ANCA-induced neutrophil F-actin polymerization: Implications for microvascular inflammation

WAI Y. TSE, GERARD B. NASH, PETER HEWINS, CAROLINE O.S. SAVAGE, and DWOMOA ADU

Department of Nephrology, University of Birmingham, United Kingdom; and Department of Physiology, University of Birmingham, United Kingdom

ANCA-induced neutrophil F-actin polymerization: Implications for microvascular inflammation.

Background. The antineutrophil cytoplasmic antibody (ANCA)-positive vasculitides are characterized by a necrotizing vasculitis of small vessels with neutrophil infiltration. The reasons behind the selectivity for small vessels remain unclear, but may relate to the necessity for neutrophils to deform in order to pass through capillaries. The resistance to deformation of neutrophils largely arises from their actin cytoskeleton. It is hypothesized that ANCA, by inducing actin polymerization, increases neutrophil rigidity and contributes to their sequestration in capillaries.

Methods. To test this hypothesis, neutrophils were treated with IgG-ANCA and the following characterizations: formation of filamentous F-actin (by flow cytometry); changes in morphology (by fluorescence and electron microscopy); and the potential to obstruct microvessels (by measuring entry times into micropipettes with comparable diameters to capillaries). The neutrophil signaling mechanisms activated by IgG-ANCA were investigated using blocking antibodies to Fcγ receptors and inhibitors of tyrosine phosphorylation. Protein tyrosine phosphorylation was examined by immunoblotting of cell lysates, and calcium fluxes were measured by spectrofluorimetry of Fura-2 pentakis (acetoxymethyl) ester (Fura 2-AM) labeled neutrophils.

Results. IgG-ANCA led to a significant dose-dependent actin polymerization over about 10 minutes. Over the same period, neutrophils became distorted in shape and more resistant to micropipette aspiration. Treatment with normal IgG caused less marked and delayed changes in these parameters. Actin polymerization required engagement of FcγRIIa receptor, tyrosine phosphorylation, and calcium fluxes.

Conclusion. These novel findings reveal signaling mechanisms that underlie ANCA-induced actin polymerization and might explain the predilection for small vessels in IgG-ANCA-associated vasculitis.

Neutrophils play a key role in mediating the inflammatory vascular lesions associated with the antineutrophil cytoplasmic antibody (ANCA)-associated systemic vasculitis. Vasculitic lesions often focus to particular organ sites, notably the lungs and kidneys. In the kidney, the vascular lesions have infiltrates of neutrophils [1, 2], and there is a strong correlation between the extent of renal involvement and the number of neutrophils present in renal biopsies [2, 3]. The reasons behind selectivity for small vessels in affected organs remain unclear, but may relate to the size difference between neutrophils (diameter \( \approx 8 \) μm) and capillaries (\( \approx 5 \) to 8 μm). In order to traverse the capillaries, neutrophils must deform their resistive actin cytoskeleton. In the resting neutrophil, monomeric, globular actin (G-actin) predominates and this polymerizes to form filamentous F-actin upon neutrophil activation [4]. This response is required for motility, translocation of cytoplasmic organelles and granules, activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, recycling of receptors and phagocytosis [4–6]. However, actin polymerization induced by a number of activators makes neutrophils rigid and more likely to be sequestered in capillaries [7–9]. We hypothesized that ANCA might induce actin polymerization and a similar increase in neutrophil stiffness, and thus impair the ability of neutrophils to traverse capillaries. Sequestration induced through mechanical changes might be exacerbated by the ability of ANCA to increase adhesiveness of neutrophils [10]. After exposure to ANCA, neutrophils might initiate microvascular injury by promoting ischemia through vascular obstruction, and also by the release of proteolytic enzymes and reactive oxygen species (ROS). This paradigm might explain the focal nature of injury seen in the ANCA-associated systemic vasculitides.

