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Modification of actin, myosin and tubulin distribution during cytoplasmic granule movements associated with platelet adhesion

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Background and Objectives. Cytoskeletal elements determine the changes in platelet cell shape which occur during adhesion, aggregation and release of granular contents as part of the activation process. The aim of this study was to characterize the changes in the distribution of actin filaments, myosin and tubulin molecules during several stages of platelet adhesion to glass and their association with granule displacement, as assessed by confocal microscopy.

Design and Methods. Platelets obtained from healthy donors were adhered to glass and cytoskeleton distribution was characterized and correlated to changes of cell shape and intracellular granule displacement by immunofluorescence assays and phase contrast microscopy. Treatment with specific cytoskeleton inhibitors such as cytochalasin D, butanedione monoxime and colchicine were used before and after the adhesion process. The spatial distribution of the cytoskeleton in association with cytoplasmic granules was analyzed in both confocal microscopy projections and three-dimensional images obtained by merging the respective projections.

Results. Our experiments revealed that as platelets contact the substrate, a sequential and simultaneous rearrangement of actin filaments, myosin and tubulin molecules occurred and this was related to cell shape, as well as to movements of cytoplasmic granules. Treatment of platelets with cytoskeleton inhibitors, modified not only the target molecule but also other cytoskeletal components with consequent alterations in the studied platelet functions.

Interpretation and Conclusions. During platelet adhesion to glass and granule displacement, a close spatial and functional relation between actin filaments, myosin molecules and microtubules was observed suggesting that these different cytoskeleton components interact in supporting the platelet functions here studied.

Key words: adhesion to glass, cytoskeleton, filopodia, granule migration, lamellipodia, platelets.

Platelets are very highly specialized, anucleated fragments whose fundamental role in hemostasis is interaction with the vascular endothelium by adhesion and aggregation. These processes, triggered by blood vessel damage, have the final result of forming a clot with the participation of plasma factors. Clot formation involves a complex series of cellular events called platelet activation. These events include mobilization and exposure of different surface receptors, changes in the shape of the platelet from a discoid to a dendritic shape, and secretion of cytoplasmic compounds.[1,2] Because of the difficulty in studying the physiologic and biochemical properties of platelet adhesion and aggregation *in vivo*, several *in vitro* adhesion systems have been developed using different substrata including plain glass slides and siliconized slides, cultured vascular endothelial cells and biomaterials such as polystyrene derivatives.[3-7] Adhesion to glass has been widely used, not only because it is an easy technique, but also because it appears to maintain some properties which can be used as a reference measure for platelet function.[8]

The platelet cytoskeleton is formed by a network of actin filaments,[1,9-12] a marginal ring of microtubules,[13-16] myosin molecules,[17-19] and actin binding proteins such as α-actinin, vinculin, talin, spectrin, and tropomyosin.[2,12,20-25] The actin is rapidly polymerized into filaments during platelet activation induced by adhesion to surfaces[26-29] with formation of filopodia and lamellipodia, and generation of the extended platelet shape, a process that is inhibited by actin filament depolymerizing drugs such as cytochalasins.[30-33] Myosin is a long, asymmetric molecule organized as bipolar filaments,[17] detected in focal adhesion zones forming stress fiber-like structures, by interaction with actin filaments and α-actinin molecules.[19] This organization provides the support for the tensile strength required in platelet adhesion and membrane

extension.[2,19,34] In activated platelets, myosin is found as an actomyosin gel within the cell body and surrounding the granulomere,[9,35-38] where it has been suggested to intervene in organelle centralization.[39,40] Platelet discoid shape is determined by a marginal ring of microtubules organized in concentric rings derived from a long coiled microtubule.[13,39,41,42] During activation of platelets, induced by exposure to agonists or by adhesion to surfaces, the microtubule ring splits and reorients in extended platelets.[27,28,43] Since ring modification does not occur immediately on activation, it has been suggested that it is not an event essential to the beginning of activation or to other platelet functions.[34,44]

The significance of the cytoskeleton in platelet function has been widely documented; however, most studies have been carried out in platelets completely activated or adhered to substrates, whereas data on the progression of the events involved, the spatial and functional interrelation between the cytoskeletal elements, and the reorganization sequence followed in the different adhesion and activation stages, including granular migration, are lacking. The present study serially analyzed the spatial distribution of actin filaments, myosin and tubulin molecules during platelet adhesion to glass and the distribution of cytoplasmic granules by confocal microscopy. Furthermore, three-dimensional images, produced by merging the respective confocal projections, were analyzed. The functional interrelation of the various cytoskeletal elements was also analyzed through platelet treatment with cytochalasin D, butanedione monoxime, and colchicine, before and after adhesion to glass.

Design and Methods

All reagents and inhibitors were purchased from Sigma, Chemical Co. (St. Louis, MO, USA) unless otherwise indicated.

Preparation of platelet suspensions

Platelets were obtained by venipuncture from healthy donors who had not received any drug during the 10 days previous to sampling and who gave consent to the procedure. Blood was immediately mixed with citrate anticoagulant including dextrose at pH 6.5 (93 mM sodium citrate, 70 mM citric acid, and 140 mM dextrose), at a ratio of 9 parts of blood per 1 part of anticoagulant. Platelet-rich plasma was obtained from total blood by centrifugation at 100 g for 20 min at room temperature, and subsequently mixed with an equal volume of citrate anticoagulant and centrifuged at 400g for 10 min.[45] The platelet pack was suspended and washed twice with Hank's balanced saline solution (HBSS) without calcium (137 mM NaCl, 5.3 mM KCl, 1 mM $MgCl_2$, 0.28 mM Na_2HPO_4.12H2O, 0.87 mM NaH_2PO_4, 0.44 mM KH_2PO_4, 4.1 mM $NaHCO_3$, 5.5 mM glucose) and counted in an hematocytometer. Platelets non-specifically activated by the washing were kept in this solution for 60 min at 37°C,[46] in order to reach a resting condition, which was evaluated by the recovery of the discoid shape. All incubations and processing were carried out at room temperature.

