Ultrastructure of Clots during Isometric Contraction

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ABSTRACT We explored the retraction or contraction of platelet-fibrin clots under isometric conditions. In the presence of micromolar calcium clots of normal platelet-rich plasma developed tension at an initial rate of 0.1 to 0.2 g/min per cm^2 (initial cross-sectional area). Electron microscopy of clots fixed after attaining a force of 1.6 g/cm^2 revealed platelets with elongated bodies and pseudopods in close apposition to fibrin strands which were oriented in cablelike fashion in the direction of tension. The development of tension could not be explained simply on the basis of platelet-platelet association and interaction alone. First, factor XIII-dependent cross-linking of fibrin fibers was critical to normal isometric contraction. Second, tension decreased linearly, rather than exponentially, when the platelet count in the platelet-fibrin clot was decreased, suggesting that platelets must be interacting with another component (i.e. fibrin). Thrombasthenic platelets, deficient in fibrinogen receptors, failed to develop tension or to align fibrin strands or pseudopods in the clot. Platelet-fibrin clots treated with vincristine to disassemble microtubules or cytochalasin B to disrupt microfilaments failed to develop tension and relaxed if these agents were added after tension had developed. Relaxation under these conditions, however, was not associated with loss of orientation of fibrin strands. Our findings suggest that platelet-fibrin interaction in clots under isometric conditions leads to orientation of fibrin strands and platelets in the direction of force generation. Tension develops as platelets simultaneously attach to and spread along fibrin strands, and contract. The contraction draws some fibrin into platelet-fibrin clumps and aligns other strands in the long axis of tension. The achievement and maintenance of maximum tension appears to depend on the development of platelet-fibrin attachments and extension of platelet bodies and long pseudopods containing bundles of microfilaments and microtubules along the oriented fibrin fibers.

The phenomenon of clot retraction has been recognized for more than two hundred years (20). Physiologically, clot retraction may enhance clot lysis and facilitate recanalization of blood vessels (6). Mechanistically, clot contraction remains poorly understood, though in platelet-rich plasma it has been clearly shown to result from the interaction of a static, nonmotile fibrin mesh and dynamic, actively motile blood platelets (5, 12, 13, 29, 39). Concepts concerning interaction of these two components can be classed into three general proposals. The first suggests that platelets reach out with their pseudopods to combine with and attach to strands of fibrin. Pseudopods then contract, pulling the fibrin mesh toward the platelets. According to one variation of this concept, the center of the platelet may be an anchoring point for pulling in the fibers (35, 43). Another modification proposes that contraction is related to platelet-platelet interaction, and occurs between adhesion sites formed by cytoplasmic protrusions from adjacent cells (12). In the second general concept, pseudopodia are envisaged as exercising sweeping movements that gather in and compress fibrin fibers (1, 8). A third proposal, called the zipper model (9), suggests that platelet-platelet stickiness is critical in clot retraction. In this model, platelets are envisaged as undergoing contraction after having extended long pseudopods which join adjacent platelets. The surfaces of these adjacent platelets are then slowly zippered together compressing the fibrin fibers inside.

Significant advances in the understanding of muscle physiology developed as a result of evaluating contractile events in muscle strips fixed under constant tension. We have applied this principle to the study of clot retraction and have investi-

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gated the process of contraction with the ends of the clot tied and maintained a fixed distance apart. Under these conditions isometric contraction of the clot generates tension (8). The effect of factor XIIIa-catalyzed cross-linking of fibrin on clot retraction was studied. Factor XIIIa, the last enzyme in the coagulation cascade is the thrombin- and Ca^{2+}-activated form of the zymogen, termed factor XIII, which is present in plasma and in the platelet cytosol. It is a highly specific transglutaminase of the endo-γ-glutamine:ε-lysine transferase type and it catalyzes the formation of the ε-(γ-glutamyl)-lysine linkages in fibrin and various cytoskeletal proteins (10, 14, 26). Niewiarowski et al. (32) determined that clot retraction induced by platelets is prevented with fully cross-linked fibrin fibers. We investigated the role of fibrin cross-linking on the development of isometric tension, as well as on the ultrastructural features of platelet-rich fibrin clots. The results of these studies provide evidence to distinguish among current models of clot retraction and to propose a new model for its mechanism.

MATERIALS AND METHODS

Platelet Suspensions

Venous blood was collected from the antecubital vein of normal donors or patients after obtaining informed consent and anticoagulated using 1 vol of citric acid-citrate-glucose (ACD) for 9 vol of blood. Platelet-rich plasma and washed platelets were prepared and maintained at room temperature and were used within 3 h of blood collection. Platelet-rich plasma was obtained after centrifugation at 125 *g* for 10 min. When washed platelets were required, platelet-rich plasma was adjusted to pH 6.5 with 0.15 M citric acid, and platelets were washed three times according to the method of Jenkins et al. (22) using 0.154 M sodium chloride, 0.0154 M Tris-HCl, 5.5 mM glucose, 6.6 mM EDTA, pH 7.35 (to be called Jenkins' buffer). Platelets were sedimented by centrifugation at 1,000 *g* for 15 min. When necessary, the platelet suspension was centrifuged at room temperature at 120 *g* for 5 min to sediment contaminating erythrocytes and leukocytes. The final pellet, consisting only of platelets, was washed in Jenkins' buffer which did not contain EDTA, and suspended in the approxiate platelet-poor plasma. Platelets were counted using a Coulter Counter (Thrombocounter C; Coulter Electronics, Inc., Hialeah, FL).

Contraction Measurements

Isometric measurements were performed as described by Cohen and de Vries (8) on thrombin-clotted platelet-rich plasma. Cylindrical clots were obtained by pouring 1 ml of platelet-rich plasma, immediately after thrombin addition (Parke-Davis, Division of Warner-Lambert, Inc., Morris Plains, NJ; 1 U/ml), into glass cylinders (7 × 0.5 cm ID) plugged at one end with parafilm. After 9 min, the clot cylinder was poured into a Petri dish containing Ca^{2+}-free Tyrode buffer (40) at 2°C to prevent clot shortening. The clot was then tied at one end with a cotton thread to a silver alloy wire holder and to the other end to a force displacement transducer (Model FT 03, Grass Instrument Co., Quincy, MA) linked to a Model 79D Polygraph System (Grass Instrument Co.). The clot was immersed into an isolated tissue bath (Metro Scientific, Farmingdale, NY) containing the aforementioned Tyrode buffer maintained at 37°C. A preload of 82 mg was applied to straighten the clot. Since the transducer developed a displacement rate of 0.02 mm/g, isometric contraction was measured in the longitudinal direction. Contraction in the absence of an external load was performed using the floating clot technique whereby the rate of shortening of a cylindrical clot freely suspended in Tyrode solution at 37°C was measured. The final platelet concentration was 4 to $5 \times 10^5/\mu l$, unless specified otherwise.

Electron Microscopy

Clots were fixed under tension using a modification of previously described methods for fixation of platelets (18, 42). The Tyrode solution surrounding the clot was drained and replaced by 20 ml of a solution containing 10 ml of the original Tyrode solution and 10 ml of 2% glutaraldehyde in White's saline (42). After 5 min of 37°C, this solution was drained and immediately replaced by a mixture of 10 ml of Tyrode and 10 ml of 6% glutaraldehyde in White's saline. After 30 min at 37°C in this fixative solution the clot was removed and cut into small fragments so that the initial orientation of the clot was preserved. The clot fragments were fixed an additional 30 min at room temperature in 3% glutaraldehyde in White's saline. The fragments were then transferred to a solution of 1% osmium tetroxide in a 15 mg/ml solution of potassium ferrocyanide in distilled water (pH 7.4) for 90 min at 2°C. After osmium fixation, the samples were dehydrated in a graded series of ethanol concentrations, then treated with propylene oxide and embedded in Epon. Sections were cut with the sample oriented either along the direction of tension (longitudinal section) or across the direction of tension (cross-section). The sections were then stained using uranyl acetate and lead citrate and viewed using either a Philips 301 electron microscope or a Philips 400 electron microscope with a goniometer stage. Sections from some clots fixed at half-maximal or maximal tension were picked up on 200, rather than 300 mesh grids to facilitate identification of serial sections.

Patients

Freeze-dried plasma from three patients lacking factor XIII were kindly supplied by Dr. Tadashi Kamiya (Nagoya University School of Medicine, Japan) and Dr. Jeanne Stibbe (University of Rotterdam, The Netherlands). The clots obtained from these plasmas solubilized within 45 min in 5 M urea and within 3 min in 1% monochloracetic acid. <1% factor XIII was detected by the antifactor XIII inhibition test (3) and no evidence of factor XIII was detected with the electroimmunoassay (24) in the two plasmas provided by Dr. Kamiya.