In order for the neutrophil to respond to chemical stimuli, occupancy of plasma membrane receptors must be linked to changes in cytoskeletal organization via the generation of second messengers. ANCA are present in the sera of patients with systemic vasculitis [11, 12] and activate neutrophils by cross-linking the antigens, proteinase...
3 (PR3), or myeloperoxidase (MPO) with Fcγ receptor [10, 13, 14]. Still, little is known of the neutrophil signal transduction pathways after ANCA binding and FcγR engagement, but we have demonstrated the involvement of tyrosine kinase C, protein kinase C, and phosphatidylinositol (PI)-3 kinase in ANCA-mediated neutrophil activation [15–17]. The purposes of this study were to determine if ANCA could induce F-actin polymerization and changes in cellular mechanics, and to dissect signaling mechanisms involved in this response.

METHODS
Isolation of human IgG and F(ab′)2 preparations
Control IgG was isolated from sera of normal subjects. ANCA-rich IgG was isolated from sera or plasma exchange samples from ANCA-positive vasculitis patients with active disease and ANCA titers of >1 in 400 dilution on indirect immunofluorescence. IgG isolation was performed using affinity chromatography on a HiTrap protein G affinity column (Pharmacia, Uppsala, Sweden). F(ab′)2 of human IgG-ANCA were prepared as described using a modified method by Lamoyi [18]. The purity of intact IgG and F(ab′)2 preparations was demonstrated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The protein content of the IgG and F(ab′)2 samples were determined by spectrometry using a Pharmacia LKB Ultraspec III (Pharmacia). ANCA antigen specificity was determined using both indirect immunofluorescence and antigen-specific enzyme-linked immunosorbent assay (ELISA) as previously described [19]. Endotoxin contamination of IgG and F(ab′)2 samples was minimal (<0.48 ng/mL) as determined by a Limulus amebocyte assay (Sigma, Survey, UK).

Neutrophil isolation and treatments
Neutrophils were isolated from six healthy donors using a method adapted from Toothill et al [20]. After washing in sterile phosphate-buffered saline (PBS), they were resuspended in PBS supplemented with 0.9 mmol/L MgCl2 and 1.3 mmol/L CaCl2. Neutrophils were activated with either 10−8 mol/L formylmethionyleucylphenylalanine ( FMLP), 10−7 mol/L phorbol myristate acetate (PMA), or 32 to 500 μg/mL of normal IgG, MPO-ANCA or PR3-ANCA for desired periods at 37°C. The doses of FMLP and PMA used were those known to induce neutrophil F-actin polymerization [21, 22]. Altogether, nine IgG samples were used: three normal IgG, three MPO-ANCA, and three PR3-ANCA. All experiments were repeated three times and two replicates of all samples. In separate experiments, neutrophils were also isolated from seven patients with active vasculitis. Of these, six patients (two were positive for MPO-ANCA and four were PR3-ANCA) were in the induction phase of therapy comprising of prednisolone and cyclophosphamide. The last patient who was PR3-ANCA-positive was in the relapsed phase and was also treated with prednisolone and cyclophosphamide. These neutrophils from patients were activated with normal IgG, MPO-ANCA, and PR3-ANCA, as described above.

In some experiments, neutrophils were incubated with blocking monoclonal antibody to FcγRI (anti-CD16 F(ab′)2) (Ansell Corporation, Bayport, MN, USA), FcγRIIa (IV.3 Fab) (Medarex, Annandale, NJ, USA) or FcγRIIIb (3G8 F(ab′)2); Medarex) for 15 minutes at 37°C before the addition of ANCA. Monoclonal antibodies were used at the saturating concentration of 5 μg/mL as determined by flow cytometry. In other experiments, 5 μg/mL of cytochalasin B (F-actin assembly inhibitor) or inhibitors of intracellular signals were added and incubated for 10 to 120 minutes before the addition of stimulus. The inhibitors used were genistein (tyrosine kinase inhibitor), ethyleneglycol-bis (β-aminoethylether)-N,N′-tetraacetic acid (EGTA) (extracellular calcium chelator) and BAPTA AM (intracellular calcium chelator). Typically inhibitors were added at the inhibition content (IC)50 dose, and also one log above and one log below the IC50. All experiments were repeated at least three times.

