed to flowing native blood: Greater in factor XII and XI than in factor VIII and IX deficiency. Blood 1984; 63: 1004–14.


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