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Actin Polymerisation Regulates Integrin-Mediated Adhesion as Well as Rigidity of Neutrophils

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Activation of adherent neutrophils causes them to convert from selectin-mediated rolling to integrin-mediated immobilisation and migration. Migration is known to depend on formation and redistribution of filamentous (F) actin, but although immobilisation in seconds parallels early cortical actin polymerisation, no link has been proven. We tested the effect of the actin-polymerising agent jasplakinolide (10μM) on adhesive and mechanical properties of neutrophils. Pretreated cells were able to adhere and roll on immobilised platelets in a flow-based adhesion assay, but whereas untreated rolling cells became immobilised in seconds when chemotactic formyl peptide (fMLP, 0.1μM) was superfused over them, the cells treated with jasplakinolide continued rolling. Pretreatment with jasplakinolide also blocked *de novo* expression of integrin CD11b and shape change which otherwise occurred in minutes after treatment with fMLP. Jasplakinolide directly caused actin polymerisation within neutrophils, evidenced by a marked increase in rigidity (resistance to aspiration into a 5μm micropipette) and increase in association of actin with the Triton-insoluble cytoskeleton. These results indicate that rearrangement of the actin cytoskeleton regulates integrin-mediated adhesion of activated neutrophils, as well as their migration and mechanical properties.

Neutrophils circulate until induced to adhere to the vessel wall when they migrate into tissue as part of the inflammatory response. Adhesion proceeds by defined steps: flowing cells are captured and induced to roll slowly via selectin adhesion molecules expressed on stimulated endothelium; surface-bound activating signal(s) cause activation of neutrophil β2-integrins, which immobilise the cells; cyclic regulation of integrin adhesivity and actin polymerisation are required for migration over and through the surface (1). The activation step is crucial in transforming the neutrophil's adhesive behaviour and in stimulating the motile apparatus underlying migration, and it may be inferred that the two processes are linked through actin polymerisation and cytoskeletal rearrangement. Upon delivery of an activating stimulus, neutrophils become rigid and switch from rolling to stationary adhesion in seconds, before marked shape change occurs (2,3), at the same time as transformation of cortical globular (G) actin to F-actin (4). Subsequently the F-actin decreases in concentration over minutes and udergoes a redistribution correlating with formation of pseudopodia (4,5), partial recovery of deformability (2) and modulation of integrin-mediated adhesion and cell spreading during onward movement (3, 6).

While cytoskeletal actin rearrangements are recognised to underlie changes in shape, deformability and motility (2,5,7), their relation to changes in integrin-mediated adhesion of neutrophils has not been demonstrated. Clustering of integrins in lymphocytes and formation of focal adhesions in fibroblasts are known to be regulated by the cytoskeleton (and impeded for instance by cytochalasins which inhibit and reverse actin polymerisation) (8-10). However, these events are slow compared to the initial neutrophil activation step. The Rho family of GTP-binding proteins have been implicated in control of several actin-dependent cell functions, and were recently shown to influence activation-dependent, integrin-mediated adhesion of leucocytes (11). We conjectured that the initial cortical actin polymerisation which has no hitherto recognised function, could play a part in transformation of neutrophil adhesion by rapidly altering anchorage, distribution or conformation of integrins. We thus examined the effects of the novel agent jasplakinolide which has a higher affinity for F-actin than phalloidin and induces actin polymerisation in solution (12). This agent has antiproliferative activity in transformed cell lines (13) and inhibits secretion by epithelial cells (14), which suggests that it is able to cross the membrane of live cells. Indeed we found that jasplakinolide caused neutrophils to become rigid in minutes and increased the association of actin with the Triton-insoluble cytoskeleton. We next

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tested the effect of treatment in a flow-based adhesion model (3). Neutrophils rolling on P-selectin presented by immobilised platelets were exposed to N-formyl-methionyl-leucyl-phenylalanine (fMLP) which normally induces rapid transformation to stationary adhesion through activation of constitutive β2-integrin (CD11b/CD18) and prolonged attachment through de novo expression of CD11b/CD18 (3). Pre-treatment with jasplakinolide allowed initial rolling but prevented immobilisation of neutrophils on exposure to fMLP, as well as blocking quantitative upregulation of CD11b and shape change. Thus ordered rearrangement of actin is essential for regulation of the integrin-mediated adhesion as well as cellular mechanical properties that confer ability to migrate out of the circulation.