Actin polymerization and cell morphology
Neutrophils (2.5 × 106 cells) were stained for F-actin using N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) (NBD)-phallacidin [4]. They were simultaneously fixed, permeabilized, and stained by mixing with 50 μL of 37% phosphate-buffered formalin containing 50 μg of phosphatidylcholine and 12.5 μL of 6.6 μmol/L NBD-phallacidin. The cell mixture was incubated at 37°C for a further 10 minutes. The intensity of fluorescence of 10,000 stained cells was analyzed using a Beckton-Dickinson flow cytometer (Oxford, UK) equipped with a water-cooled argon laser emitting at 488 nm and fluorescence was measured through a 550 ± 30 nm bandpass filter. Results were expressed as the mean intensity for the experimental sample divided by the untreated control cells (i.e., relative F-actin content). In studies with inhibitors, unless otherwise stated, results were expressed as percentage of the response without inhibitor.

Fluorescent photomicrographs were made of neutrophils treated with NBD-phallacidin to examine the cellular distribution of F-actin. Neutrophils were allowed to settle on glass slides previously coated with 0.03% poly-L-lysine, then mounted with coverslips and the edges sealed with nail polish.

To examine cell morphology, neutrophils were fixed in 2.5% glutaraldehyde in phosphate buffer (pH 7.4) and left overnight at 4°C. They were then dehydrated through
a graded acetone series. Critical point drying was carried out in an Emscope CPD 750 critical point drier and the samples were mounted onto copper stubs coated with platinum in an Emscope SC 500 sputter coater (East Sussex, UK). They were then examined in a Jeol 100CX II scanning electron microscope (Garden Welwyn City, UK). Alternatively, neutrophils were examined unfixed, by videomicroscopy during micropipette aspiration experiments. Video recordings were made at chosen times after treatment with IgG, and on playback, neutrophils were classified as active if they showed marked distortion with pseudopodia, as opposed to spherical “passive” cells.

**Micropipette aspiration**

Neutrophils were suspended at a concentration of 10^6 cells/mL in PBS containing 5% autologous EDTA-plasma at 37°C. At time zero, ANCA or control IgG was added (100 or 500 µg/mL), then 200 µL of the neutrophil suspension was placed in a chamber made up of two coverslips separated by a U-shaped gasket and placed on a microscope stage held at 37°C. A micropipette with an internal diameter of 4.7 µm or 5.3 µm (in different experiments) was introduced into the chamber from the open side, and a fixed aspiration pressure was applied by lowering a water reservoir connected to the pipette. Video recordings were made of neutrophils aspirated into the pipette at chosen times. Subsequently, on playback, the time taken to enter the pipette was measured using an overlaid time signal (accuracy 1/100 seconds). Typically 60 neutrophils were tested in each sample. Cell morphology of aspirated and nearby cells was simultaneously noted. The pressure was adjusted so that control neutrophils entered the pipette within a period of ≃0.1 to 1.0 second.

**Analysis of neutrophil phosphotyrosyl proteins and cytosolic calcium concentration**

Neutrophil phosphotyrosyl proteins were detected by blotting with PY20, a mouse monoclonal antiphosphotyrosine antibody (Sigma) using published methods [16]. Concentration of ionized calcium was monitored in neutrophils loaded with 2 µmol/L Fura-2 pentakis (acetoxymethyl) ester (Fura 2-AM), as previously described [23]. Intensity of fluorescence emission at wavelength 505 nm was measured at excitation wavelengths of 340 and 380 nm, and the ratio of intensities at 340/380 nm used a relative measure of ionized calcium concentration. Changes were followed as a function of time after treatment with chosen activators in a spectrofluorimeter thermostated at 37°C.

**Statistical analysis**

All results are expressed as the mean ± SEM. Changes in F-actin, morphology, and pipette entry time with time was analyzed by analysis of variance (ANOVA). Individual treatments were compared by paired t test, and a value of P < 0.05 was accepted as statistically significant.