Sequence of platelet adhesion to glass and fixation

The platelets (1×10^8/mL) were settled on glass coverslips for 30 s, 3, 7, and 20 min, removing those which did not adhere by washing with HBSS. Platelets were fixed with 2.5% glutaraldehyde in HBSS for 60 min, washed and micrographed in a Zeiss Axioskop II phase contrast microscope. For immunofluorescence assays, the same concentration of cells were allowed to adhere to glass slides, fixed and permeabilized at the same time with a mixture of 2% *p*-formaldehyde, 0.05% glutaraldehyde, and 0.04% NP-40 in the cytoskeleton stabilizing solution PHEM (100 mM PIPES, 5.25 mM HEPES, 10 mM EGTA, 20 mM $MgCl_2$) for 20 min.[47]

Immunofluorescence

The fixed and permeabilized glass-adhered platelets were incubated with 0.1 μg/mL FITC-phalloidin to stain actin filaments, with an anti-myosin rabbit polyclonal antibody (Cat. M-7523, Sigma Chemical Co., St. Louis, MO, USA) or with an anti-β-tubulin monoclonal antibody (Cat. T-4026, Sigma Chemical Co.) diluted in PBS with 0.1% bovine sera albumin, for 2 h with gentle agitation. Glass coverslips were washed with PHEM and incubated for 1 h with Alexa-Fluor 488-conjugated secondary goat anti-rabbit antibodies (Cat. A-11008, Molecular Probes, Eugene, OR, USA), Alexa-Fluor 568-conjugated secondary goat anti-mouse antibodies (Cat. A-11004, Molecular Probes), or Alexa-Fluor 568-conjugated secondary goat anti-rabbit antibodies (Cat. A-11011). Coverslips were washed several times and mounted in Vectashield (Vector laboratories Inc. Burlingame, CA, USA). The slides were observed in a Zeiss Axioskop microscope coupled to a Bio-Rad MRC600 confocal system equipped with a Kr/Ar laser. A C-Planapochromat 100X N.A. 1.4 oil-immersion objective with 3.0 zoom (0.051 μm pixel dimension [x,y]) was used. Optical sections [z] were performed at 0.18 μm.

The filters used were adequate for double staining with FITC/Alexa Fluor 488 and RITC/Alexa Fluor 586 fluorophores. The power of the laser was between 0.3 and 3%. Negative controls included platelets without primary antibodies, incubated with normal rabbit serum or with an irrelevant IgG monoclonal antibody against the tachyzoite form of the parasite *Toxoplasma gondii* (produced in the laboratory). Controls with a secondary antibody did not show a conspicuous signal with the enhancement and power used. Likewise, platelets incubated with 0.1% DMSO were processed for 1 h as the solvent control.

The distribution of cytoplasmic granules was determined by phase contrast microscopy while cytoskeletal distribution was observed by fluorescence in a Zeiss Axioskop II microscope and micrographed with a Zeiss Plan-Neofluar-Ph 100 X objective with 1.3 N.A.

Three-dimensional reconstruction

The three-dimensional reconstruction of the datasets was performed with Voxel View 2.5 software (Vital Images, Inc. Playmouth, MN, USA), in a Silicon Graphics O_2 workstation. Optical sections were linearly interpolated before reconstruction, and opacities (function a) of both channels were manually edited to increase details. The images were processed by Adobe (Mountain View, CA, USA) Photoshop software.

Preparation of inhibitors

A 20 μM cytochalasin D solution (2X) was prepared in HBSS from a 9.8 mM concentrated solution. The inhibitors butanedione monoxime and colchicine (Research Organics Inc, Cleveland, OH, USA) were directly dissolved in HBSS as 2X solutions.

Adhesion of platelets previously treated with cytoskeleton inhibitors

Resting platelets (1×10^8/mL) in suspension were mixed with the same volume of the drugs in order to get final concentrations of 10 μM of cytochalasin D (CD),[48] 30 mM butanedione monoxime (BDM),[49] or 10 mM colchicine.[50] Platelets were incubated for 60 min and later allowed to adhere to glass slides for 20 min, fixed and then processed for immunofluorescence as formerly described.

Adhesion of platelets to a glass surface and post-treatment with cytoskeleton inhibitors

In this series of experiments, 1×10^8/mL resting platelets in suspension were adhered to glass coverslips for 20 min and than incubated with 10 μM cytochalasin D, 30 mM butanedione monoxime, and 10 mM colchicine for 60 min. The inhibitors were then removed and the platelets were fixed and processed for immunofluorescence.

Results

Sequential rearrangement of actin, myosin and tubulin cytoskeleton during platelet adhesion to glass

To determine the sequence of rearrangement in the platelet cytoskeleton during the process of adhesion, actin filament distribution was examined by phalloidin-FITC staining in platelets adhered to glass, and myosin and tubulin distribution through specific antibodies at different adhesion times and compared with the distribution of these molecules in platelets in suspension. The cytoskeletal distribution was analyzed in at least 30 platelets for each defined time and at least three different assays were carried out for each condition. We observed a high efficiency of platelet adhesion to glass, which was explained in detail in a previous report[51] to be the result of strong interaction forces between the plasma membrane and the surface of the substrate.