The patient with Glanzmann's thrombasthenia is from a previously described family, and his platelets have been well characterized and shown to be markedly deficient in glycoproteins IIb and III (17, 33). The patient with storage pool deficiency has been described previously (16) and has been shown to be markedly deficient in dense bodies and their contents of ADP and serotonin. The patient with the gray platelet syndrome has been well characterized as having a deficiency of alpha granules and their contents of thrombin-sensitive protein, platelet factor 4, beta-thromboglobulin and fibrinogen (19).

Preparation of Plasma Factor XIII

Plasma factor XIII was prepared according to the method of Loewy et al. (25) as modified by Curtis and Lorand (11).

Vincristine was obtained from Eli Lilly & Company (Indianapolis, IN) and cytochalasins B and E from the Sigma Chemical Company (St. Louis, MO). Stock solutions of 8×10^{-3} M of the cytochalasins were made in absolute ethanol.

RESULTS

Noncontracted Clots

Samples of platelet-rich plasma were allowed to clot under conditions used to prepare clots for study in the absence of an external load or in isometric conditions, but were fixed immediately after clot formation. Ultrastructural evaluation revealed platelets to be far apart, usually single but sometimes in small clumps (Fig. 1). Most of the platelets appeared degranulated and showed signs of pseudopod formation. The pseudopods were oriented randomly and few associations of platelets with fibrin strands were seen. The orientation of the fibrin strands was random throughout the clot.

Clots Contracted in the Absence of an External Load

Clots allowed to contract in the absence of an external load revealed the morphological features described by many previous investigators (5, 12, 43) (Fig. 2). Platelets and fibrin were gathered into large clumps and appeared relatively spherical in thin sections. Fibrin tended to be concentrated toward the center of the clumps while platelets were more prominent at the periphery. Some of the peripherally oriented platelets or pseudopods appeared swollen into spherical forms. Platelet bodies, pseudopods, and fibrin strands were organized in a purely random fashion in clots contracted in the absence of an external load.

Clots Contracted Under Isometric Conditions

Clots of normal platelet-rich plasma contracting under isometric conditions developed tension at an initial rate of 0.1 to

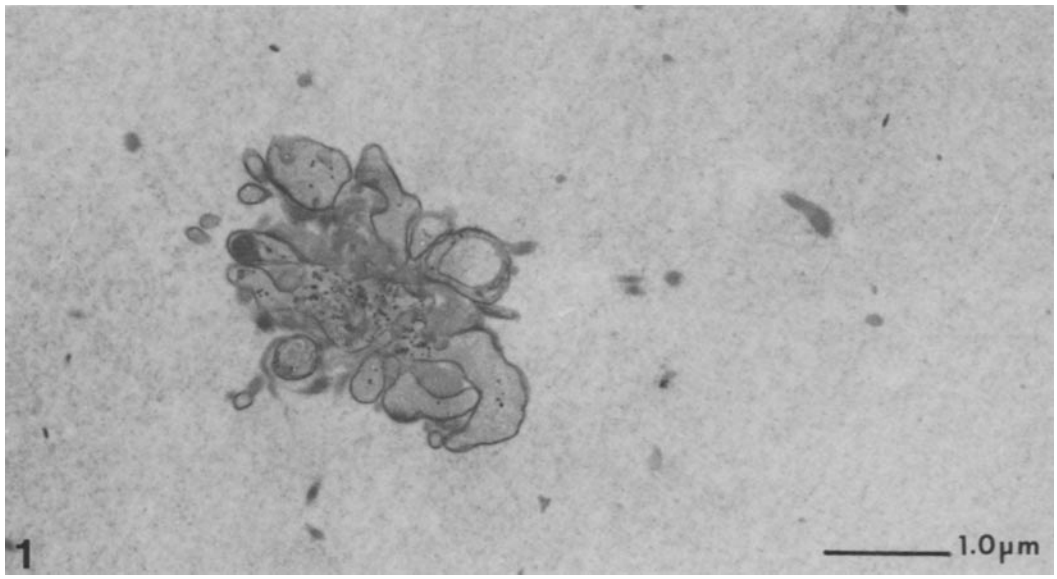


FIGURE 1 Platelet-rich plasma clot before tension development. Platelets have changed their shape, forming short pseudopods, and have secreted their granule contents. Fibrin strands are not oriented. Longitudinal section, uranyl acetate and lead citrate stain. × 10,900.

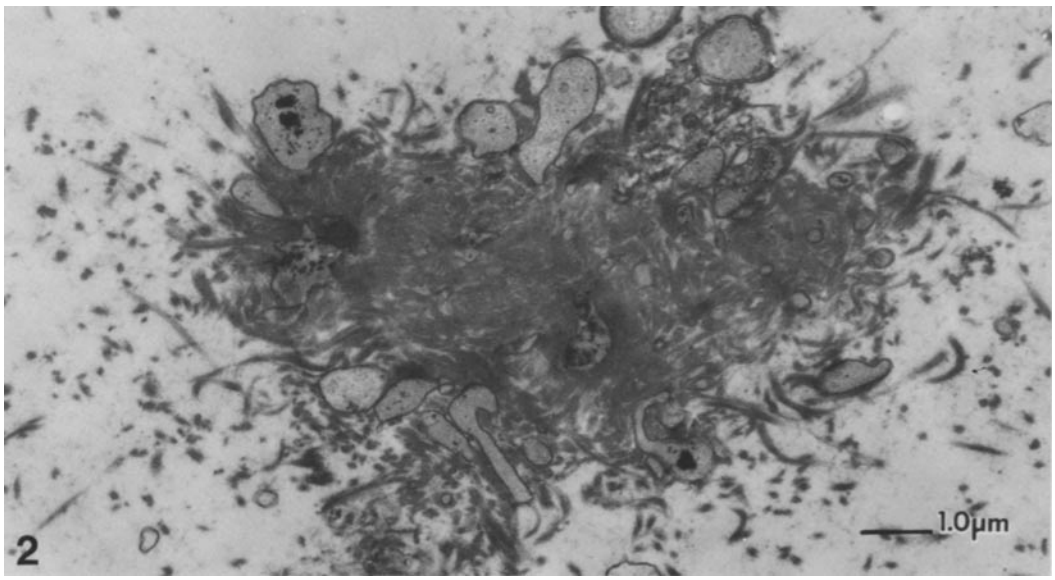


FIGURE 2 Platelet-rich plasma clot contracting in the absence of an external load. Large platelet-fibrin clumps have formed, however, no particular orientation of fibrin strands or platelets can be seen. Longitudinal section, uranyl acetate and lead citrate stain. × 9,000.

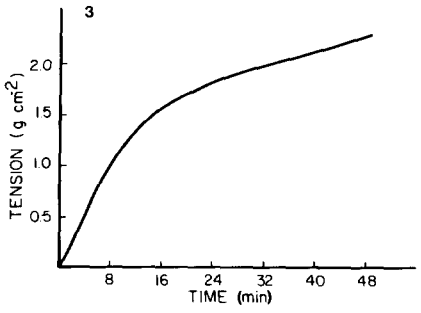


FIGURE 3 Tension development in thrombin-clotted platelet-rich plasma. Citrated platelet-rich plasma, 0.9 ml, containing 3.3×10^8 platelets/ml was clotted with 1 U thrombin (Parke-Davis). The tension recorded is expressed in g/cm^2 (initial cross-sectional area).