**RESULTS**

**Time course and concentration dependence of ANCA-induced actin polymerization**

PR3-ANCA and MPO-ANCA induced dose-dependent actin polymerization after 10 minutes of treatment using both neutrophils from normal donors (Fig. 1A). Both PR3-ANCA and MPO-ANCA caused significantly greater actin polymerization than normal IgG at concentrations of 125, 250, and 500 µg/mL (P < 0.05). The time course of formation of F-actin

![Fig. 1. Neutrophil F-actin polymerization, in response to human normal IgG or antineutrophil cytoplasmic antibody (ANCA) IgG. F-actin response in 2.5 x 10^6 neutrophils stimulated with human normal IgG, proteinase 3 (PR3)-ANCA or myeloperoxidase (MPO)-ANCA. Neutrophil F-actin was stained with N-(7-nitrobenz-2-oxa-1,2-diazol-4-yl (NBD)-phallacidin, and measured by fluorescence-activated cell sorter (FACS) analysis. The neutrophil F-actin dose response, measured at 10 minutes is shown (A), and shows a concentration-dependent effects of ANCA. The neutrophil F-actin time response in response to 100 µg/mL of human normal IgG, PR3-ANCA, or MPO-ANCA is shown (B). Maximum ANCA-induced F-actin polymerization was at 10 minutes, while that induced by normal IgG was at 20 minutes. F-actin increase induced by both PR3-ANCA and MPO-ANCA was significantly greater than that induced by normal IgG [analysis of variance (ANOVA), P < 0.05]. All experiments were repeated three times using neutrophils from six different donors, three normal IgG, three PR3-ANCA, and three MPO-ANCA and two replicates of all samples. Results show mean ± SEM of data pooled.

![Image 336x483 to 531x569]

![Image 337x608 to 524x698]
at 100 µg/mL of IgG is shown in Figure 1B. The IgG-ANCA–induced F-actin responses peaked at around 10 minutes. In contrast, F-actin increase induced by normal IgG peaked at around 20 minutes. F-actin increase induced by both PR3-ANCA and MPO-ANCA was significantly greater than that induced by normal IgG (ANOVA, \( P < 0.05 \)). These responses are much slower than actin polymerization induced by FMLP [22]. All experiments were repeated three times using neutrophils from six different normal donors: three normal IgG, three PR3-ANCA, and three MPO-ANCA and two replicates of all samples. Similarly, both PR3-ANCA and MPO-ANCA induced dose-dependent actin polymerization using neutrophils from patients with vasculitis which was comparable in magnitude to that from normal donors (data not shown). The addition of 5 µg/mL of cytochalasin B abolished the ANCA-induced F-actin increase (not shown). Following these observations, 100 µg/mL of IgG and 10 minutes of exposure were used in subsequent experiments to examine mechanisms of actin polymerization.

**Neutrophil morphology and F-actin distribution**

Unstimulated neutrophils were spherical and showed weak fluorescence with NBD-phallacidin, with little accumulation toward their periphery (Fig. 2). Responses to treatment with \( 10^{-8} \) mol/L FMLP are also shown in the same figures. Thirty seconds after the addition of FMLP, the majority of the cells were distorted with small lamellipodia and the fluorescence was concentrated in these protrusions. With increasing time, the cells became polarized, with fluorescence concentrated in the
pseudopodia. After treatment with IgG-ANCA, fluorescence microscopy showed F-actin formation and gradual distribution into surface irregularities (Fig. 2A). Scanning electron microscopy showed corresponding pseudopod formation and membrane ruffling (Fig. 2B). MPO-ANCA and PR3-ANCA induced similar morphologic changes. Normal IgG induced similar changes in shape which lagged behind those induced by ANCA. The F-actin intensity was less than that of ANCA-treated neutrophils (Fig. 2).

Light-microscopic assessment of the proportion of neutrophils which were “active” (with distorted shape) showed similar trends (Fig. 3). The majority of neutrophils treated with ANCA were activated by 5 minutes, with a peak at about 10 minutes. Normal IgG caused a lesser and slightly delayed morphologic response. Shape tended to recover partially between 25 and 30 minutes after treatment.

