Micropipette Aspiration of Human Platelets: Influence of Microtubules and Actin Filaments on Deformability

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The deformability of human platelets has been evaluated by micropipette aspiration. Control discoid platelets were about ten times as resistant to deformation in the micropipette as red blood cells. Under a constant negative pressure of 10 cm H₂O, control platelets developed extension lengths of 0.74 ± 0.1 μm. Prior treatment with vincristine, colchicine, or low temperature, all of which remove platelet microtubules, was associated with marked increases in lengths of aspirated segments. Taxol or heavy water, which stabilize microtubules, prevented the increased deformability caused by agents that dissolve microtubules. Cytochalasin B, an agent that inhibits assembly of actin microfilaments, also caused an increase in lengths of aspirated segments that could not be prevented by taxol. Vincristine and cytochalasin B, together, caused a greater increase in deformability than either agent alone. These results indicate important roles for microtubules and microfilaments in platelet deformability.

MATERIALS AND METHODS

Blood was collected by venipuncture from normal human donors after informed consent. The blood samples were mixed immediately with 3.8% trisodium citrate or citrate-citric acid, pH 6.5 (93 mmol/L sodium citrate, 70 mmol/L citric acid, and 140 mmol/L dextrose) in a ratio of 9 parts blood to 1 part anticoagulant. Platelet-rich plasma (PRP) was separated from whole blood by centrifugation at 100 g for 20 minutes at room temperature. Samples of PRP were mixed with an equal volume of the citrate anticoagulant and centrifuged at 400 g for 15 minutes at 20°C to pellets. Supernatant was discarded and the pellets resuspended in phosphate-buffered saline containing bovine serum albumin (0.1%). Adenosine (5 mmol/L) and theophylline (3 mmol/L) were incubated in the suspending media to inhibit activation on contact with foreign surfaces. Washed platelets were maintained at room temperature until used in specific experiments.

Vincristine sulfate was obtained from Eli Lilly, Indianapolis, Ind. Colchicine and heavy water were obtained from the Aldrich Chemical Co, Milwaukee, Wis. Taxol was obtained from the Natural Products Branch, Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Bethesda, Md. The agent was dissolved in dimethylsulfoxide (DMSO) at a concentration of 10 mmol/L. A volume of 10 μL added to 1 mL of washed platelets yielded a final concentration of 10⁻⁶ mol/L taxol and 1.0% DMSO. Cytochalasin B was obtained from Sigma, St Louis, Mo. Micropipettes were prepared in a two-step process. Initially, boron silicate glass capillary pipettes, 1 mm in external diameter, were heated and drawn on a horizontal micropipette puller (model M-1, Industrial Science Association, Ridgewood, NY) into long, needle-like configurations. Then, using a manual stereoscopic microforge (De Forbrune, Aloe Scientific, St Louis, Mo), a second interval of tension drew the pipettes to diameters sufficiently narrow for platelet studies. Internal diameters of the pipettes were measured on the television monitor that was standardized with a 10-μ grid, described below. The internal diameters ranged from 0.6 to 0.8 μm. The tips of the pipettes were filled with phosphate-buffered saline by immersion under lyophilizer vacuum for two hours, and the large diameter portions were backfilled by use of a thin wire guide. The filled pipette was connected by saline-filled, clear plastic tubing to a pressure transducer (Beckman model 4-327), a pressure adjustment system, and a water manometer. The pipette was mounted in a Defonbrune micromanipulator (Aloe Scientific, St Louis, Mo). A second interval of tension drew the pipettes to diameters sufficiently narrow for platelet studies. The pipette was mounted in a Defonbrune micromanipulator (Aloe Scientific, St Louis, Mo) and a water manometer. Negative and positive pressure was exerted through the pipette by a screw-driven syringe and the pressure was continuously recorded on a Beckman R511 A dynograph.

Drops of washed platelets were placed on siliconized glass slides and inserted on the stage of an upright Reichert Zetopan microscope (Vienna, Austria) equipped with Zeiss Nomarski interference.
Deformity of Platelet Cytoskeleton

Fig 1. Micropipette aspiration of human platelets. A normal platelet in this example has been drawn into a micropipette with an internal diameter of 0.6 μm under constant negative pressure of 10 cm H2O. The length of the aspirated segment is approximately 0.74 μm (540×).

The tip of the pipette was advanced under direct vision, while a constant negative pressure of 10 cm H2O was maintained. Preliminary studies revealed that this pressure uniformly deformed discoid platelets without causing passage through the pipette. When a platelet was contacted, a portion of the cell was immediately drawn into the shaft of the pipette, and the bulk of the platelet remained outside (Fig 1). The aspiration was recorded on videotape until equilibrium in the length of the platelet projection was attained.