0.2 g/min per cm^2 (initial cross-sectional area) (Fig. 3). When fixed under tension after having attained a force of 1.6 g/cm^2, the isometrically contracting clots differed strikingly in ultrastructural appearance from clots contracting in the absence of an extrenal load (Figs. 4–7). Longitudinal sections revealed many long, thin fibrin strands oriented like cables in the axis of tension. Platelet-fibrin clumps in the same sections tended to be elongated or spindle-shaped. Long platelet bodies or pseudopods extended in the axis of tension along similarly oriented fibrin strands. Bundles of microtubules and microfilaments present in the extended platelet segments were also oriented in the direction of force generation. The pattern of extended platelet segments containing longitudinally arranged microtubule or microfilament bundles in close apposition to long fibrin cables in the axis of tension predominated in

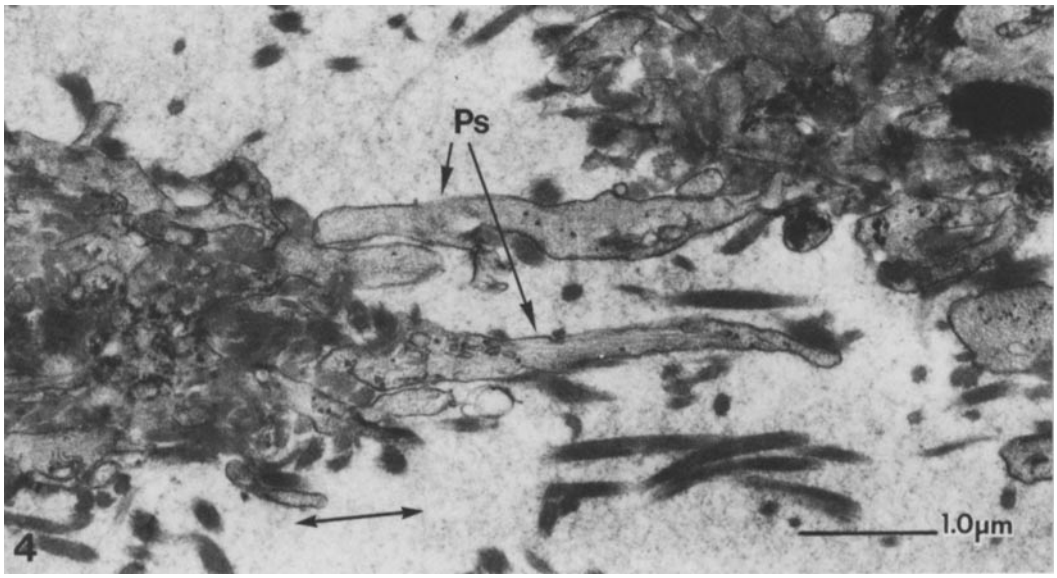


FIGURE 4 Platelet-rich plasma clot fixed under tension. A clot formed using platelet-rich plasma was tied at its ends and inserted in an apparatus to allow isometric contraction and to measure tension development. It was then allowed to contract until half-maximal tension was achieved and fixed. Pseudopods (*Ps*) can be seen extending out from the platelet-fibrin clumps. The section is cut longitudinally as described in the text and shows the alignment of pseudopods and beginning alignment of fibrin strands in the direction of tension. Longitudinal section, uranyl acetate and lead citrate stain. × 18,000. The bidirectional arrow, where present, indicates the direction of tension.

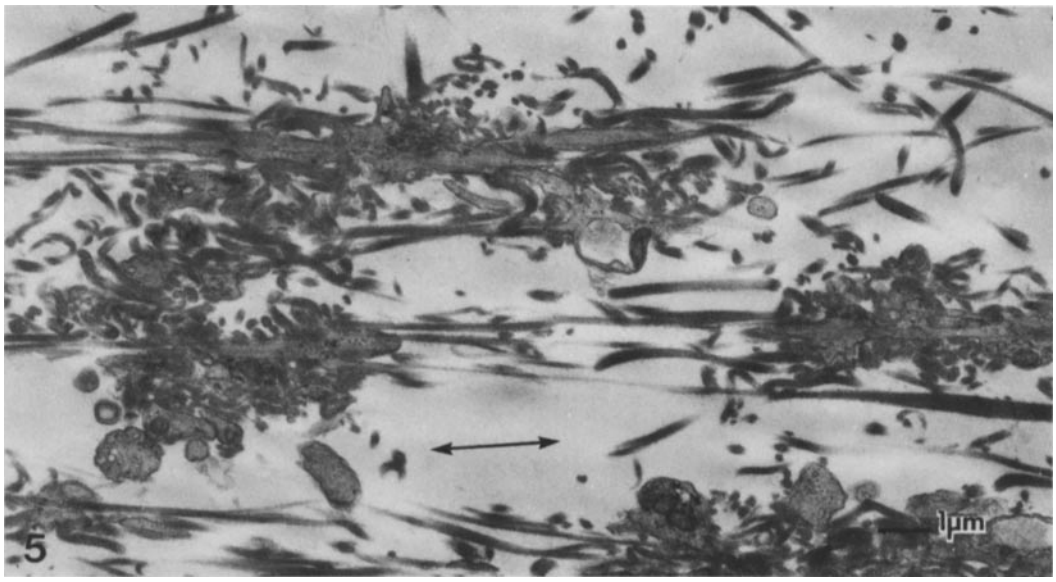


FIGURE 5 A clot was formed using platelet-rich plasma and then allowed to undergo contraction isometrically until half-maximum tension was achieved. Platelet pseudopods and fibrin strands can be seen aligning in the direction of tension. Longitudinal section, uranyl acetate and lead citrate stain. × 7,800.

longitudinal sections. Cross sections of the isometrically contracted clots (Fig. 8) yielded the contrasting appearance. Cables of fibrin similar to those in longitudinal sections were virtually absent. Portions of platelets in close apposition to fibrin strands contained hollow-cored circular profiles of microtubules. Platelet segments with longitudinally oriented bundles of tubules or microfilaments were as rare in cross sections as they were common in sections cut in the coronal plane.

Groups of 3 to 10 serial and adjacent longitudinal sections were examined to determine if only pseudopods or entire platelet bodies assumed extended configurations in isometrically contracted clots (Figs. 9 and 10). Extended portions of the cells in close apposition to the long fibrin strands away from aggregates appeared most often to be single pseudopods. However, no clear demarcation between pseudopods and platelet bodies could be identified in the aggregates. Therefore, the entire platelet appears involved in establishing the longitudinally oriented platelet-fibrin associations identified in clots fixed under isometric conditions.

To further assess the role of platelets, in particular the requirement for platelet-platelet attachment, clots were formed using platelet-poor plasma and a concentration of platelets varying from 0 to $4.8 \times 10^5/\mu l$. The rate of tension produced was roughly proportional to the number of platelets present (Fig. 11). With increasing numbers of platelets, the size of the platelet clumps increased and the distance between them de-

creased. The orientation of fibrin fibers in the direction of tension, seen under isometric conditions, occurred only when force was generated in the presence of platelets. Clots of platelet-poor plasma showed randomly oriented fibrin fibers and the number of fibrin strands aligned in the direction of tension increased as platelet numbers were increased. A section from a clot made from plasma containing 3×10^4 platelets/μl is shown in Fig. 12. Clots made from plasma containing 4.8×10^5 platelets/μl were similar to those shown in Figs. 4–7. As platelet numbers were varied from $3 \times 10^4/\mu$l of $4.8 \times 10^5/\mu$l, the tension increased linearly.

Role of Fibrin Cross-linking on Isometric Clot Contraction

When normal platelets were washed and resuspended in factor XIII-deficient plasma, the tension developed in the thrombin-generated clot was about tenfold diminished (Fig. 13 *b*) as compared to the control containing washed platelets resuspended in normal plasma (Fig. 13 *a*). The clot formed in the absence of factor XIII was much softer than usual, showed a loss of elasticity and care had to be taken when tieing the thread or it would cut right through the clot. Tension was restored to normal when pure factor XIII was added to the factor XIII-deficient system before clotting with thrombin (Fig. 13 *c*). Platelets in factor XIII-deficient clots were in small to medium size nonelongated clumps (Fig. 14). The most striking change was seen in the fibrin fibers which were randomly oriented and formed only a few thick, striated fibers. Preparations of factor XIII-deficient plasma from two different sources were evaluated. In one, striated fibrin strands were absent and in the other they were present, but strikingly reduced. Platelets within these clots clumped together to some extent, but the distance between clumps remained large. Upon restoration of

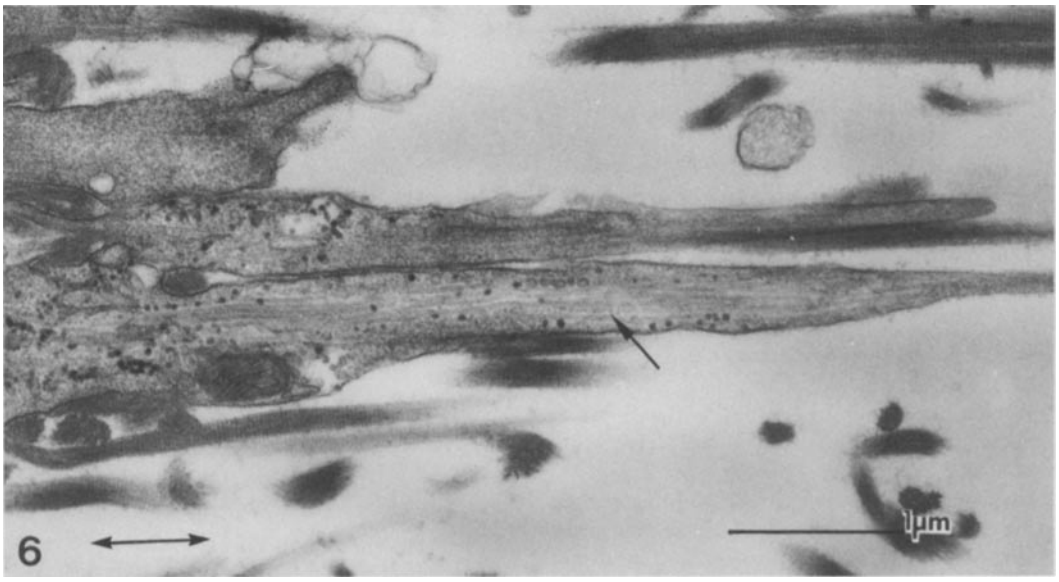


FIGURE 6 A higher power view of a sample of platelet-rich plasma contracted isometrically until maximum tension was achieved. A long platelet pseudopod with numerous microtubules (arrow) can be seen. Longitudinal section, uranyl acetate and lead citrate stain. × 27,000.