Distribution of actin filaments

Platelets in suspension presented a diffuse distribution of the phalloidin label in the cytoplasm with some aggregates in the center of the platelet (Figure 1a). After 30 s of adhesion to glass, platelets produced filopodia extending over the substrate, the actin filament label increased in the center of the platelet, and there was diffuse labeling in the filopodia (Figure 1b). As the adhesion process advanced, the filopodia expanded laterally over the substrate (Figure 1c), becoming lamellipodia by 10 and 20 min (Figure 1d,e). The actin filament label was distributed from the center to the cortical portion of the plasma membrane, particularly at the advancement front of the membrane. Once the adhesion had been completed at 20 min, the cell had reached its typical extended shape with actin filaments associated with the granulomere, bundles extending to the front of the plasma membrane, and well-defined label on the platelet cortical membrane (Figure 1e). An interesting observation was the presence of a central circular region associated with the granulomere (asterisk), which contained actin filaments in some platelets, while in others had a label-free zone as shown in Figure 1e. This was related to the presence or absence of cytoplasmic granules in the granulomere zone, which was evaluated by phase contrast microscopy (Figure 2 a,c,e).

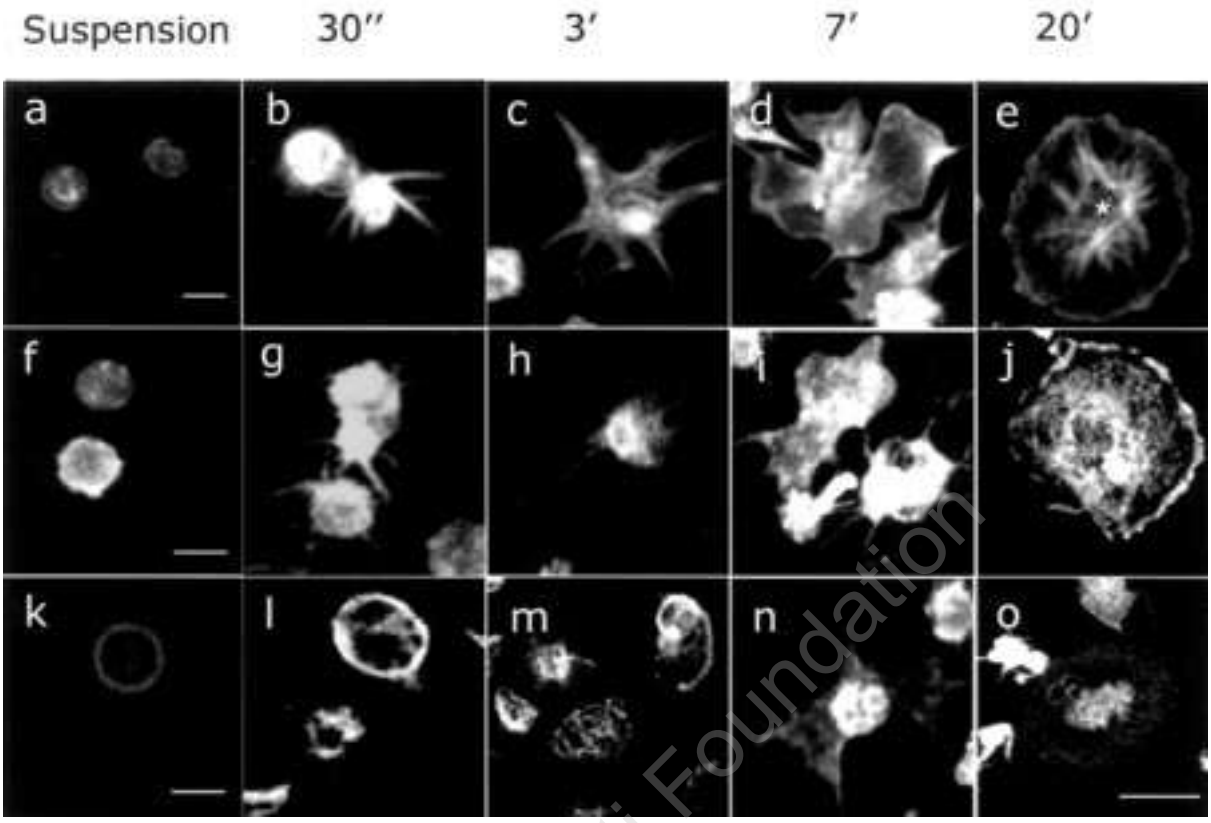

Figure 1. Reorganization of the actin, myosin, and tubulin cytoskeleton during platelet adhesion to glass. Resting platelets were allowed to adhere to glass coverslips for 30s, 3, 7 and 20 min, then fixed and processed for fluorescence studies. Images correspond to confocal optical sections of platelets in suspension or on the substrate. Micrographs a, f and k, are of resting platelets in suspension stained for actin filaments, myosin and tubulin molecules, respectively. Micrographs b to e show actin filament staining, g to j myosin staining and l to o tubulin staining at the different times of platelet adhesion. The granulomere zone is marked with an asterisk in e. Experiments were performed at least 4 times for each tested condition. Bar = 5 μm.

Distribution of myosin

The labeled myosin was diffusely distributed with some cytoplasmic aggregates in platelets in suspension (Figure 1f). Adhered platelets showed a myosin distribution comparable with that described for actin filaments, including centralized label at the start of adhesion and labeling in the filopodia (Figure 1g). The label was distributed diffusely within the cytoplasm with concentration zones in the center of extended platelets (Figure 1g-i). After 20 min of adhesion, the myosin surrounded the granulomere zone, having a cortical localization associated with the front of the lamellipodium, as described for actin filaments. Myosin, however, was not found to be associated with the actin bundles but was distributed as discrete aggregates throughout the cytoplasm (Figure 1j). Like actin, myosin showed a differential distribution in the platelet with centralized or spread granules. Figure 1j shows only the last stage.