MATERIALS AND METHODS

Preparation of neutrophils and platelet-coated surfaces. Human venous blood was collected from healthy volunteers into buffered citrate anticoagulant (CPDA, 8:1) or into preservative-free sodium heparin (5U/ml: Leo Laboratories Ltd. Princes Risborough, UK). Neutrophils were isolated from citrated blood using a two-step density gradient of Histopaque-1077 and Histopaque-1119 (Sigma Chemicals Co. Ltd, Poole, UK.) as described (3), washed and suspended at 1×10^6 cells/ml in phosphate-buffered saline (PBS) containing 1mM Ca^{++}, 0.5mM Mg^{++} (ICN Flow Labs., High Wycombe, UK) and 0.1% bovine serum albumin (Sigma). Heparinised blood from the same donor as used in neutrophil isolation was centrifuged at 290g for 5 minutes and the platelet-rich plasma collected. Platelets were counted and diluted to 2×10^8 cells/ml with PBS, and pipetted into glass capillaries with a rectangular cross-section of 300μm by 3mm, and a length of 50mm (Microslides: Camlab Ltd, Cambridge, UK). Microslides had been acid-washed and coated with 3-aminopropyltriethoxysilane (15). Platelets settled, adhered, and spread in 30 minutes to form an essentially confluent layer on the lower surface of the microslide (15).

Jasplakinolide (Molecular Probes, Leiden, The Netherlands) was dissolved in ethanol at 1mM and added to neutrophils for various periods at 37°C before analysis. Initial studies indicated that treatment for 30 minutes with 10μM but not 1μM jasplakinolide inhibited shape changes induced by addition of fMLP (0.1μM; Sigma), and 10μM was used thereafter. Control samples were treated with the same volume of ethanol alone, which had no effect on measurements compared to untreated samples.

Flow-based adhesion assay. The adhesion assay was similar to that recently described (3). The platelet-coated microslide was glued to a glass microscope slide, mounted on a microscope stage and viewed by video microscopy. One end of the microslide was attached by silicon rubber tubing to a syringe pump which controlled the flow rate through the microslide to provide a constant wall shear stress of 0.1Pa. The other end of the microslide was attached by silicon tubing to an electronic valve which allowed smooth switching between the neutrophil suspension and cell-free suspending medium. The apparatus was maintained at 37°C.

A timed bolus of neutrophils was perfused over the immobilised platelets followed by a brief washout period with cell-free medium. The number of adherent neutrophils was counted by direct microscopic observation of a number of complete microscope fields along the center-line of the microslide, and expressed as cells adhered per square millimetre per 10^6 cells perfused. fMLP (0.1μM in PBS) or cytochalasin B (10μM in PBS) was perfused over the rolling neutrophils and their response recorded by video. Video recordings were analysed off-line using a computerised image analysis system to determine the percentage of adherent neutrophils which were stationary or rolling, and the rolling velocity (3,15). For categorisation of adhesion, stationary cells were defined as those with velocity less than 0.4μm/s, which represents the minimum velocity resolved by the analysis system for 5-10s observation periods. Activated neutrophils typically migrate at about 0.2μm/s in this system (6).

Micropipet analysis of resistance to deformation. Neutrophils were aspirated into a micropipette with internal diameter of 5.3μm as previously described (16) using a microscope/micromanipulator enclosed in a temperature-regulated perspex box. Cells were treated with jasplakinolide and either placed immediately into a transparent chamber into which the pipette was inserted or held at 37°C for a desired period before analysis. The time taken for neutrophils to enter the pipette was measured immediately and compared to measurements on untreated cells. A fixed aspiration pressure of 1000Pa was used, so that entry time was $<$1s for untreated neutrophils.

Expression of CD11b on neutrophils. The level of expression of CD11b on neutrophils was quantitated by direct immunofluoresence, using RPE-conjugated antibody (R841; Dako Ltd) compared to an isotype-matched RPE-conjugated antibody to an irrelevant antigen (X928, Dako Ltd.), and a FACScan flow cytometer (model 440, Becton-Dickinson Ltd.) as described (17). Neutrophils were incubated with or without jasplakinolide (10μM) for 60 minutes, with antibody for 20 minutes and then with or without fMLP (0.1μM) for a further 10 minutes, all at 37°C. Cells were fixed in 2% formaldehyde, washed in PBS, and intensity of fluoresence labelling was measured.