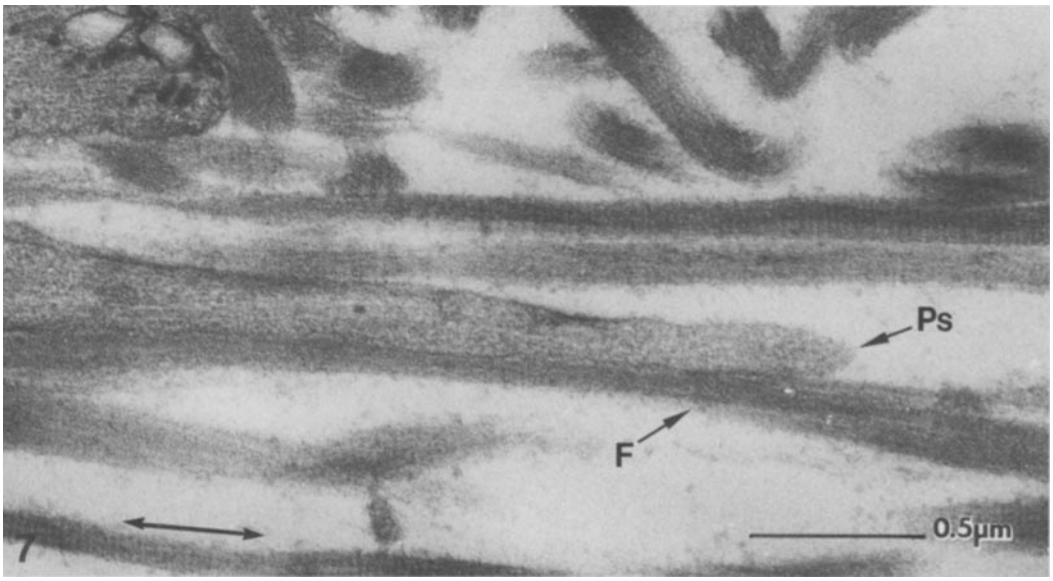


FIGURE 7 Another view of the sample shown in Fig. 6 to show close interaction between a platelet pseudopod (*Ps*) and a fibrin strand (*F*). Longitudinal section, uranyl acetate and lead citrate stain. × 46,000.

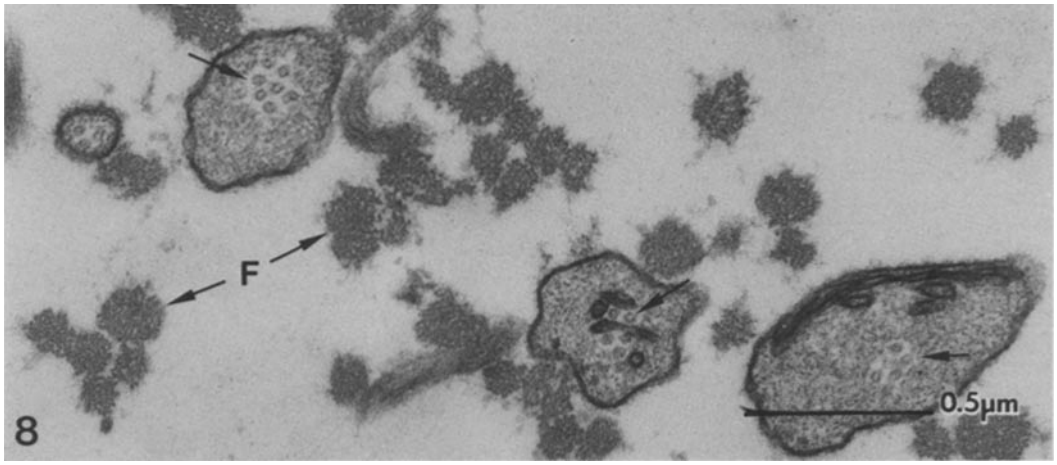


FIGURE 8 A cross section of the clot formed in platelet-rich plasma and allowed to contract isometrically to reach maximal tension. Fibrin (*F*) strands are cut almost exclusively in cross section as are platelet pseudopods. Microtubules (arrows) are present within all cross sections of pseudopods. Cross section, uranyl acetate and lead citrate stain. × 50,000.

pure factor XIII, thick fibrin strands oriented in the direction of tension were produced and platelets developed pseudopods (Fig. 15*a* and *b*) which extended along the fibrin strands. Deficient clot retraction was not restricted to isometric conditions, but was also found in the absence of an external load in the floating clot technique.

Role of Abnormal Platelets in Clot Retraction

Clots were made from platelet-rich plasma from a patient with Glanzmann's thrombasthenia. Ultrastructurally, these clots did not show an orientation of the fibrin strands in the direction of isometric tension (Fig. 16) and the platelets did not form clumps.

Clots were also made from platelet-rich plasma from patients with either absent dense bodies or markedly decreased alpha granules. In both cases, clots formed normally, had a normal ultrastructural appearance, and generated tension normally.

Effects of Inhibitors

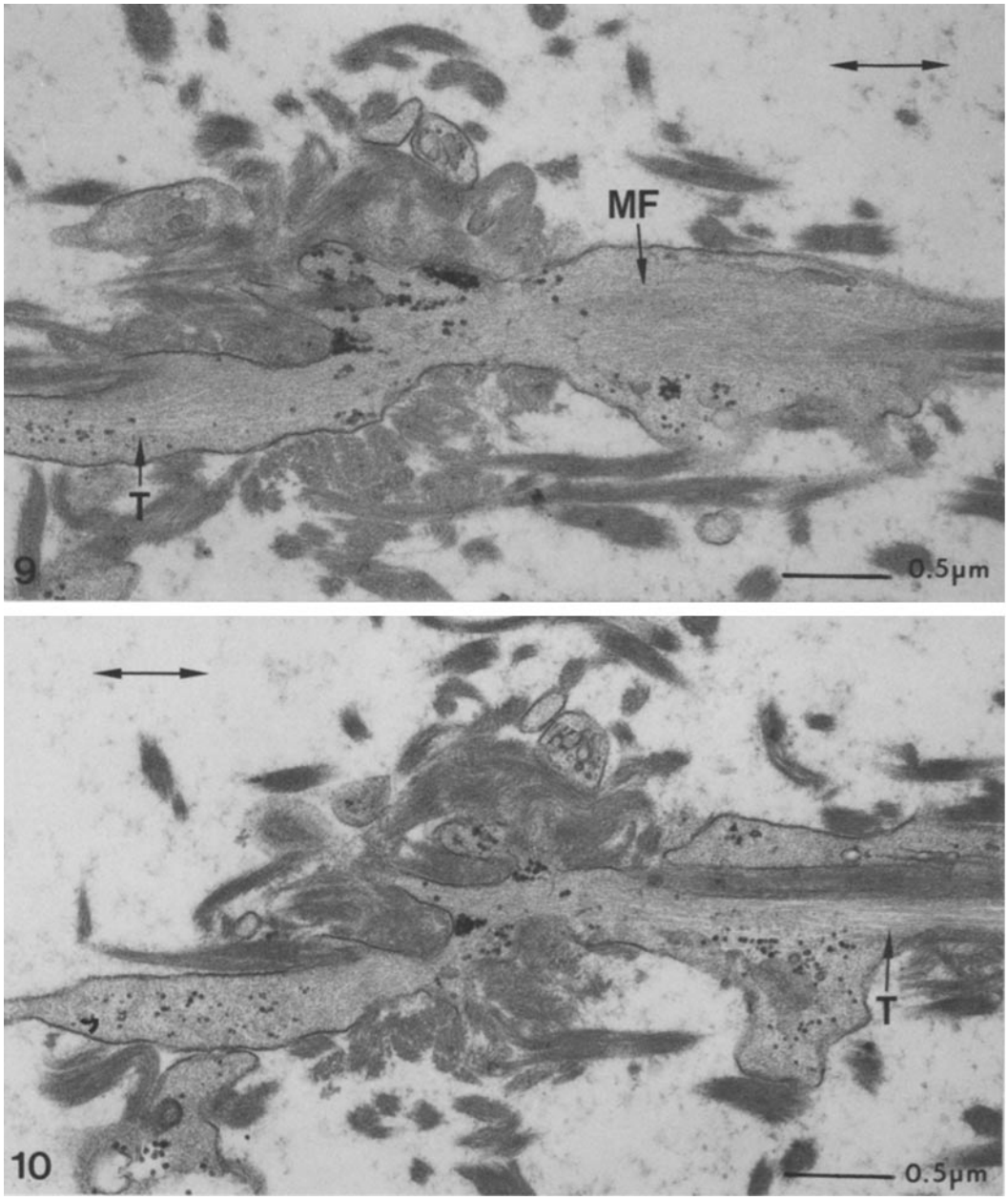
Addition of prostaglandin E_1 (2.8×10^{-6} M) with theophylline (2×10^{-3} M), EGTA (10^{-2} M), cytochalasin B (4×10^{-5} M), or vincristine (10^{-4} M) before clot formation, prevented the generation of tension and the development of platelet-fibrin clumps.