Resistance to deformation of neutrophils

Treatment of neutrophils with ANCA caused a significant increase in the time taken to enter a micropipette with diameter comparable to a blood capillary. The increase was maximal about 10 minutes after treatment, and was more rapid and greater than that induced by normal IgG (Fig. 4). By 25 to 30 minutes, entry time had largely recovered and there was no significant difference in micropipette entry time between ANCA- and normal IgG-treated neutrophils. When ANCA and normal IgG were compared at 500 μg/mL in one experiment, entry time response was similar to 100 μg/mL (not shown). If cells were treated with cytochalasin before ANCA, the increase in entry time was abolished (e.g., after 9 minutes entry time was 1.26 ± 0.22 seconds for ANCA-treated cells, but 0.23 ± 0.02 seconds for ANCA + cytochalasin B; means ± SEM for >20 cells). Preincubation with cytochalasin B prevented the ANCA-induced neutrophil morphologic changes.

Within the neutrophil population, a heterogeneous response was apparent in micropipette entry times. Microscopic observations showed that wide cell to cell variation in flow resistance was partly due to variation in cell shape. Some neutrophils could rapidly enter the pipette if their bipolar shape was oriented parallel to the pipette axis, whereas others entered slowly or could block the tip by bridging it while fluid flowed around them. In this situation, other cells slowly flowed to the pipette tip and became trapped there or temporarily pushed the trapped cell to one side while they entered. The rigid cell then became retrapped, so that activated neutrophils could become firmly trapped by bridging the entrance. Thus, pipette analysis revealed that variation in flow resistance could depend on geometric factors as well as structural rigidity, with the behavior of apparently rigid neutrophils being dependent on whether their shape allowed alignment and rapid flow into pores. All these factors served to prolong entry into and flow through micropipettes.

Role of Fcγ receptor in ANCA-induced actin polymerization

As previous studies have suggested a role for Fcγ receptor engagement in the ANCA-induced superoxide
Fig. 5. Human IgG-induced tyrosine phosphorylation in neutrophils and the effect of genistein on tyrosine phosphorylation. Neutrophils (5 × 10⁶/mL) were stimulated at 37°C with 500 μg/mL normal IgG, myeloperoxidase (MPO)-ANCA or proteinase 3 (PR3)-ANCA, blocked at the indicated times, lysed, and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted with anti-phosphotyrosine PY20. In some experiments, neutrophils were pretreated for 15 minutes with 150 μmol/L genistein at 37°C. Genistein used at this dose inhibited IgG-induced (normal IgG, MPO-ANCA, and PR3-ANCA) tyrosine phosphorylation. Only a representative experiment showing the effect of genistein on PR3-ANCA induced tyrosine phosphorylation is shown. All experiments were repeated three times.

Fig. 6. Effects of tyrosine kinase inhibition on antineutrophil cytoplasmic antibodies (ANCA)-induced neutrophil F-actin polymerization. Effects of genistein (50 μmol/L and 150 μmol/L) preincubation on 10⁻⁸ mol/L formyl-methionylleucylphenylalanine (FMLP), 10⁻⁷ mol/L phorbol myristate acetate (PMA), 100 μg/mL proteinase 3 (PR3)-ANCA or 100 μg/mL myeloperoxidase (MPO)-ANCA-induced F-actin polymerization in 2.5 × 10⁶ neutrophils. Results are expressed as percentage inhibition of the maximal effect seen with each stimulus. Data are shown as mean ± SEM of at least three individual experiments performed in duplicate. *P < 0.05; **P < 0.005.

Role of tyrosine phosphorylation

Signaling via Fcγ receptors recruits src family kinases [24], so evidence for tyrosine phosphorylation was sought. Treatment of neutrophils with ANCA caused tyrosine phosphorylation of a number of proteins, particularly bands with molecular weights between 86 and 28 kD (Fig. 5). Increase in tyrosine phosphorylation induced by ANCA was apparent at 5 minutes, was more marked at 10 minutes, and still present at 15 minutes. In contrast, although normal IgG induced a similar profile of tyrosine phosphorylation, the intensity of bands was low compared to that induced by ANCA. Pretreatment of the neutrophils with genistein, a tyrosine kinase inhibitor, essentially abolished tyrosine phosphorylation in response to subsequent challenge with normal IgG or ANCA.