Isolation of neutrophil cytoskeleton and polyacrylamide gel electrophoresis (PAGE). Triton-insoluble cytoskeletal and soluble protein fractions were derived from neutrophils under conditions designed to maintain the state of F-actin (18,19). Neutrophils were pelleted centrifugally after treatment with 10μM jasplakinolide for 5 or 60min, or with fMLP for 1min, and lysed by addition of ice-cold 10mM PIPES buffer with 0.3M sucrose, 10mM NaCl, 2mM MgCl2, 1mM EGTA, 0.2mM dithiothreitol, 0.2mM PMSF, 5μg/l aprotinin, 0.5μg/ml leupeptin, 0.5% Triton X-100 (all from Sigma) and held on ice for 20min. The triton-insoluble pellet and supernatant were separated after centrifugation at 12,000g for 5 minutes, boiled with 1% SDS, 1% mercaptoethanol, 5% sucrose, 0.125M TRIS-HCL and analysed by PAGE with 9% polyacrylamide gels (20).

RESULTS

Inhibition of transformation from rolling to stationary adhesion by jasplakinolide. Neutrophils perfused over immobilised platelets at a wall shear stress of 0.1Pa adhered efficiently (460 adherent cells/mm^2/10^6 perfused averaged over 11 experiments). More than 90% of adherent cells rolled continuously until superfused with fMLP, when they stopped in seconds (see e.g., Fig. 1) as previously reported (3). Pre-treatment with jasplakinolide for between 10 and 120 minutes did not alter attachment of neutrophils (number adherent was 99% of control for comparisons averaged over the complete range of times of pretreatment) or the initial percentage of adherent cells rolling (Fig. 1). However, pre-treatment did markedly inhibit the ability to immobilise in response to fMLP. The time course was biphasic, with essentially total blockade of immobilisation for 10 or 120 minutes pre-treatment with jasplakinolide, and somewhat less efficient effect at 40 or 60 minutes pre-treatment (Fig. 1). Interestingly, the

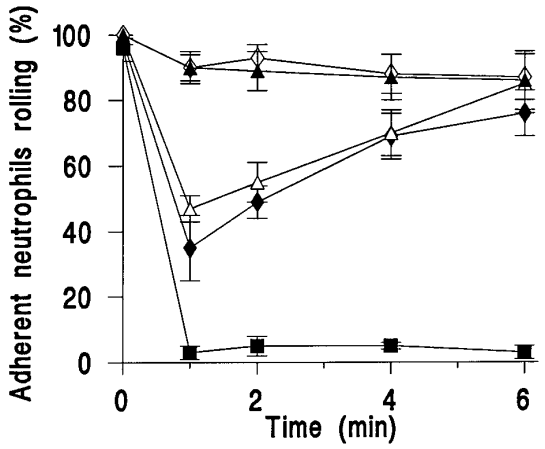

FIG. 1. Effect of pre-treatment with jasplakinolide on the response of adherent rolling neutrophils to superfusion with fMLP (0.1μM). Neutrophils were preincubated at 37°C with 10μM jasplakinolide for 10 (◇), 40 (◆), 60 (△) or 120 minutes (▲), or without jasplakinolide (■), before initial perfusion over immobilised platelets. Data are means ± SEM from 3 experiments where the percentage of adherent neutrophils that were rolling was determined as a function of time after initiation of superfusion with fMLP. Data at time zero were obtained before superfusion of fMLP. Analysis of variance (ANOVA) showed that there was a significant effect of treatment with jasplakinolide ($p<0.01$), and that rolling after 1 minute was only time dependent for 40 and 60 minutes of preincubation with jasplakinolide.

intermediate time treatments allowed some cells to stop immediately after delivery of fMLP but they gradually started rolling again over 2-6 minutes perfusion with fMLP. At no time did the jasplakinolide-treated cells obviously change their shape in response to fMLP, in contrast to the marked distortion and spreading shown by stationary control neutrophils.