Distribution of tubulin

Tubulin in platelets in suspension formed the typical microtubule marginal ring limiting the rounded shape characteristic of non-activated platelets, and there was very little or no diffuse staining for this protein in the cytoplasm (Figure 1k). As adhesion initiated, the microtubule ring was modified, showing discontinuous staining with a central diffuse label associated with the remnant ring (Figure 1l). Once platelet extension had progressed, the microtubule ring presented a visible opening or was ruptured (Figure 1m), with a somewhat diffuse distribution of the label through the cytoplasm, and intense labeling at the platelet center, which even limited the granulomere central vesicles. There was

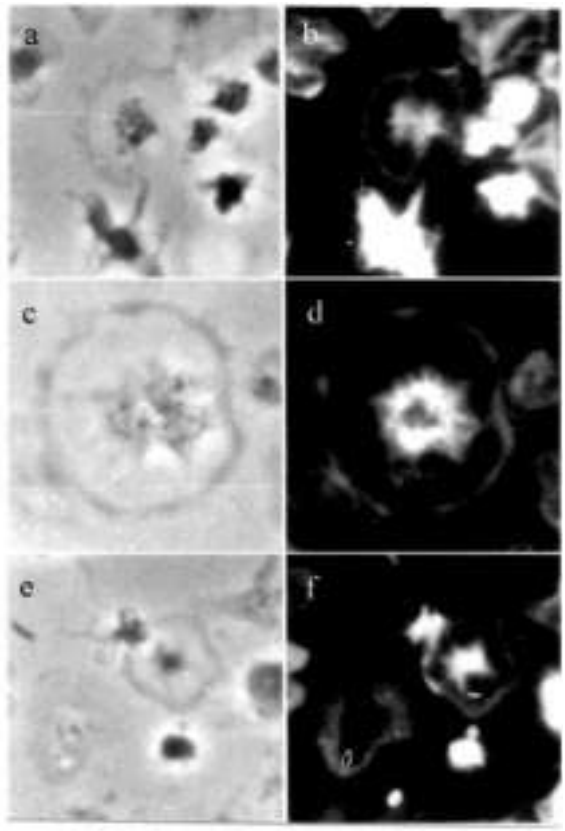

Figure 2. Relationship between granule migration and actin filament distribution in platelets adhered to glass. Platelets were allowed to adhere to glass coverslips, and actin filaments were labeled with FITC phalloidin, and observed in an Axioskop-II fluorescence phase contrast microscope. The distribution of cytoplasmic granules in the cells was determined by changing observation conditions to a phase contrast optics. The left column shows phase contrast images of different adhered platelets at 7 min (a), 10 min (c) and 20 min (e) of adhesion, and the right column (b,d, f) corresponds to the respective fluorescence images of actin filaments. The adhered platelet in micrographs a and b has centralized granules, c and d show granules partially spread in a centripetal pattern and e and f show an adhered platelet with complete spreading of the granules (arrow). Assays were performed three times and images are representative of the relation between the degree of granule dispersion and actin filament distribution. Bar = 5 µm.

no label in the lamellipodium (Figure 1n). The tubulin central label was found in platelets with centralized and spread granules. Figure 1o shows only the latter stage. Not all the adhered platelets were totally extended, some remained in a pseudopodial condition showing a centralized distribution of actin filaments, myosin and tubulin, as well as label in filopodia with absence of the microtubule marginal ring.

Relation between granule displacement and actin filament distribution

The distribution of granules, detected by phase contrast microscopy, and of actin filaments, evaluated by fluorescence, were compared in order to determine the spatial relation between these elements in adhered platelets. Adhered platelets forming a radial lamellipodium initially presented the granules associated with the granulomere zone as a well-defined dense structure (Figure 2a), while actin filaments were distributed, as formerly described, in the front of the membrane, and centralized in the granulomere region (Figure 2b). As the platelet extended, granules spread centripetally towards the front of the cell membrane (Figure 2c), and the actin filaments initially located in the central zone rearranged producing a label-free central circular region, while filament radial bundles and cortical label in the front of the lamellipodium were not modified (Figure 2d). Some platelets with completely spread or barely visible granules showed actin filament distribution only in the cortical front of the membrane in zones close to the cytoplasm and with a large central region with no visible labeling of actin filaments (Figure 2f, arrow).

Double labeling of cytoskeletal components in adhered platelets

The spatial distribution of cytoskeletal elements in the adhered platelets was determined by double labeling, and analyzed in three-dimensional confocal projections of dorsal, transversal to the adhesion axis, and ventral views. Figure 3a-c shows labeling of actin filaments (green) and myosin molecules (red) in a platelet with spread granules, revealing in ventral and dorsal views the presence of filament bundles from the label-free central zone towards the front of the plasma membrane, as well as in those located in the advancement front. Both distributions showed a spatial localization mainly towards the apical surface of the platelet membrane, although with diffuse staining in the basal zone. Myosin was present in a series of dense aggregates associated with the granulomere zone both in the basal and apical zones of the platelet, coinciding with the central circular zone limited by actin filaments (Figure 3a-c), and weakly but diffusely distributed throughout the cytoplasm. As a result of granule dispersion, the platelet showed a flat shape in transversal views (Figure 3b).

