IMMUNOELECTRON MICROSCOPY OF FODRIN IN THE UNROOFED SPECIMEN OF THE ADHERENT HUMAN NEUTROPHIL

TOYOSHI FUJIMOTO AND KAZUO OGAWA

Department of Anatomy, Faculty of Medicine, Kyoto University, Konoe-cho, Yoshida, Sakyo, Kyoto 606

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We reported previously that fodrin is concentrated in the posterior portion of the human neutrophil stimulated with the chemotactic peptide (Fujimoto and Ogawa, *J. Cell Sci.*, 96: 477-486, 1990). In the present study, the mechanically unroofed specimen of glass-adherent neutrophils was examined to define finer details of fodrin distribution. By immunofluorescence microscopy, F-actin and fodrin were positively labeled; after stimulation with the chemotactic peptide, fodrin was found more densely in the posterior half of the cell as in the whole cell observation. In ultrathin sections of the unroofed and immunogold-labeled specimen, two dimensional distribution of fodrin could be observed. Some gold particles were observed with filaments of about 5 nm in diameter, but others were on amorphous materials. In the stimulated neutrophil, labeling for fodrin was mainly found between straight filaments of about 10 nm in diameter which were densely distributed in the posterior portion of the cell. The results indicate that the redistribution of fodrin in the stimulated neutrophil is concurrent with other cytoskeletal changes.

Cell migration is enabled by a combination of multiple factors, but obviously cytoskeleton is the major player in the phenomenon (17). In moving cells, various cytoskeletal proteins show redistribution, and accumulation either in the anterior or in the posterior portion has been reported: F-actin (10, 14), myosin II and actin-binding protein (18) in neutrophils and myosin I in amoebae (9) are seen anteriorly, whereas myosin II in amoebae is in the posterior (9).

In the previous study, we observed that fodrin is concentrated in the posterior portion of migrating neutrophils stimulated with the chemotactic peptide (8). Moreover, fodrin was not localized to the cell membrane itself, but in the peripheral cytoplasm appearing highly filamentous in thin sections. We therefore concluded that fodrin is a cytoskeletal rather than a peripheral membrane protein and may be related to the dynamic reorganization of the cytoskeleton in the neutrophil. A question raised by the result was where and how fodrin is localized in the macromolecular architecture of the cell. Presently we utilized a cytochemical technique which allows distinct visualization of the membrane associated cytoskeletal components, and localized fodrin in direct correlation with them.

MATERIALS AND METHODS

Unroofing of Neutrophils

Human neutrophils were isolated from healthy human donors by dextran sedimentation and Ficoll-Hypaque centrifugation (3). The cells were suspended in Hank's balanced salt solution buffered with 10 mM Hepes (pH 7.4), either untreated or treated with 10^{-8} M N-formyl-L-methionyl-leucyl-phenylalanine (FMLP; Sigma Chem. Co.) for 5 min at 25°C, and placed on clean glass coverslips for 5 min. The coverslips were rinsed briefly with ice-cold phosphate-buffered saline (PBS) and overlaid with a sheet of nitrocellulose membrane prewet in the cold PHEM buffer (16): 60 mM Pipes, 25 mM Hepes, 10 mM EGTA, 2 mM $MgCl_2$, pH 6.8. After excess liquid was blotted with paper towel, several glass slides were laid on top of the membrane and pressed gently with a finger. The stack of glass and membrane was separated and rinsed in the PHEM buffer containing 0.1 mM phenylmethylsulfonyl fluoride (Wako Chem. Co.), immediately transferred to 3% formaldehyde plus 0.1% glutaraldehyde in the same buffer and left for 10 min at 4°C. Specimens were observed under an inverted phase-contrast microscope and only

specimens showing efficient unroofing were used for the following experiments.

Immunofluorescence Microscopy

Coverslips carrying unroofed cells were treated with 1 mg/ml NaBH₄ for 5 min, then with 2% gelatin for 10 min, and incubated with affinity-purified rabbit anti-rat brain fodrin antibody (7) for 30 min at 37°C. After rinses, they were doubly labeled with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG antibody (Cappel) and rhodamine-phalloidin (Molecular Probes) and observed under a Zeiss Axiophoto epifluorescence microscope.

Immunoelectron Microscopy

For immunogold labeling, the specimens were processed in a similar manner as above up to the primary antibody step; 5 nm colloidal gold-conjugated goat anti-rabbit IgG antibody (Amersham, UK) was used as the secondary antibody. They were then fixed with a mixture of 2% glutaraldehyde and 0.2% tannic

acid for 10 min, post-fixed with 1% osmium tetroxide for 30 min at 4°C, dehydrated, and embedded in Epon 812. After polymerization, coverslips were detached from the epoxy block by plunging into liquid nitrogen and hot water alternately, and ultrathin sections were cut in parallel to the adherent cell surface.