Mechanism of action of jasplakinolide. As an indicator of polymerisation of actin, we measured resistance of neutrophils to deformation. Untreated neutrophils all entered a 5.3μm pipette in less than 1s (Fig. 2a), and mean entry time was 0.35 ± 0.02s (mean ± SEM from 60 cells in 3 experiments). In the period 2-4 minutes after treatment with 10μM jasplakinolide (the earliest time testable), a few neutrophils slowly entered the pipette (mean entry time 11.5s; only 4 cells would enter in 3 experiments) but thereafter neutrophils would not enter and blocked the aperture (Fig. 2b). Small clear protuberances similar to membrane blebs appeared on many treated neutrophils over minutes, and it was evident that some blebs could be smoothed out and a membrane tongue drawn into the pipette (Fig. 2b), but a rigid granular core within the cell prevented further deformation. Over longer periods the blebs tended to be reabsorbed, and a clear cytoplasmic 'halo' appeared around a darkened cortex. After 60 minutes, for instance, neutrophils were still highly rigid compared to controls. If pressures were greatly elevated by a syringe attached to the pressure system, cells could enter without rupture, showing that there was no geometrical constraint on their ability to adapt to the cylindrical opening.

F-actin could not be quantified using fluorescent derivatives of phalloidin because jasplakinolide displaces this agent (12). However, direct evidence of changes in the actin cytoskeleton was obtained by SDS-PAGE of Triton-insoluble (cytoskeletal) and soluble protein fractions. There was a marked increase in the actin associated with the cytoskeleton within 5 minutes of treatment of neutrophils with 10μM jasplakinolide (Fig. 2c). This shift was more marked than the effect occasioned by treatment with fMLP (Fig. 2c), persisted after 60 minutes of treatment with jasplakinolide, and was evident on three occasions.

Additional evidence that jasplakinolide acted through an effect on actin polymerisation was gained by perfusing cells with cytochalasin B instead of fMLP. As previously reported (21), cytochalasin B caused control neutrophils to deform to tear-drop shape and stop rolling (Fig. 3), an effect not mediated through integrins. However, when neutrophils were pretreated with jasplakinolide for 60minutes, they did not change shape and continued rolling when cytochalasin B was perfused over them (Fig. 3). Jasplakinolide thus inhibited the ability of cytochalasin B to shift equilibrium from F- to G-actin.

Finally we checked whether jasplakinolide altered expression of integrin CD11b. Neutrophils treated with jasplakinolide showed a small but not significant reduction in surface expression of CD11b as measured by immunofluoresence (Table 1). However, when fMLP was subsequently added, the neutrophils that had been treated with jasplakinolide showed no significant increase in expression in 10 minutes, compared to a greater than four-fold increase for the control neutrophils (Table 1).

DISCUSSION

In the model of transformation used here, neutrophils roll on P-selectin presented by platelets until fMLP is delivered, and then stop in seconds through activation of constitutively-expressed CD11b/CD18; prolonged attachment requires this integrin to undergo continual *de novo* expression and activation as the previously-activated integrin is down-regulated (3). The main new finding is that disturbance of cytoskeletal regulation destroys the ability of neutrophils to rapidly activate integrin mediated-adhesion in response to the fMLP. The slower increase in integrin expression was also blocked if neutrophils were pretreated with jasplakinolide. The treated, rolling cells were unable to change shape in response to fMLP, and were unaffected when cytochalasin B was perfused over, implying that the cytoskeleton was 'locked' in a rigid conformation.