Figure 3d-f shows actin distribution in relation to microtubules. This figure shows an adhered platelet

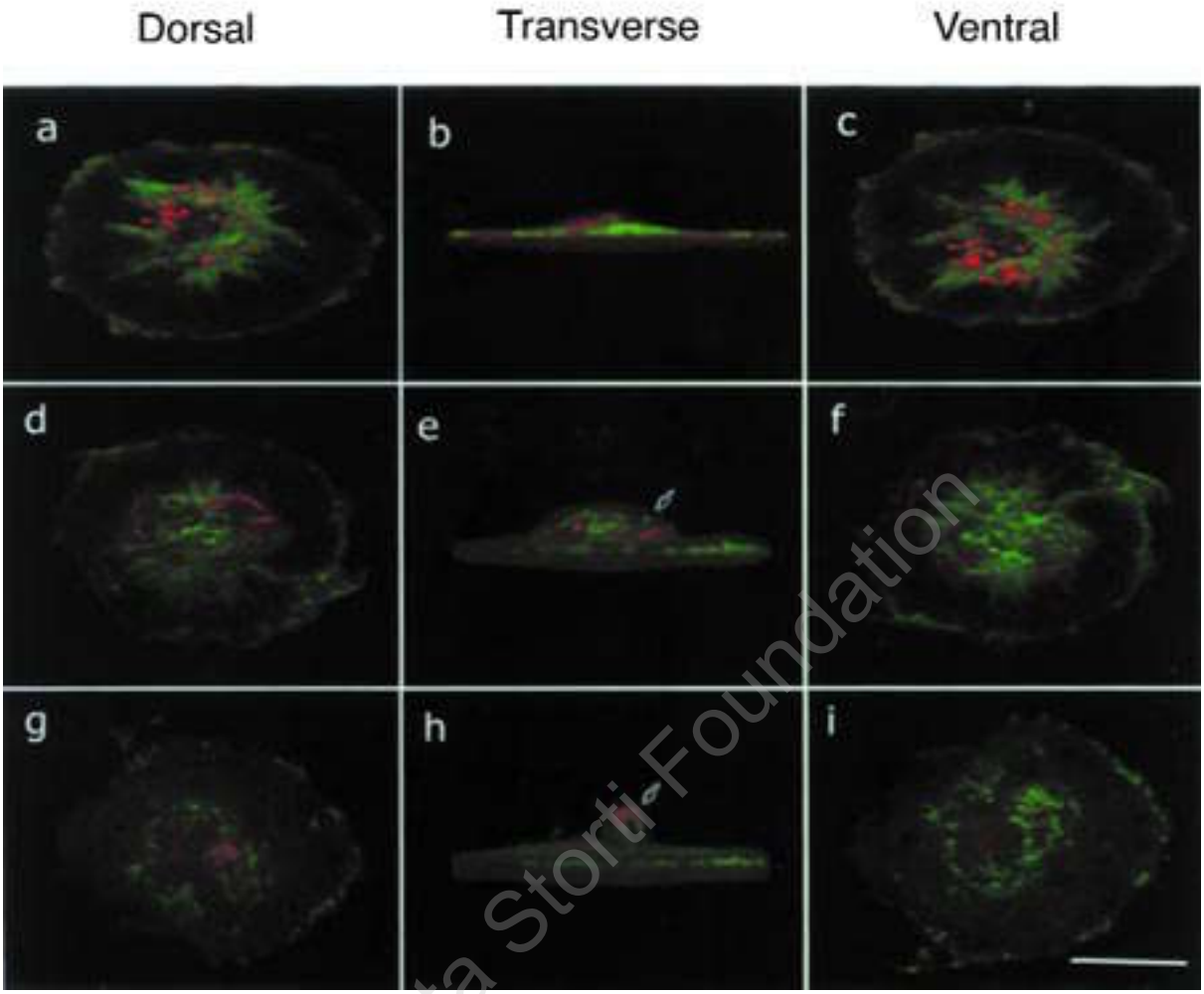

Figure 3. Three-dimensional spatial distribution of the cytoskeleton of platelets adhered to glass. Three-dimensional constructs were formed by merging the respective confocal optical sections projected from a dorsal view (a,d,g), from a transverse view to the axis of platelet adhesion (b,e,h) and from a ventral view (c,f,i). Series a-c show a platelet stained for actin filaments (green) and myosin molecules (red); d-f show a platelet stained for actin filaments (green) and tubulin molecules (red); and g-i show double staining for myosin (green) and tubulin (red) molecules. The arrow in (e) indicates a protuberance associated with aggregated granules in the platelet central zone, while the arrow in (h) shows a non-activated platelet settled over the apical face of an adhered platelet with spread granules. Analyses in three-dimensional reconstructions were performed in at least five platelets for each of the double stainings. Bar = 5 μm.

with dense granules associated with the granulomere zone coinciding with the central aggregate of actin filaments (green). Actin label was abundant in the basal and apical zones of the platelet. Tubulin molecules (red) were found as aggregates associated with the granulomere and embedded in the actin filaments with a certain tendency to be found towards the platelet apical zone, and to a lesser extent in a diffuse form throughout the platelet cytoplasm. No microtubule marginal ring was observed. The platelet presented an evident protuberance in the transversal view due to the central location of granules (Figure 3e, arrow). Figure 3g-i shows the simultaneous distribution of myosin and tubulin in an adhered platelet with spread granules. Myosin molecules (green) were distributed in the granulomere zone, limiting the aforementioned label-free central region, and with less intensity, in the front of the lamellipodium, and in platelet cytoplasm, while the tubulin label (red) remained aggregated in the platelet center even in the myosin-free central zone, with a diffuse label in all of the cytoplasm but not in the front of the lamellipodium. The aggregated or spread gran-