When EGTA (10^{-2} M, not shown), cytochalasin B (4×10^{-5} M), or vincristine (10^{-4} M) were added at half-maximal tension, they all produced relaxation (Fig. 17). Whereas the tension returned to the baseline in the presence of EGTA, it was reduced to about 70 and 50% respectively, in the presence of cytochalasin B and vincristine at the concentrations used. Full tension was restored upon washing the EGTA and cytochalasin B-treated clot with Tyrode buffer. Lower concentrations of vincristine were ineffective. Whereas 4×10^{-6} M cytochalasin B did not cause relaxation but prevented tension to develop further, relaxation produced by 4×10^{-6} M cytochalasin E was similar to that caused by a tenfold concentrated cytochalasin B (Fig. 17). Associated with the decrease in tension produced by EGTA, aggregates of fibrin and platelets within the clot became more loosely organized and orientation of fibrin strands was lost (Fig. 18). When cytochalasin B was added at half-maximal tension, the platelet-fibrin clumps persisted, though the platelets within the clumps became less tightly packed (Fig. 19). Fibrin strands remained largely oriented in the direction of tension, but platelet bodies and pseudopods at the edge of a clump became more rounded in appearance. These changes were associated with disappearance of oriented bundles of microfilaments in the platelet cytoplasm, and their replacement by a granular appearance, as described earlier (41). When vincristine was added at half-maximal tension, the relaxation was associated with the loss of platelet microtubules and an apparent decrease of the pseudopod length (Figs. 20 and 21). The fibrin fibers remained oriented in the direction of isometric tension.

DISCUSSION

The results of the present study can best be evaluated in the light of earlier studies and models of clot contraction (Fig. 22). The essential importance of platelets as the actively contracting or force-generating component necessary for contraction was well documented in the studies of Budtz-Olsen (5). Noteworthy also was the observation by Sokal (39) of pseudopods emerging radially and over a considerable distance from platelet masses and attaching to fibrin fibers. The role of fibrin in clot contraction has been less clear. One concept has been that fibrin serves primarily as a load to be collected or gathered together by the platelets. Development of this concept produced the zipper model of clot contraction (9), according to which platelets extend pseudopods until they come into contact. This is followed by a "zippering" together of the membranes of adjacent platelets to contract the clot (*Model 1*). Fibrin would be gathered and compacted into the space between platelet membranes but would not provide an essential structural component, except as a mesh for platelets to extend pseudopods and contact other platelets. Support for the idea that fibrin serves primarily as a load to be compressed by platelets came from studies with clots prepared from factor XIII-deficient plasma. These studies appeared to show little change in the contraction of clots made from non-cross-linked fibrin compared to clots formed from normal fibrin (36). One study in which cross-linking appeared to delay retraction was used as further support for the fibrin = load concept (32).

The methods used to assess clot retraction in these earlier studies could have led to artifactual results, and it was essential to reassesses the role of factor XIII. In our studies, under



FIGURES 9 and 10 Adjacent serial sections in the longitudinal plane through the same platelet. The body and pseudopods extend in the axis of tension. Microtubules (*T*) are prominent in one pseudopod and masses of microfilaments (*MF*) in the opposite extension in Fig. 9. The microfilaments are replaced by microtubules (*T*) in a deeper section of the same pseudopod in Fig. 10. Longitudinal sections, uranyl acetate and lead citrate stain. Fig. 9, × 30,500; Fig. 10, × 30,000.

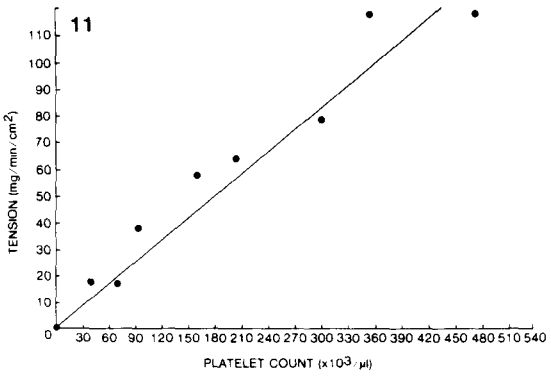


FIGURE 11 Variation in the rate of tension development in a plasma clot with increasing concentrations of washed platelets.

conditions of isometric contraction, cross-linking of fibrin was quite clearly critical. Factor XIII deficient clots failed to develop tension and this could be corrected by addition of pure factor XIII. Cross-linking of fibrin may function either to provide an improved support for the extension of platelet pseudopods or to provide fibrin with high enough tensile strength so that it does not fall apart when subjected to tension by platelets.

Another approach for evaluating the relative role of platelets and fibrin was to vary the platelet concentration. If platelet-platelet interaction was the important component for force generation, as suggested by Model 1, small platelet numbers should be much less able to make pseudopod-pseudopod contact. Tension should fall off exponentially with a decrease in platelet numbers, unless the platelets had some way of com-

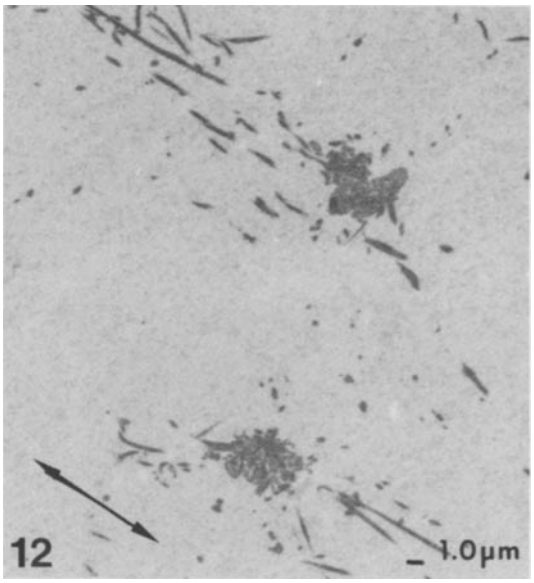


FIGURE 12 Clot formed using a low concentration of washed platelets suspended in homologous platelet-poor plasma (3×10^4 platelets/μl) and allowed to contract isometrically. Some alignment of platelets and fibrin is apparent but less than at higher platelet concentration. Longitudinal section, uranyl acetate and lead citrate stain. × 2,000.