In order to calibrate the magnification of the total optical system and micropipettes, a Zeiss 10-μm standard grid was projected onto the monitor from the microscope stage in the same manner as for platelets. A vernier caliper was used to measure the lengths of the images of platelet projections into micropipettes. These measurements were extrapolated to actual dimensions by comparison with caliper measurements of the 10-μm grid. Values were expressed in micrometers. Means and standard deviations were derived from these measurements, and the statistical significance was determined using Student’s t test. Each mean was based on measurements of 15–20 aspirated platelets.

Control cells were studied with the same pipettes used to evaluate modified platelets. The slight variations in pipette diameter between experiments had little influence on the reproducibility of the resultant extension lengths of control or modified platelets. The cells were retained in the pipette for 15–30 seconds and then extruded. Observations on the extruded cells continued for 5 minutes.

RESULTS

Deformability of Control Platelets

Samples of platelets from 15 normal donors were evaluated. Aspiration of a control platelet into a pipette is shown in Fig 1. The average extension length of discoid untreated platelets under a constant negative pressure of 10 cm H2O was 0.74 ± 0.1 μm (Fig 2). Extension lengths were the same whether generated at the edge or in the center of the disc. Upon expulsion from the pipette, platelets regularly regained their discoid form within 1 minute.

Influence of Microtubules

Vincristine and colchicine are well known antimitotic agents. At concentrations that remove platelet microtubules completely, both drugs resulted in a marked increase in extension lengths on aspirated cells (Figs 2 and 3). Vincristine (10⁻³ mol/L) and colchicine (10⁻³ mol/L) treated platelets failed to retract aspirated segments after expulsion from the pipette and remained deformed throughout the 5-minute period of observation.

Taxol is an interesting new chemical that stabilizes microtubules and prevents their disassembly. Exposure to taxol alone had no measurable effect on platelet deformability (Fig 2). Thus, even though taxol stabilizes microtubules, it does not appear to increase their inherent resilience. Taxol was shown in previous studies to prevent disassembly of platelet microtubules by antimitotic chemicals. At similar concentrations in this study (10⁻⁴ mol/L), pretreatment with taxol inhibited the softening influence of vincristine and colchicine on the platelet (Fig 2). When added after vincristine (10⁻⁵ mol/L) or colchicine (10⁻⁵ mol/L), taxol did not affect the marked increases in extension lengths on aspirated cells (Fig 2). Platelet deformability: role of microtubules (−10 cm H2O). Suspensions of washed platelets, prepared as described in the text, were aspirated into glass micropipettes with a mean internal diameter of 0.7 μm. The lengths of aspirated segments on platelets drawn into the micropipette were measured on images projected from videotapes. Each cell was watched for 5 minutes following extrusion from the pipette to observe the ensuing morphological changes. Fifteen to 20 cells were analyzed to obtain each data point. The vertical bars indicate the mean extension lengths in micrometers, and the linear extensions represent standard deviations. Untreated control platelets developed extension lengths of 0.74 ± 0.1 μm and rapidly recovered their discoid form after extrusion from the micropipette. Taxol (10⁻⁴ mol/L), a microtubule stabilizing agent, had no effect on the lengths of aspirated segments (0.79 ± 0.7 μm). Disassembly of microtubules by vincristine at 10⁻⁴ mol/L resulted in a marked increase in the lengths of aspirated sleeves (1.53 ± 0.1 μm), which remained extended for more than 5 minutes after extrusion. Taxol, at a concentration of 10⁻⁴ mol/L that prevents disassembly of microtubules by vincristine (10⁻⁴ mol/L), prevented softening of the cell membrane by the antimitotic agent. Chilling at 4°C for 10 minutes also dissociates platelet microtubules. Chilled platelets were just as soft (1.85 ± 0.2 μm) as those treated with vincristine.
lengths caused by the antimitotic drugs. Heavy water (deuterium D₂O), another microtubule stabilizing agent, also prevented the increased deformability caused by effects of vincristine and colchicine. These findings suggest that the circumferential microtubule has a strong influence on platelet deformability, despite its apparent lack of direct contact with the cell surface.

The antimitotic drugs are known to have effects on cell membrane function in addition to and separate from their action on microtubules. To obviate such effects, the influence of chilling on platelet deformability was evaluated, as shown in Figs 2 and 3. Disassembly of microtubules by exposure to low temperature softened platelets to the same extent as vincristine and colchicine. The chilled platelets remained deformed for several minutes after expulsion from the pipette. This was expected, as it takes almost 30 minutes for the circumferential microtubule to reform when chilled platelets are warmed to 37°C or room temperature.