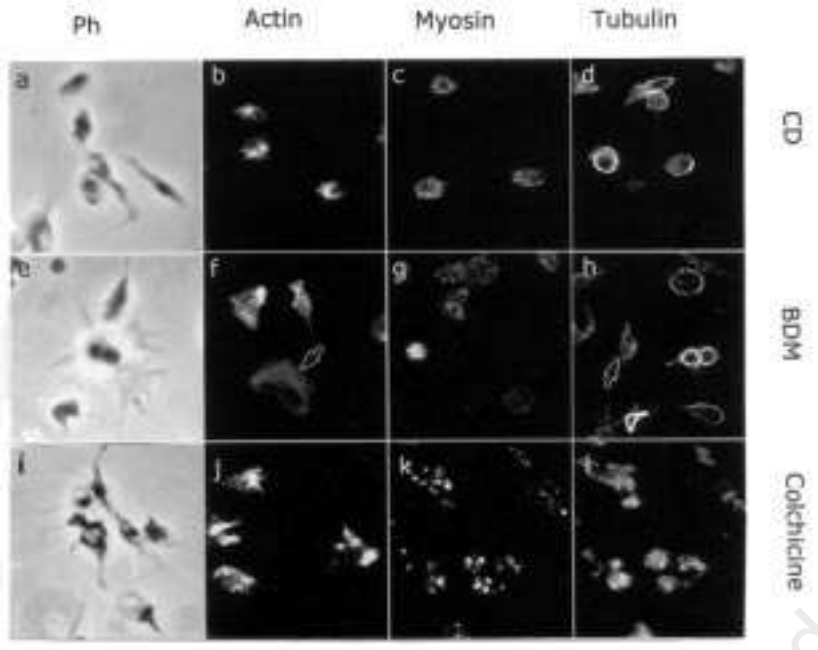

Figure 4. Topographic distribution of actin filaments, myosin and tubulin molecules in platelets treated with cytoskeleton inhibitors and then adhered to glass. The platelet suspensions were incubated with 10 μM CD (series a-d), 30 mM BDM (series e-h), or 10 mM colchicine (series i-l) for 60 min, adhered to glass, stained respectively for actin filaments (b,f,j), myosin molecules (c,g,k) and tubulin molecules (d,h,l), and then observed by confocal microscopy. Morphologic changes induced by the inhibitor treatment in adhered platelets were assessed in similarly treated cells, which were observed and micrographed in a phase contrast microscope (a,e,i). Controls were made by incubating the cells with the respective solvent diluted in HBSS. Bar = 5 μm.

ules related to actin and myosin distribution, whilst tubulin was located in the platelet center (Figure 3d, g). The small protuberance detected in Figure 3h (arrow) corresponds to a second platelet adhered to the apical face of the extended platelet.

Effect of cytoskeleton inhibitors during adhesion of platelets to substrate

Platelets in suspension were treated with the cytoskeleton inhibitors CD for actin filaments, BDM for myosin, and colchicine for microtubules, and then allowed to adhere to determine changes in the cytoskeletal distribution. The treatment of platelets in suspension with any of the three evaluated inhibitors, and the subsequent sedimentation on glass slides, seemingly did not inhibit the process of adhesion to the slide, which was verified by the presence of platelets after the processing (Figure 4 a,e,i). However, extension over the substrate was inhibited as a result of a modification in the organization of all the studied cytoskeletal elements regardless of the inhibitor used. Platelets treated with CD presented rounded and elongated shapes during the adhesion (Figure 4a). Actin filaments formed aggregates within the cytoplasm and associated with the plasma membrane (Figure 4b). The few filopodia that did extend contained the label for actin filaments. Myosin was located diffusely and as aggregates within the cytoplasm and associated with the plasma membrane (Figure 4c), while tubulin formed a marginal ring characteristic of cells in suspension, although decorated with tubulin aggregates, and with a clear diffuse label throughout the cytoplasm (Figure 4d). Platelets treated with BDM mostly had pseudopodial shapes (Figure 4e) and a central aggregate of actin filaments surrounded by other small aggregates distributed in the cytoplasm, as well as in filopodia (Figure 4f). Some platelets presenting a partial membrane extension showed actin filaments surrounding the plasma membrane and distributed in the cytoplasm (arrow). Myosin label was located as fine clumps apparently lacking in the plasma membrane (Figure 4g), while tubulin distribution was comparable to that described for the CD treatment, that is, a microtubule marginal ring decorated with a tubulin patch pattern, although the microtubule ring fragmented in some partially extended platelets (Figure 4h, arrow). Platelets incubated with colchicine presented partial membrane extension, showing short filopodia associated with the substrate (Figure 4i). Actin filaments were distributed as spread aggregates in the cytoplasm and in diffuse form in filopodia: the intensity of the label was weaker in association with the plasma membrane (Figure 4j). Myosin molecules were found exclusively as cytoplasmic clumps, and with limited staining in the plasma membrane (Figure 4k). The microtubule marginal ring disappeared with colchicine treatment, leaving only diffuse label with some floccules in the cytoplasm (Figure 4l).