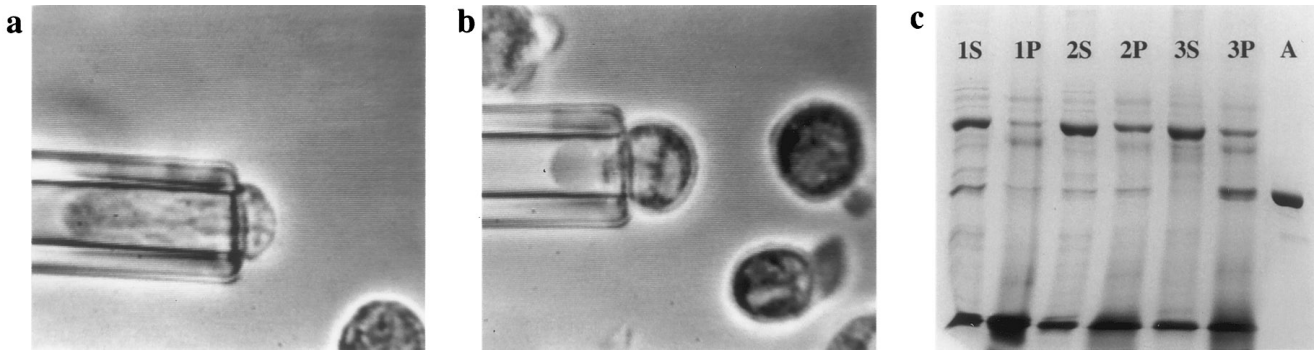

FIG. 2. Effects of jasplakinolide on deformability of neutrophils and on actin association with the Triton-insoluble cytoskeleton. Comparison of micropipette aspiration of (a) an untreated neutrophil (in the final stage of entry) and (b) a neutrophil treated with jasplakinolide (blocking the tip). The pipette diameter was 5.3μm and pressure was 1000Pa; by 5 minutes after treatment with jasplakinolide, no cells entered the pipette at this pressure. In (c), SDS-PAGE is shown for supernatant (S) and pellet (P; Triton insoluble cytoskeleton) fractions of neutrophil protein extracts: 1=untreated neutrophils; 2=neutrophils treated with fMLP (0.1μM) for 1 minute; 3=neutrophils treated with 10μM jasplakinolide for 5 minutes. A=purified actin from rabbit skeletal muscle.

That jasplakinolide had caused actin polymerisation was evident from a shift in actin from the cytoplasmic pool to the Triton-insoluble cytoskeleton, and a marked increase in cellular rigidity.

Jasplakinolide has previously been shown to promote actin polymerisation in solution and to have a higher affinity for F-actin that phalloidin (12). In vitro studies showed that growth of carcinoma cells was inhibited and their cytoskeleton disrupted during prolonged exposure to about 0.1μM jasplakinolide (13) while secretion by epithelial cells and rearrangement of F-actin fibres in response to c-AMP was inhibited within minutes of treatment with 1μM jasplakinolide (14). Here, jasplakinolide at 10μM was taken up within minutes, judging from the changes in cell rigidity and association of actin with the cytoskeleton. This is the first direct demonstration that jasplakinolide causes polymerisation inside cells; labelling of F-actin with fluorescent phalloidin decreased in previous studies because of competitive inhibition of its binding by the jasplakinolide (14).

Rigidification, stabilisation of cell shape and inhibition of movement of receptors to the cell surface might be predicted to result from exposure to an agent that essentially irreversibly transforms G- to F-actin. However, only indirect evidence previously suggested that the ability of neutrophils to activate surface integrins was dependent on cytoskeletal rearrangement. A connection between integrin adhesion receptors and cytoskeletal structures has been well documented in fibroblasts, where the linkage regulates adhesion by drawing receptors into focal adhesions sites (9). In lymphocytes, activation induces clustering of integrins, which increases the avidity of cell-cell attachment, a

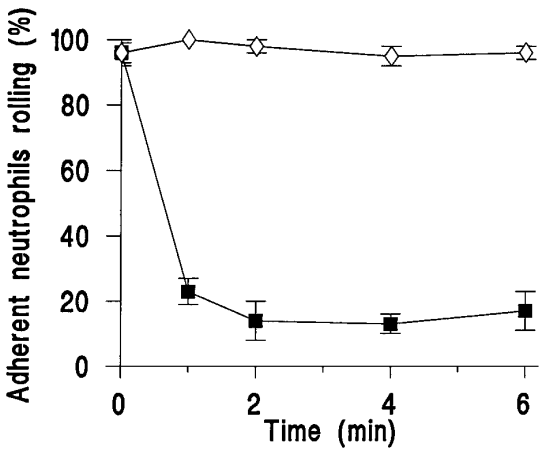

FIG. 3. Effect of pre-treatment with jasplakinolide on the response of adherent rolling neutrophils to superfusion with cytochalasin B (10μM). Neutrophils were preincubated at 37°C without (■) or with (△) 10μM jasplakinolide for 60 minutes before initial perfusion over immobilised platelets. Data are means ± SEM from 3 experiments where the percentage of adherent neutrophils that were rolling was determined as a function of time after initiation of superfusion with cytochalasin B. Data at time zero were obtained before superfusion of cytochalasin B. ANOVA showed a significant effect of treatment with jasplakinolide ($p<0.01$).