Role of intracellular calcium transients

Actin polymerization is typically dependent on calcium [25], so we checked whether this was also true for IgG-ANCA–induced actin polymerization. The effects of FMLP, normal IgG, and ANCA on neutrophil intracellular calcium were examined by spectrofluorimetry. Results of representative experiments are shown in Figure 7. FMLP caused a rapid increase in intracellular ionized calcium which peaked in less than 1 minute. Both MPO- and PR3-ANCA caused increase in intracellular calcium, which was somewhat slower than the response to FMLP. The calcium transient in response to normal IgG was slower again, and peaked only after 3 minutes.

We tested the effects of extracellular and intracellular calcium chelation on actin polymerization (Fig. 8). Preincubation of neutrophils with 5 mmol/L EGTA for 30 minutes at 37°C inhibited the both the MPO-ANCA and PR3-ANCA responses (to 40 ± 5% and 26 ± 4%, respectively, both P < 0.005), while the same dose of EGTA did not inhibit the FMLP-induced response (94 ± 5%). Pretreatment with 50 μmol/L BAPTA-AM for 30 minutes at 37°C also inhibited both ANCA-induced response (MPO-ANCA 35 ± 11%, PR3-ANCA 26 ± 15%, both
DISCUSSION

Actin is a key component of the neutrophil cytoskeleton, and its reversible polymerization from its globular monomeric form, G-actin to its filamentous form, F-actin is central to many of the processes involved in inflammation [5, 27, 28]. The stiffness of the neutrophil, both in its quiescent [9] or stimulated state [7], is dependent on the assembly of F-actin. Although much less deformable than red cells, neutrophils successfully circulate through microvessels with diameters less than their cellular dimensions [29]. Activation of neutrophils by chemotactic agents leads to a reduction in their deformability, accompanied by an increase in structural rigidity that is related to F-actin polymerization [22, 30]. Studies in which activated neutrophils have been perfused through the lungs of animals or in which activating stimuli have been infused into the animals show lung sequestration is associated with a reduction in cellular deformability [30, 31]. Microvascular sequestration of neutrophils has also been implicated in impaired microcirculatory perfusion of animal models of shock and acute ischemia [32, 33]. These changes in deformability have been generally attributed to in vivo activation of the neutrophils and may promote further microvascular obstruction.

In this study we show for the first time that IgG-ANCA can induce a concentration-dependent increase in F-actin assembly and reorganization in neutrophils. This was associated with a reduction in neutrophil deformability as measured by the micropipette entry times. Entry times of neutrophils into pipettes observed in the current study were similar to those observed for neutrophils flowing through human capillaries [32]. The resultant change in cell stiffness may hinder the ability of the neutrophil to...
traverse the capillary network. IgG-ANCA compared to normal IgG significantly prolonged micropipette entry times at 10 minutes, the time of peak F-actin increase. Our findings on micropipette entry times agree with the only other study which has examined the effects of ANCA on neutrophil deformability [8]. In the current study, pretreatment with cytochalasin B prevented ANCA-induced morphologic changes and normalized the entry times, confirming that IgG-ANCA–induced neutrophil rigidity was dependent on the assembly of the actin cytoskeleton. Within the neutrophil population, a heterogeneous response was apparent in micropipette entry times, in part at least depending on whether distended, activated cells could align with the pipette axis. Thus, the circulatory effects of neutrophil activation can be predicted to depend on the stimulus, and on the local vascular architecture. ANCA, by inducing a nonorganized and dysregulated F-actin assembly, may increase neutrophil rigidity, which then promotes neutrophil sequestration in the microvasculature. Delay in passage through microvessels might also allow binding through integrins activated by ANCA [10]. These entrapped neutrophils may induce deleterious effects in the lungs and other major organs both by impairing microvascular perfusion and by releasing toxic factors after becoming trapped. This paradigm may explain the focal nature of injury seen in the ANCA-associated systemic vasculitides.