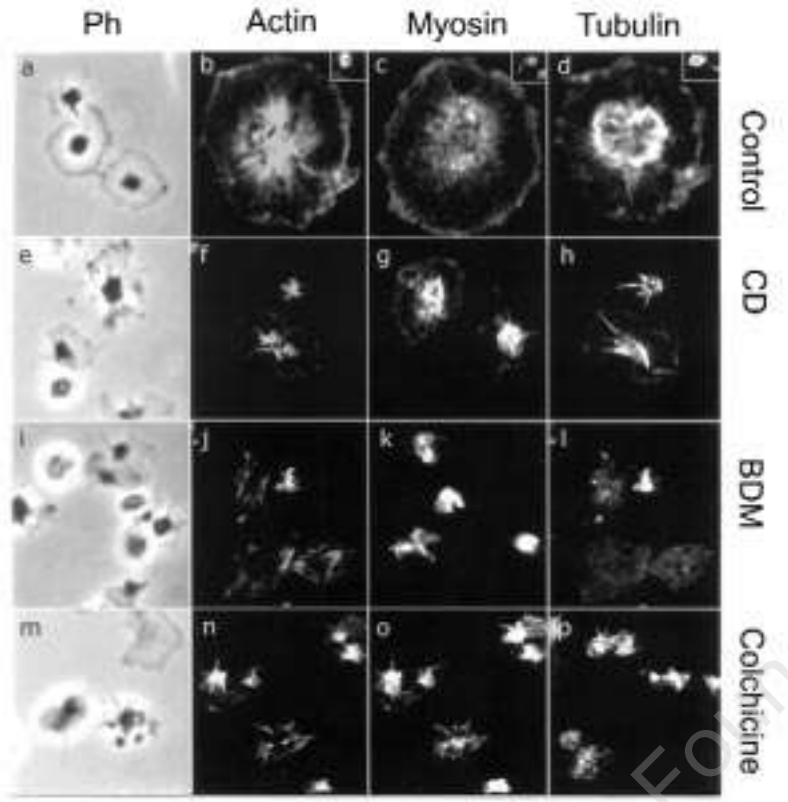

Figure 5. Topographic distribution of actin filaments, myosin and tubulin molecules in platelets previously adhered to glass and post-treated with cytoskeleton inhibitors. Platelets in suspension were alllowed to adhere to glass for 20 min, and then incubated with 10 μM CD (series e-h), 30 mM BDM (series i-l), or 10 mM colchicine (series m-p) for 60 min, and then fixed, processed and observed by confocal microscopy for actin filaments (f,j,n), myosin (g,k,o) and tubulin molecules (h,l,p). Respective morphologic changes were evaluated by phase contrast (Ph) microscopy (a,e,i,m). The series a-d shows adhered platelets not treated with drugs, but stained for actin filaments (b), myosin molecules (c) and tubulin molecules (d). Bar= 5 μm.

Effect of the cytoskeleton inhibitors on adhered and extended platelets

Previously adhered and extended platelets were exposed to CD, BDM and colchicine to determine the role of cytoskeletal elements in the *maintenance* of cell shape. Figure 5a shows the cell shape evaluated by phase contrast microscopy, and Figure 5b-d shows the distribution of actin filaments, myosin and tubulin molecules, assessed by fluorescence, of the adhered platelets prior to their exposure to inhibitor. Some platelets (shown in the inserted figures) remained with dendritic shapes forming pseudopodia on the substrate with the corresponding fluorescent label location. Treatment of extended platelets with CD, BDM and colchicine induced partial retraction of various intensities in the platelet plasma membrane with subsequent modification of cell shape (Figure 5 e,i,m). Treatment with CD produced a redistribution of actin filaments towards the platelet center with a decrease of actin label in the plasma membrane (Figure 5f). The distribution of the myosin label was not apparently altered with respect to the distribution in the control (Figure 5g), although it was modified by the shape change produced by the partial retraction of lamellipodium. Tubulin became organized as radial fibers advancing from the center towards the front of the lamellipodium (Figure 5h) and there was a lack of cytoplasmic and cortical labeling. The central aggregate of actin filaments disappeared in platelets treated with BDM (Figure 5j), leaving just filament bundles distributed in the cytoplasm and associated with the plasma membrane. Myosin molecules were aggregated in the platelet center, with a decrease of the label in the front of the lamellipodium (Figure 5k). In fully extended platelets, the central aggregate of the tubulin label disappeared leaving diffuse staining all over cytoplasm, while pseudopodial platelets concentrated the label in the central body (Figure 5l). Treatment with colchicine produced actin filament aggregation in the center of the platelet, in filopodia, as well as in the plasma membrane. Myosin was aggregated in the platelet center with some diffuse labeling in the cytoplasm. Tubulin was modified as aggregates in the platelet center and absent in the front of the lamellipodium (Figure 5p).

Discussion

In an attempt to understand the physiologic events occurring during hemostasis, *in vitro* models have been developed which try to reproduce and to simulate the phenomena of platelet adhesion to damaged vascular endothelium and clot formation.[3,5] Although several substrates have been used to study the different platelet adhesion properties, there are no reports which specifically determine a direct and complete correlation of *in vitro* systems with physiologic processes occurring *in vivo*. Nevertheless, the results obtained have provided a broad outline of the events of adhesion and platelet activation including the role of the cytoskeleton, the participation of surface receptor molecules, and the mobilization and release of the contents of cytoplasmic granules.[2,4,7,22,26,52–55] Substrates used for these studies include plain glass, siliconized surfaces and glass slides covered with extracellular matrix components.[56,57] According to the interaction forces between platelets and glass itself, it has been possible to determine, in isolated systems, the role of proteins and cations such as fibrinogen, calcium and magnesium ions in platelet adhesion[51,53,58] and the effect of agonists in platelet activation.[3,59] In our study we used platelet adhesion to glass to investigate platelet shape changes as well as migration of intracellular granules and their relationship with the arrangement of the cytoskeleton.