Influence of Microfilaments

Actin filament assembly can be blocked or partially reversed by cytochalasin B. Incubation of washed platelets with cytochalasin B (10⁻⁵ mol/L) resulted in increased extension lengths on aspirated platelets, though not as long as after exposure to vincristine or colchicine (Fig 4). The deformity induced by the pipette on cells treated with cytochalasin B remained after expulsion, despite preservation of discoid shape. These findings suggest that the microfilament system in platelets contributes to the resistance of the cell membrane to deformation and plays a role in recovery from mechanical distortion.

Combined Effects of Tubules and Filaments

The combined influence of microtubules and microfilaments on platelet deformability was evaluated by treating the cells with both cytochalasin B and vincristine. Exposure to the two agents caused increases in extension lengths greater than those resulting from either chemical alone (Fig 4). This observation suggests that intact microtubules and microfilaments both contribute to the structural integrity of the platelet, and when both systems are depolymerized, the cells have almost no resistance to deformation in the micropipette under constant negative pressure. This interpretation is supported by the observation that about one-third of the doubly treated, soft platelets tend to fragment spontaneously while in the pipette.

DISCUSSION

The present investigation has employed micropipette aspiration to evaluate some of the factors that influence platelet deformability. Micropipette aspiration has been used extensively to define surface characteristics of red blood cells, but, to our knowledge, has not been applied previously to platelets. The small size of the platelet and its tendency to become sticky on contact with glass have limited this approach in the past. Preparation of pipettes with internal diameters below 1 μm, and the addition of agents that inhibited activation of washed platelets, appeared to overcome these problems. Aspiration of normal platelets into the micropipette under these conditions did not appear to
damage the cells significantly. The lengths of the sleeves of platelet membrane drawn into the pipette did not change during the 15–30-s period of observation, and after extrusion, they were reincorporated into the discoid cell.

The extensibility of the platelet into the micropipette may depend on several aspects of platelet structure. Lengths of aspirated segments could reflect the protein–lipid character of the surface membrane, attachment of the membrane to the underlying cytoskeletal network, the state of organization of mobile microfilamentous structures of the cytoskeleton, or integrity of the circumferential band of microtubules. Slight variations in platelet size and shape observed in these studies had a minimal influence on membrane deformability. The dramatic increases in platelet extension caused by agents affecting microfilament and microtubule assembly suggest an important role for these structures in platelet resistance to deformation.

The circumferential microtubule has been shown to play an important role in supporting the discoid shape of blood platelets. Results of the present study demonstrate that the presence of the microtubule band contributes significantly to platelet resistance to deformation in the micropipette. Platelets exposed to low temperature, vincristine, or colchicine to remove their microtubules were twice as deformable as control cells. Taxol and heavy water stabilize microtubules and prevent their disassembly by cold and antimitotic agents. Neither agent made platelets significantly more resistant to deformation, indicating that their action does not make microtubules stiff. Both drugs, however, prevented the increased deformability caused by vincristine and colchicine. These observations suggest that circumferential microtubules exert a force on the platelet surface that contributes significantly to the cell’s resistance to deformation.

Cytochalasin B is a fungal metabolite that inhibits assembly of actin microfilaments and, to some degree, promotes their disassembly. Actin filaments are a major constituent of the detergent-resistant cytoskeleton of activated platelets and, therefore, may influence deformability of the cell surface. Micropipette aspiration of platelets following exposure to cytochalasin B caused a marked increase in extension lengths, though not as great as that caused by removal of microtubules. Taxol which only affects microtubules, had no influence on the softening caused by cytochalasin B. Thus, actin microfilaments appear to contribute significantly to the resistance of platelets to deformation independent of the influence of microtubules. It was to be expected, therefore, that removal of microtubules with antimitotic drugs, combined with inhibition of microfilament assembly by cytochalasin B, produced a greater increase in deformability of the platelet than resulted from either influence alone.

In conclusion, the present study has demonstrated that human platelets possess characteristics that make them resistant to deformation in micropipettes following aspiration under constant negative pressure. Removal of the circumferential microtubule and/or inhibition of actin microfilament polymerization cause a marked decrease in resistance of the cell manifested by aspirated segments of increased length. Recovery following extrusion from the pipette is also affected by microtubule and microfilament disassembly. Thus, the state of assembly of critical components in the platelet cytoskeleton, singly and together, play important roles in the resistance of the cell to deformation.

REFERENCES

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