We found that whole IgG-ANCA induced neutrophil F-actin polymerization, whereas F(\text{ab}')\textsubscript{2} fragments of ANCA resulted in little F-actin polymerization. These observations suggest that Fc\gamma receptor engagement by the Fc portion of ANCA is instrumental in mediating the F-actin increase. To elucidate further the role of Fc\gamma receptors, blocking studies were performed using monoclonal antibodies to Fc\gamma receptors. First, an anti-FcRI receptor blocking antibody had little effect on the ANCA-induced neutrophil sequestration. Since neutrophils only express FcRI on induction by cytokines [34], the lack of impact of Fc\gamma RI receptor blockade was not surprising. In contrast, blockade of the Fc\gammaRIIa receptors almost completely abrogated the ANCA-induced neutrophil F-actin polymerization. Blocking Fc\gammaRIIb had little inhibitory effect on the ANCA-induced neutrophil F-actin polymerization. However, activation of neutrophils leads to shedding of this receptor and continual mobilization of intracellular Fc\gammaRIIb receptors to the cell surface may have offset the degree of receptor occupancy [35]. Thus, a contributory role for Fc\gammaRIIb receptor in ANCA-neutrophil F-actin polymerization cannot be excluded altogether. Indeed, we have shown in tumor necrosis factor-\alpha (TNF-\alpha)-primed neutrophils that blockade with Fc\gammaRIIa or Fc\gammaRIIb antibodies inhibited ANCA-induced superoxide production [15].

The magnitude of the ANCA-induced neutrophil F-actin responses may be influenced by the amount of ANCA antigens (PR3 and MPO) present on neutrophils’ surface accessible for cross-linking by Fc\gamma receptors. Membrane PR3 expression on human neutrophils is genetically determined and expresses a polymorphism [36]. The high PR3-expressing phenotype was significantly increased in patients with ANCA-associated vasculitis [37], and higher amounts of membrane PR3 expression correlates with disease activity [38, 39]. In contrast, no skewing of the G-463A MPO gene polymorphism was found in patients with MPO-ANCA associated vasculitis [40]. The in vivo relevance of ANCA antigen expression on neutrophil F-actin polymerization and deformability is not known and could be the focus of future studies.

Fc\gammaRIIa is a transmembrane receptor containing an immune receptor tyrosine-based activation motif (known as the ITAM or Reth motif) [41] in the cytoplasmic tail, which has been shown to be involved in the signaling of Fc\gammaRIIa [42, 43]. Tyrosine kinases are involved in the Fc\gammaR-mediated signal transduction pathways through phosphorylation of tyrosine residues within the ITAM of Fc\gammaRs by src family protein tyrosine kinases [24]. We confirmed that, both ANCA and normal IgG induced an increase in tyrosine phosphorylation of a number of proteins. Further, genistein, a tyrosine kinase inhibitor, inhibited F-actin polymerization by ANCA. It is possible that the 40 kD phosphoprotein is Fc\gammaRIIa, which is known to undergo tyrosine phosphorylation [34]. An earlier study from our group also showed tyrosine phosphorylation of proteins in response to ANCA [16]. The present study differs since neutrophils were not deliberately primed and a different profile of proteins was phosphorylated. There is a clear requirement for calcium during actin polymerization. Indeed, ANCA-induced calcium fluxes were demonstrated using spectrofluorimetry in this study. Inhibition of the ANCA-induced F-actin polymerization by EGTA and BAPTA AM support the need for both extracellular and intracellular calcium. It is recognized that triggering of either Fc\gammaRI or Fc\gammaRIIb results in a rise in intracellular calcium [42, 45–47]. The interaction between calcium and the actin-based cytoskeletal network are likely to underlie the mechnancochemical transduction that is basic to the movement of neutrophils toward inflammatory sites as well as to the engulfment of pathogenic organisms and cellular debris.