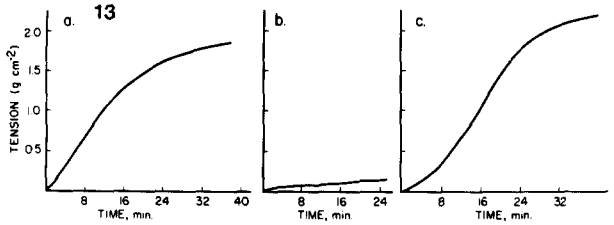


FIGURE 13 The role of factor XIII in tension development. Washed platelets were resuspended at a final concentration of 5.5×10^8 platelets/ml in normal plasma (*a*), in factor XIII-deficient plasma (*b*), or in factor XIII-deficient plasma supplemented with 24 μg pure factor XIII/ml (*c*). Factor XIII-deficient plasmas from two other patients gave the same result. Clots *a* and *c* were insoluble in 5 M urea or 1% monochloracetic acid whereas, clot *b* was soluble under the same conditions.

pensating by extending longer pseudopods. If platelet-fibrin interaction was the crucial factor for the generation of force, as suggested by other models (Fig. 22), tension development should fall off linearly with a decrease in platelet concentration. We found that the rate of tension development decreased linearly with a decrease in platelet concentration. The observation argues strongly for the importance of platelet-fibrin interaction, rather than platelet-platelet interaction in force generation.

Ultrastructural studies of clots prepared at low platelet concentrations revealed some alignment of fibrin strands but less than at high platelet concentrations. Pseudopods appeared shorter, rather than longer, when low numbers of platelets were used to form the clots. It is possible that long pseudopods not in the plane of section were missed. However, the presence of some aligned pseudopods should have meant the visibility of at least a few very long pseudopods if these had been present. None were seen. Therefore, it is unlikely that the platelets were able to compensate for decreased numbers by extending longer pseudopods. These findings provide support for the concept that platelet-fibrin interaction, rather than platelet-platelet interaction is responsible for generation of tension in contracting clots and Model 1 should be rejected.

A further evaluation of the role of platelet-fibrin interaction was done using platelets from a patient with thrombasthenia. These platelets lack glycoproteins IIb and III and lack fibrinogen receptors (2, 30). There is no firm evidence that the platelet-fibrin bond is identical to the platelet-fibrinogen attachment. However, this seems likely. Clots prepared using thrombasthenic platelets failed to generate tension or align fibrin or pseudopods. This is consistent with the critical role of platelet-fibrin interaction in this process. In contrast to the defective clot retraction in thrombasthenia, congenitally abnormal platelets with deficient dense bodies or alpha granules showed normal clot contraction, suggesting that granule contents or secretion are not essential.

We next considered the two models for clot contraction involving platelet-fibrin interaction. The waving pseudopod model (8), *Model 2* (Fig. 22), would predict many pseudopods oriented opposite to the direction of tension. Longitudinal sections of clots fixed during isometric contraction, however, revealed little evidence to support this hypothesis. Platelet-fibrin clumps, platelet bodies and pseudopods were aligned in the axis of tension, and pseudopods arranged across the longitudinal axis were rarely observed. Therefore, Model 2 should be rejected.

Model 3 (Fig. 22) in which platelet pseudopods pull in fibrin strands (35) was more consistent with our findings, but needed some revision. We found that long pseudopods and extended platelet bodies, aligned along fibrin strands, were still present under conditions of maximal tension, suggesting that pseudopod extension and retraction was a continuous process rather than just one extension and retraction as earlier envisaged. The

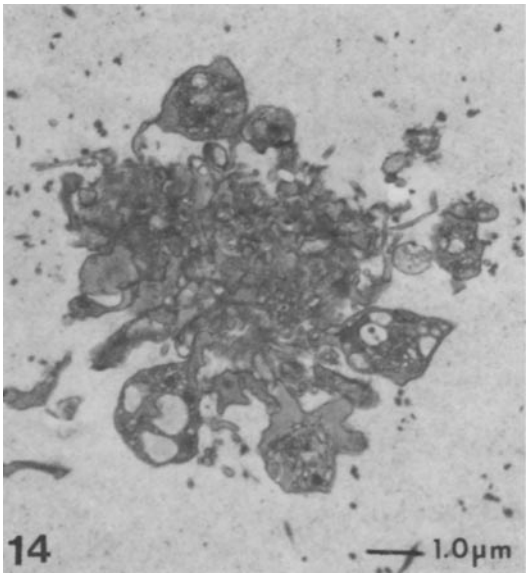


FIGURE 14 A clot was formed using washed platelets suspended in factor XIII-deficient plasma (7×10^5 platelets/μl) and allowed to contract isometrically. Platelet-fibrin clumps form as shown, but these consist primarily of platelets and the fibrin is present only in short scattered segments. No orientation or alignment of platelets or fibrin is seen. Longitudinal section, uranyl acetate and lead citrate stain. × 8,000.

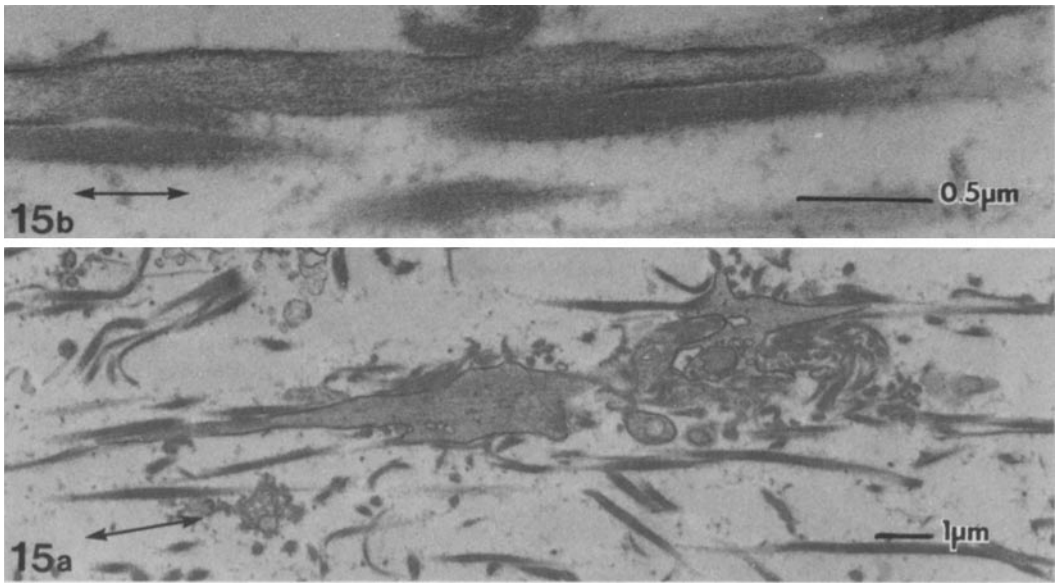


FIGURE 15 A clot was formed using washed platelets ($7 \times 10^5/\mu l$), suspended in factor XIII-deficient plasma to which was added 24 μg/ml purified factor XIII. The clot formed after thrombin addition (1 U/ml) was allowed to contract isometrically. Long fibrin strands formed and platelet pseudopods and fibrin strands aligned in the direction of tension as shown. Longitudinal sections, uranyl acetate and lead citrate stain. *a*, × 8,000; *b*, × 36,000.

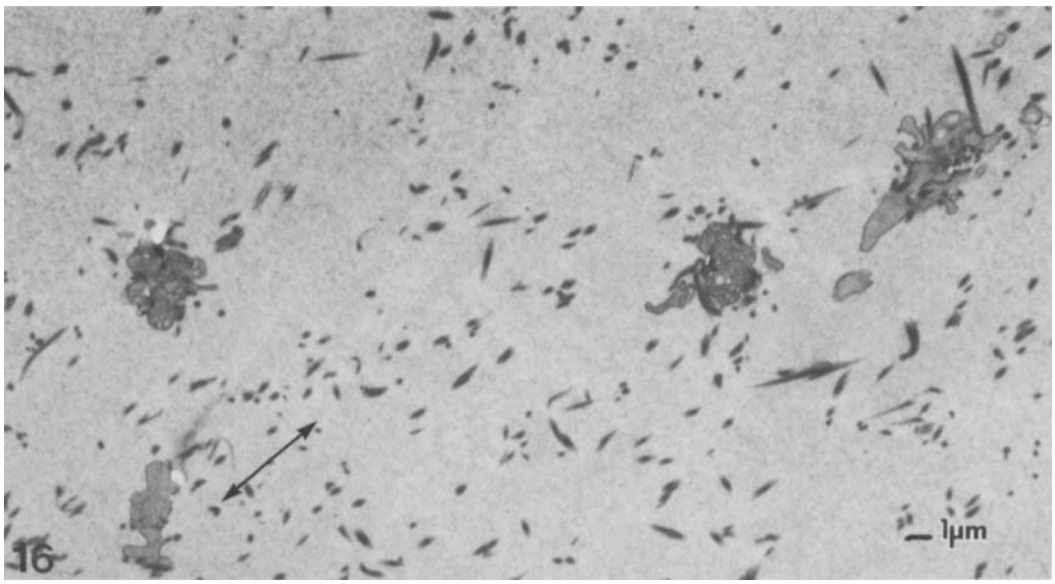


FIGURE 16 A clot was formed using a sample of platelet-rich plasma from a patient with Glanzmann's thrombasthenia and allowed to contract isometrically. No tension was produced. Platelets remained single and did not form platelet-fibrin clumps. No alignment of platelet pseudopods or fibrin strands in the direction of tension was seen. Longitudinal section, uranyl acetate and lead citrate stain. × 3,750.

tips of pseudopods were often less closely attached to the fibrin than regions near the base. This finding suggested the possibility that the membrane at the tip may continue to flow and extend outward even as the portions near the base are being pulled inward by contractile microfilaments.