TABLE 1

Effect of Jasplakinolide on Surface Expression of CD11b by Neutrophils

	Basal expression	Response to fMLP
Untreated	21 ± 4	4.3 ± 0.9
Jasplakinolide	16 ± 2	1.2 ± 0.1**

Note. Data are means ± SEM from 4 experiments. Mean intensity of fluorescence was measured by flow cytometry for cells labelled with RPE-conjugated monoclonal antibody against CD11b, and expressed relative to mean intensity of fluoresence obtained using isotype-matched irrelevant antibody (basal expression) or relative to basal expression, after treatment with fMLP (10^{-7} M for 10 minutes).

** $p < 0.01$ compared to untreated cells by paired t test.

process inhibited by cytochalasins (7). However, these phenomena occur on a much slower time scale than the adhesive transformation from rolling to stationary adhesion studied here. Studies with C3-transferase which inhibits ribosylation of G-protein Rho showed inhibition of an adhesive response to fMLP in leucocytes (11). This implies that actin rearrangement is involved in activation-dependent modification of integrin adhesion. Here we have directly shown that rapid activation of integrins in neutrophils is indeed dependent on the state of the actin cytoskeleton.

The mechanism(s) of rapid integrin 'activation' are not well understood but may reflect conformational changes in the receptor, enzymatic modification of the receptor or an associated protein, or movement of receptors into clusters (22,23). These effects could depend on physical interactions of integrins with the cytoskeleton or on active, regulated movement of co-factors. Slower *de novo* expression of CD11b was also blocked by jasplakinolide. This might represent physical obstruction of movement to the cell surface of the granules that contain this receptor (22), inhibition of a regulatory message or possibly failure to insert or stabilise the newly-arrived receptor. To investigate these possibilites it would be desirable to perturb the link between the integrin and the cytoskeleton while leaving the skeleton otherwise intact and cell mechanics unaltered. In principle this might be done by disrupting association of actin-binding proteins, such as α-actinin or talin, known to link the cytoskeleton with integrins in other types of cells (22-24).

We found an unexpected bi-phasic time-dependence in the effect of jasplakinolide on fMLP-induced immobilisation. Pretreatment with jasplakinolide for 10 or 120 minutes totally inhibited immobilisation, while after 40 or 60 minute of pretreatment, 50-60% of the neutrophils did stop rolling in seconds when fMLP was delivered. These cells then gradually started to roll again in minutes. The latter phenomenon is similar to that observed when newly expressed CD11b/CD18 failed to become activated and bind to ligand (3). Thus after the intermediate periods of pretreatment, some constitutive integrin may have become activated on delivery of fMLP, but this was gradually down-regulated and new integrin did not emerge to prolong immobilisation.

These studies provide direct evidence previously lacking in favour of a link between integrin activation and rearrangement of the actin skeleton in neutrophils immediately following stimulation. Initial selectin-mediated binding and rolling by neutrophils was not altered by jasplakinolide. Since treated cells were rigid and somewhat distorted in shape, it seems that these changes did not have major influence on selectin-mediated adhesion. On the other hand, a number of studies have found that rolling adhesion may be disrupted by cytochalasins which tend to cause a shift from F- to G-actin (21, 25-28). Results of such studies have not been entirely consistent, in that capture was eliminated in some studies (25,28) but not others (21,26,27), and rolling was slowed (26) or converted to stationary adhesion (21). The studies reported here confirm our previous observation that superfusion of cytochalasin B alone caused neutrophils to stop rolling following deformation to teardrop shape (18), and showed that jasplakinolide can inhibit these effects. The jasplakinolide thus protects F-actin against the action of cytochalasin. In summary, it appears that regulation of the cytoskeleton may influence each stage of neutrophil adhesion, from capture to rolling adhesion to immobilisation, as well modulating attachment during onward migration.

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