CONCLUSION

Our finding that ANCA induce neutrophil F-actin polymerization is intriguing, since the resultant change in cell stiffness may hinder the ability of the neutrophil to traverse the capillary network. Our observations suggest that for vessels as wide as 5.3 \textmu m, some distorted and rigid neutrophils could become firmly trapped by bridging the entrance of the micropipette after stimulation by ANCA. We infer that activation of neutrophils by ANCA is
capable of causing capillary blockage. The exact signaling sequence triggered by ANCA after binding of ANCA antigens and Fcγ receptor remains to be delineated, but findings from the present study support a role for FcγRII receptors, tyrosine phosphorylation, and calcium fluxes. An understanding of the signaling mechanisms behind ANCA-induced actin polymerization may lead to the development of newer and specific therapy. The geometric constraints imposed by the microvasculature coupled with neutrophil activation by ANCA may underlie the predilection of for small vessels, recognizing that other factors may promote small vessel injury in diseases such as systemic lupus erythematosus and cryoglobulinemia.

ACKNOWLEDGMENTS

This work was funded by a Medical Research Council Training Fellowship for W.Y.T., and by a grant from the Arthritis Research Campaign to CO.S.S. and G.B.N.

Reprint requests to Dr. Wai Y. Tse, Department of Nephrology, Derriford Hospital, Plymouth, PL6 8DH, United Kingdom.

E-mail: yee-wai.tse@phnt.swest.nhs.uk

REFERENCES


   Am J Kid Dis 7:95–110, 1986


   Current Diag Pathol 2:73–77, 1995

4. HOWARD TH, MEYER WH: Chemotactic peptide modulation of actin assembly and locomotion in neutrophils. 


5. SOUTHWICK FS, STOSSEL TP: Contractile proteins in leukocyte function. 


7. BAZIL V, STROMINGER JL: Metalloprotease and serine protease are involved in cleavage of CD43, CD44 and CD16 from stimulated neutrophils. 

   Relative contributions of adhesive and cellular mechanical properties. 


9. SOUTHWICK FS, HAYDEN TP: Contractile proteins in leukocyte function. 


10. HOWARD TH, MEYER WH: Chemotactic peptide modulation of actin assembly and locomotion in neutrophils. 


11. SOUTHWICK FS, STOSSEL TP: Contractile proteins in leukocyte function. 


16. RAPIDFORD DJ, LORD JM, SAVAGE COS: The activation of the neutrophil respiratory burst by anti-neutrophil cytoplasm autoantibody (ANCA) from patients with systemic vasculitis requires tyrosine kinase and protein kinase C activation. 


17. HENSON P, WILLIAMS JM, WAKELAM MJ, SAVAGE COS: Activation of Syk in neutrophils by antineutrophil cytoplasm antibodies occurs via Fc gamma receptors and CD18. 


18. LAMOYI E: Preparation of F(ab)2 fragments from mouse IgG of various subclasses, in Methods of Enzymology (vol 121), edited by 


22. HOWARD TH, ORLESAO CO: The kinetics of chemotactic peptide-induced change in F-actin content. F-actin distribution, and the shape of neutrophils. 


23. GRYNIKEWSKI G, POENIE M, TSUKIYAMA R: A new generation of Ca2+ indicators with greatly improved fluorescence properties. 


25. HOWARD TH, WANG D: Calcium ionophore, phorbol ester, and chemotactic peptide-induced cytoskeleton reorganization in human neutrophils. 


27. NATHAN CF: Neutrophil activation on biological surfaces. Massive secretion of hydrogen peroxide in response to products of macrophages and lymphocytes. 


28. SOUTHWICK FS, DABIRI GA, PASCHETTO M, ZIGMOND SH: Polymorphonuclear leukocytes adherence induces actin polymerization by a transduction pathway which differs from that used by chemotactic tracants. 


    Adv Microcirc 7:1–17, 1977


    Science 245:183–186, 1989


32. PETRONI KC, SHIN L, GUYRE PM: Modulation of human neutrophil Fcγ receptor function by IFN-γ and glucocorticoids. 


33. BAZIL V, STROMINGER JL: Metalloprotease and serine protease are involved in cleavage of CD43, CD44 and CD16 from stimulated


44. Dusi S, Domini M, Della Bianca V, et al: In human neutrophils the binding of immune complexes induces the tyrosine phosphorylation of Fc gamma RII but this phosphorylation is not an essential signal for Fc-mediated phagocytosis. Biochem Biophys Res Commun 201:30–37, 1994