Studies with the inhibitors, vincristine and cytochalasin B, suggested that microtubules and microfilaments are important for isometric contraction of clots. Earlier investigations demonstrated that concentrations of vincristine lower than those used in the present study will dissolve platelet microtubules without inhibiting clot retraction (44). However, the same low doses of colchicine and vinca alkaloids which are ineffective in platelet-rich plasma will inhibit clot retraction in diluted samples of platelet-rich plasma (7). Similar differences in amounts of the mitosis inhibitor required to inhibit platelet secretion compared to minimum concentrations necessary to remove microtubules have been noted in other studies (28, 37, 38).

As similar dichotomy exists with regard to effective concentrations of cytochalasins B and E. Concentrations similar to those used in the present study can inhibit total polymerization of actin in platelets (15). These amounts, necessary to block clot retraction or cause relaxation, do not appear toxic to other cell functions (23, 41). Removal of cytochalasin B by washing the clot several times restores normal contractile activity and

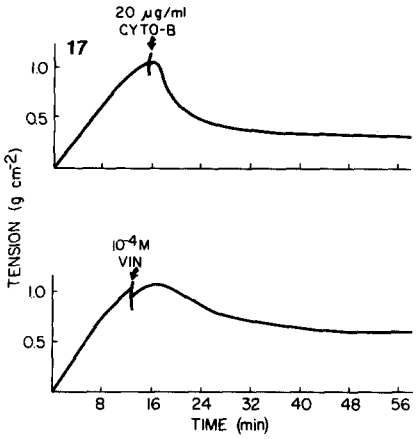


FIGURE 17 The influence of cytochalasin B and vincristine on tension development. The experimental conditions were the same as in Fig. 3, except that the bath was drained and cytochalasin B (20 μg/ml) or vincristine (100 μM) in Tyrode buffer were added as shown. No effect was seen when ethanol, the cytochalasin solvent, was added at 0.5% concentration in Tyrode buffer.

FIGURE 18 A clot formed using platelet-rich plasma was allowed to contract isometrically to reach half-maximal tension at which point the bath was drained and then 10 mM EGTA in Tyrode buffer was added. The clot relaxed until no remaining tension was measureable and was then fixed for study. Platelets continued to be associated in small platelet-fibrin clumps, however, the association of platelets and fibrin was loose. Fibrin strands and platelet pseudopods were no longer aligned longitudinally within the clot. Longitudinal section, uranyl acetate and lead citrate stain. × 13,000.

full isometric tension. Concentrations of cytochalasin B similar to those used in the present study were also required to fully inhibit several functional activities of monocytes (45). Thus, the higher concentrations of vincristine and cytochalasin B required in the present study to influence clot retraction compared to amounts needed to disassemble microtubules or prevent actin polymerization in vitro may not be cytotoxic and consistent with concentrations used to inhibit other cell systems. It is possible that preservation of fibrin orientation and platelet-fibrin interactions in clots treated with vincristine or cytochalasin B may result in significant residual tension. Higher concentrations of the two agents to produce maximal relaxation of individual platelets may be necessary under these conditions to cause significant changes in isometric tension.

Pseudopod and platelet body lengths appeared decreased in clots to which vincristine or cytochalasin B had been added at half maximal tension, despite persistence of the alignment of fibrin strands. The observed changes in pseudopod length and rounding-up of platelet extensions under these conditions were unlikely to represent artefacts due to the plane of section, since fibrin strands remained oriented in the longitudinal axis. It is probable that microtubules are important in the process of extension and stabilization of pseudopods and platelet bodies along fibrin strands in the axis of tension. By acting as a cytoskeletal support system the microtubules may facilitate contraction by oriented bundles of microfilaments.

The loss of fibrin orientation associated with total loss of tension upon addition of EGTA suggests a crucial role of calcium in platelet-fibrin interaction by analogy to platelet-fibrinogen interaction (27). EGTA may act through dissocia-

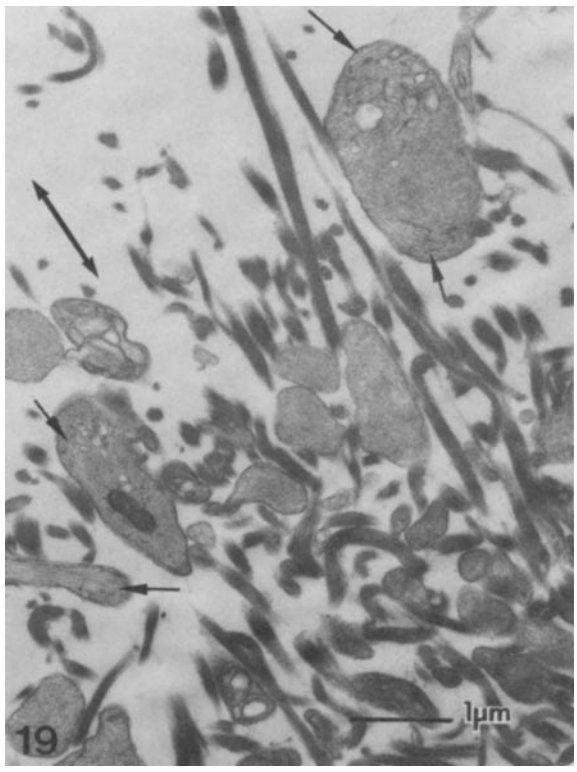
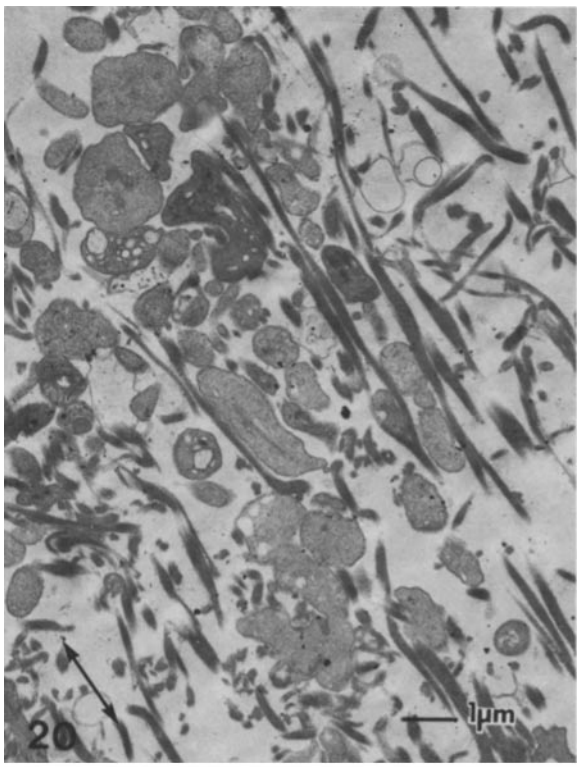
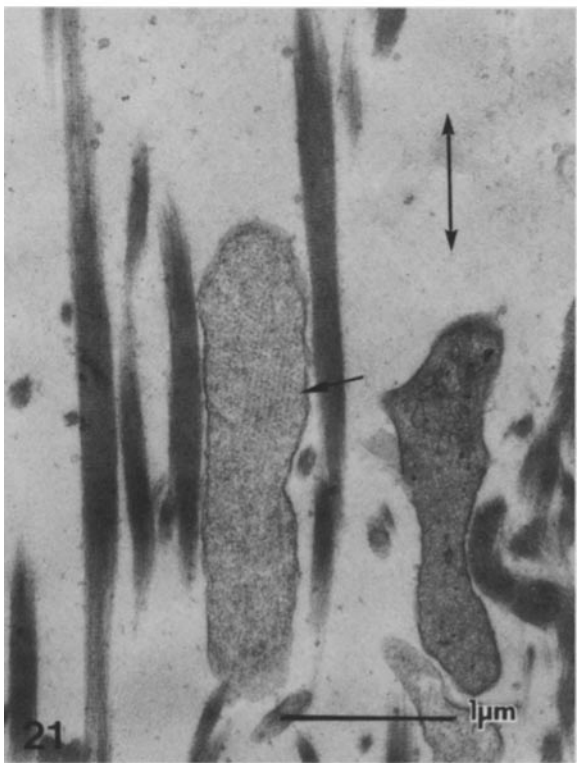


FIGURE 19 A clot formed using platelet-rich plasma was allowed to contract isometrically to reach half-maximal tension at which point the bath was drained and then 4×10^{-5} M cytochalasin B in Tyrode buffer was added. The clot relaxed as shown in Fig. 17 and was then fixed for study. Platelets are in loose clumps and are rounded with a noticeable absence of long pseudopods. Fibrin orientation in the direction of tension is maintained. Microtubules (arrows) are still present within the platelets. Longitudinal section, uranyl acetate and lead citrate stain. × 13,000.