The platelet cytoskeletal elements, including actin filaments, myosin, microtubules and associated proteins, intervene in the maintenance of cell shape and changes that result from cell activation triggered by the platelet's adhesion to substrates as well as by platelet aggregation.[3,59] Adhesion-induced polymerization of actin filaments produces an asymmetrical reorganization of the cytoskeleton into a peripheral network of filaments cortically distributed,[29] as well as in parallel filament bundles in pseudopodial or dendritic platelets.[60] Our experiments allowed us to characterize the sequential rearrangements in the platelet cytoskeleton induced by adhesion and their relation with granule migration, whereas other authors reported the distribution of cytoskeletal elements once adhesion had finished.[22] At the beginning of adhesion to glass, platelets had a dendritic shape with filopodia projected over the substrate and actin filaments inside, as well as in the platelet center. A remarkable observation was the lateral expansion of filopodia to form an early lamellipodium-type structure. This expansion was associated with the presence of cortically distributed actin and a central actin aggregate. In this intermediate stage, myosin was only found diffusely in the cytoplasm; interestingly, once platelet extension had been completed, myosin co-migrated with actin to the front of the lamellipodium, and was associated with the granulomere zone, as formerly reported[22,27,60] but some molecules also remained as aggregates in the cytoplasm. Modification of filopodia to lamellipodia suggests that the cytoskeleton changes from filament bundles in filopodia towards actin-myosin cortical contractile networks in lamellipodia in a way similar to that proposed for dorsal membrane arch formation.[61] In relation to microtubules, some studies have shown that these depolymerize after activation,[28] while others have suggested that their rupture is not required for platelet shape changes,[34] or granular release.[43,44] We suggest that the alteration of the microtubule ring in the first stages of adhesion to the substrate although not essential, might facilitate further membrane expansion, filopodia formation and granule migration. However a relationship has been reported between actin filaments and the microtubule ring rupture in activated platelets in suspension.[22,28] Although there are no reports about the molecules responsible for microtubule rupture in platelets, katanin-like molecules, such as those described in fibroblasts, might play a role.[62] The analysis of the three-dimensional images suggested that myosin molecules are closely related to the position of cytoplasmic granules and this depends on actin filament organization. In extended platelets in which granules remain centralized, actin is detected in the platelet basal surface, as well as in the granulomere zone in apparent co-localization with myosin molecules; however, when granules were dispersed, actin was redistributed towards the platelet apical surface, with an evident modification of the central aggregate in a hollow zone lacking actin filaments and myosin molecules. Actin, which is associated with myosin through contractile-type interactions in the advancement front of the membrane,[63,64] was not modified by the granule position. Similarly, the microtubule distribution was unchanged by the migration of the granules, suggesting that this dynamic process does not necessarily depend on microtubule rearrangement. In addition, the coincidence of radial bundles of actin toward the granulomere zone might suggest the existence of actin filament organizing molecules or anchor molecules located there. Although several molecules that polymerize and regulate platelet actin have been

reported, suggesting some contribution in the formation of lamellipodia, filopodia and of the general platelet shape,[65] there are no reports about the presence of cytoskeleton-associated proteins in the granulomere zone. In all cases treating the platelet with cytoskeleton inhibitors CD, BDM and colchicine affected the spatial distribution of the target molecule but also of other cytoskeletal elements, suggesting a close spatial and functional relationship between the three cytoskeletal elements studied, such that modification of any one of them results in a global change of the cytoskeleton. Radial filament bundles projecting from the granulomere zone, detected in the elongated pseudopodial forms, were resistant to CD treatment, while network-organized filaments, located mainly in the lamellipodia, were sensitive to the drug. This effect indicates that sensitivity to inhibitor depends on the organization of the actin filaments, as indeed former reports have suggested.[1,36] Myosin molecule distribution was also affected by CD treatment, which could be the result of an indirect effect reflecting a stable interrelation between both actin filaments and myosin molecules. CD did not, however, modify the typical ring form of the microtubules, although ornamenting tubulin patches were formed, suggesting a secondary aggregation of tubulin molecules from the cytoplasm. Treatment with BDM, an inhibitor of myosin ATPase,[66] diminished membrane extension in platelets adhered to glass, modifying their cell shape, suggesting that myosin could play a role in maintaining lamellipodium shape and organization, as was recently proposed.[49] BDM also modified the distribution of actin filament bundles, microtubule ring appearance, and induced the disappearance of actin filament aggregates associated with the granulomere zone, indicating that myosin may contribute to the spatial organization of both cytoskeletal elements, and to granular movement through an actino-myosin complex in the center of the platelet. This conclusion was based on the finding of concentrated granules in platelets treated with this inhibitor. A remarkable observation about tubulin distribution, as a central aggregate, was its modification by BDM treatment, suggesting that myosin might somehow be involved in tubulin distribution. Platelets adhered to glass and treated with colchicine became completely round with retraction of their extended membrane, implying that microtubules play a role in membrane extension, perhaps functioning as a scaffold on which actin filaments and myosin could interact to achieve platelet expansion. Retraction and loss of the extended shape in platelets previously adhered to glass, regardless of the inhibitor used, reinforces the idea that actin filaments, microtubules, and myosin molecules all interact to determine cell shape and to support intracellular granule, migration probably through accessory molecules with molecular crossover ability.

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DC: directly developed the project and participated in the writing of the manuscript; RS: acquisition of confocal images and three-dimensional reconstructions; SG: provided help and access to the electron and optical microscopy facilities; ER: proposed the original project from which this one was derived; RM: precise concept of the project and its design, analysis of data, writing of the paper, in collaboration with DC. RM takes prime responsibility for the manuscript writing. DC and SG; primary responsibility for Figures 1-2; DC and RS: primary responsibility for Figure 3; DC, SG and RS; primary responsibility for Figures 4-5.

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Disclosures

Conflict of interest: none.

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Peer Review Outcomes

Manuscript processing

This manuscript was peer-reviewed by two external referees and by Professor Carlo Balduini, who acted as an Associate Editor. The final decision to accept this paper for publication was taken jointly by Professor Balduini and the Editors. Manuscript received May 16, 2002; accepted September 6, 2002.

What is already known on this topic

Cytoskeleton plays a fundamental role in platelet function, being involved in shape change, adhesion, aggregation and clot retraction. However, the sequence of cytoskeleton modifications induced by platelet activation is poorly known.

What this study adds

This study characterizes the three dimensional localization of several cytoskeleton components during platelet adhesion to glass and identifies their role in displacement of cytoplasmic granules.

Potential implications for clinical practice

A better understanding of the mechanisms involved in platelet activation could be useful for the identification and characterization of new inhibitory drugs.

Carlo Balduini, Associate Editor (Pavia, Italy)