FIGURES 20 and 21 A clot formed using platelet-rich plasma was allowed to contract isometrically to reach half-maximal tension at which point the bath was drained and then 10^{-4} M vincristine in Tyrode buffer was added. The clot relaxed as shown in Fig. 17 and was then fixed for study. Platelets and fibrin are in relatively loose clumps. In Fig. 20 platelets have lost their long pseudopods and are relatively rounded in appearance, although orientation of the fibrin strands in the direction of tension is maintained. At higher magnification in Fig. 21, platelets have lost their microtubules and instead show crystalloid inclusions (arrow) typical of vincristine-treated platelets. Longitudinal section, uranyl acetate and lead citrate stain. 20, × 7,500; 21, × 23,000.

tion of the bonds between platelets and fibrin. Alternatively, the thrombin-activated platelets within a clot may undergo membrane alterations associated with ATP degradation (21) and causing leakage of calcium. This cation is then trapped by EGTA added externally, preventing, therefore, intracellular contractile protein interactions required for tension development. Since cytochalasin B influences the platelet contractile mechanism through depolymerization of actin filaments (4) without affecting the platelet-fibrin relationships, it is likely that EGTA influences both contraction and the interaction of platelets with fibrin.

The findings of the present study, taken in the context of present knowledge of platelet contractile proteins, suggest that the process may take place as shown in Fig. 23. The main features of this model involve the development of a platelet contractile activity and of platelet-fibrin interaction. In this concept platelets activated by thrombin initially undergo polymerization of actin (31, 34), change their shape, extend pseudopods, and have fibrinogen binding sites generated on their surfaces. The polymerization of fibrin thus enables platelet-fibrin attachments to develop provided external calcium is present. With the formation of long fibrin strands, platelet pseudopods now have a structure along which they can spread or crawl. We suggest that fibrin strands cross-linked in the presence of factor XIII form such a surface structure for extension of pseudopods. External attachment of fibrin may enhance internal actin polymerization. Orientation of pseudopods may be dependent on such polymerization associated with the reorganization and extension of microtubules. Concurrently with pseudopod extension, polymerized actin in the pseudopod base may begin to interact with myosin in the cell center initiating retraction. The gradual pulling of pseudopods bound to fibrin, perhaps even as they continue to extend at the tips, provides a mechanism for the platelet to gather in fibrin strands so that strands remaining outside the developing platelet-fibrin clumps become stretched and aligned in the direction of tension (Fig. 24). The process thus achieves the picture seen at maximal tension of large platelet-fibrin clumps with tightly stretched fibrin strands in between.

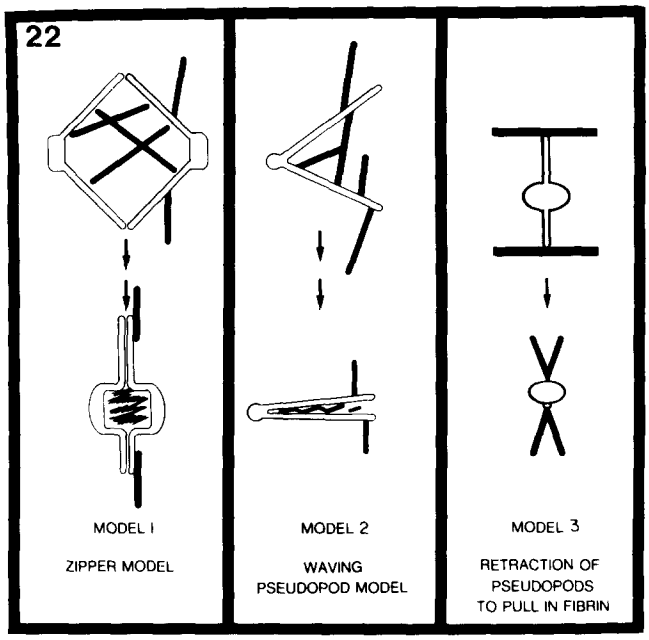


FIGURE 22 Earlier models of clot contraction. Heavy lines represent fibrin fibers; light lines delineate platelets.

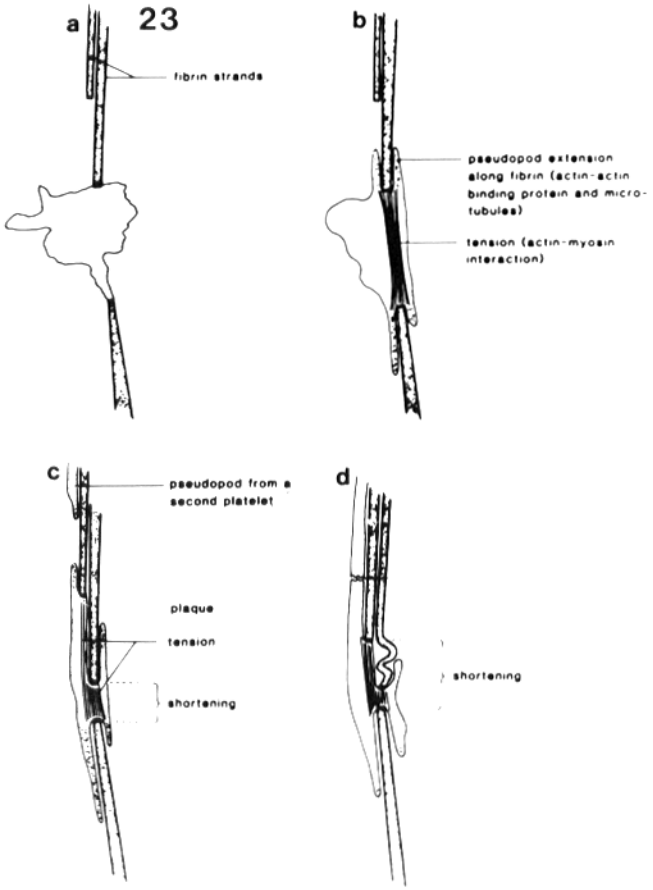


FIGURE 23 A proposed model for the interaction of platelets and fibrin in an isometrically contracting clot. The platelet initially forms attachments to fibrin strands (*a*). Pseudopods then start to crawl along the fibrin strands (*b*). We speculate that such pseudopod extension may involve the interaction of actin with actin binding protein and microtubules. In addition, we speculate that actin-myosin interaction within the body of the platelet (*b*, *c*, *d*) is responsible for drawing fibrin strands attached on opposite sides of platelet closer together. Compaction of a fibrin strand in association with the platelet is beginning in *d*. As more fibrin strands are compacted within such platelet-fibrin aggregates, the strands that remain outside such aggregates are pulled to create the tension measured and to align them in the direction of tension as shown in Fig. 24.

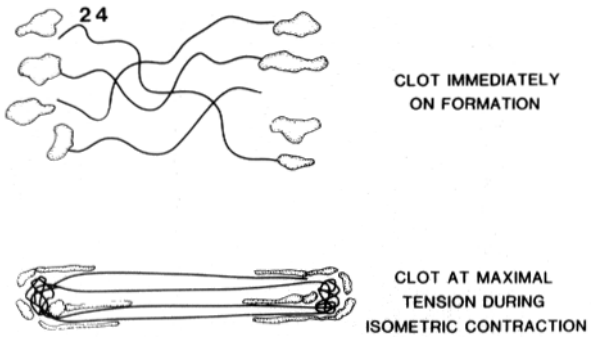


FIGURE 24 Alignment of fibrin strands during tension development as it would occur postulating the model of clot retraction shown in Fig. 23.

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