RESULTS

Among several unroofing procedures tried, the method using nitrocellulose membrane gave the most consistent result for neutrophils. By ordinary immunofluorescence microscopy of whole cells, fodrin and F-actin are distributed homogeneously in untreated neutrophils adherent on glass, but after stimulation with the chemotactic peptide, the two proteins are polarized to the opposite ends (8). The unroofed specimen of neutrophils in the two states were examined for more precise localization of fodrin

FIG. 1. Immunofluorescence micrographs of (a) fodrin and (b) F-actin. Unroofed specimens of control neutrophils. Fodrin is observed evenly in the ventral plasma membrane, while the labeling for F-actin makes bright spots. $\times 970$

FIG. 2. Immunofluorescence micrographs of (a) fodrin and (b) F-actin. Unroofed specimens of neutrophils stimulated with the chemotactic peptide. Fodrin is concentrated in the posterior half of the cell, but F-actin is labeled diffusely. Note slender F-actin-positive 'tails' in the rear of the cells (arrows). $\times 970$

FIG. 3. Immunogold electron microscopy of fodrin. Unroofed specimen of the control neutrophil. Filaments, about 10 nm in diameter (double arrow), and coated membrane plaques (arrowheads) are not labeled particularly. Some gold particles are found with thinner (about 5 nm in diameter) filaments (arrows). $\times 78,000$

in relation to the cell membrane. In control cells, the isolated ventral membrane was almost round in shape and fodrin was seen diffusely without segregation (Fig. 1a). On the other hand, F-actin was seen as bright spots, which probably correspond to star-like foci of actin filaments observed previously (1), but accumulation of the foci to any one portion of the cell was not observed (Fig. 1b). After stimulation, the shape of the ventral cell membrane became elongated and concentration of fodrin was seen in the posterior portion in many cells (Fig. 2a). Distribution of F-actin appeared homogeneous in those cells (Fig. 2b). Notably thin 'tails' emanating from the posterior end of the cells were recognized by their positive labeling for F-actin (arrows in Fig. 2b).

For immunogold electron microscopy of the unroofed specimen, sectioning was started directly from the glass-adherent surface and only the first several ultrathin sections were used for observation. In the control cell, the labeling was scattered without making large accumulations (Fig. 3). Some im-

munogold particles were associated with short thin filaments of about 5 nm in diameter (arrows in Fig. 3). Filaments of about 10 nm in diameter and coated plaques were also observed in the specimen (Fig. 3), but they were not particularly related with the labeling for fodrin.

In the unroofed specimen of the stimulated neutrophil, morphological differentiation along the front-rear axis was obvious (Fig. 4a). The anterior half was filled with amorphous materials, and immunogold particles were rarely seen (Fig. 4b). The posterior portion contained bundles of straight filaments of about 10 nm in diameter, which seem to run mostly in an encircling direction. The labeling was densely observed in the amorphous material between the thick filaments (Fig. 4c).

DISCUSSION

The unroofed specimen is appropriate for immunolabeling because antigenic sites are freely accessi-

FIGS. 4a-c. Immunogold electron microscopy of fodrin. Unroofed specimen of the stimulated neutrophil. a. The anterior (left) and posterior (right) halves of the cell appear different even at low magnification. b. Amorphous materials in the anterior portion are not labeled for fodrin intensely. c. Filaments of about 10 nm in diameter are seen in bundles in the posterior portion. They are interposed with amorphous materials containing many immunogold particles. a. $\times 8,000$ b, c. $\times 78,000$

ble to antibodies. The present study showed that the unroofing technique is useful for visualizing the overall change in the cytoskeleton and localization of specific antigens in the same specimen. The technique has been used for several cultured cells (2, 6, 13, 15), but not for the neutrophil to our knowledge. The neutrophil is a difficult material for applying the method because they are not adhered to the substrate firmly and tend to fall off when unroofing is attempted. In the present study, by using clean glass in the protein-free medium, the neutrophil was made strongly adhesive and thus left the ventral membrane efficiently with the unroofing procedure. On the other hand, the firm adherence prohibits changes in cell shape and cell migration. Therefore, cells were incubated with the chemotactic peptide in suspension and thus allowed to go through reorganization in the cytoplasm before adhesion.

By pre-embedding immunoelectron microscopy we previously observed that most fodrin exists in the filamentous cytoplasm (8). In the present experiment, a majority of the fodrin labeling was associated with amorphous materials. A likely explanation is that the material is derived from actin filaments which were not preserved through osmium fixation (12). In fact, some immunogolds were observed with short thin filaments, which may be remaining actin filaments. A network of thin (2–3 nm) filaments which was found positive for fodrin in the bovine chromaffin cell (6) was not observed in the present study.

The nature of thicker straight filaments seen in the posterior portion of the stimulated neutrophil is uncertain. They are not likely to be actin filaments, because of the diameter and lack of filamentous labeling by rhodamine-phalloidin in the corresponding region. Other possible candidates are vimentin intermediate filaments and myosin filaments, although the former is more likely because the latter is seldom preserved in non-muscle cells.

In both the control and stimulated specimen, although the overall distribution of fodrin was different, the labeling was mainly present in the amorphous material. If the material actually represents degraded actin filaments, it might indicate that fodrin binds only actin filaments in the control cell but crosslinks actin and intermediate filaments after the stimulation. In any way, since fodrin can interact with various cytoskeletal filaments and affect their organization (4, 5, 11), the rapid redistribution of fodrin (8) is possibly a causative factor rather than a result of changes in other components.

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REFERENCES

1. Aggeler, J., Takemura, R. and Werb, Z.: High-resolution three-dimensional views of membrane-associated clathrin and cytoskeleton in critical-point-dried macrophages. *J. Cell Biol.* 97; 1452–1458, 1983.
2. Avnur, Z. and Geiger, B.: Substrate-attached membranes of cultured cells. Isolation and characterization of ventral cell membranes and the associated cytoskeleton. *J. Mol. Biol.* 153; 361–379, 1981.
3. Boyum, A.: Isolation of mononuclear cells and granulocytes from human blood. *Scand. J. Clin. Lab. Invest.* 21 (Suppl. 197); 77–89, 1968.
4. Burridge, K., Kelly, T. and Mangeat, P.: Non-erythrocyte spectrins: actin-membrane attachment proteins occurring in many cell types. *J. Cell Biol.* 95; 478–486, 1982.
5. Fach, B. L., Graham, S. F. and Keates, R. A. B.: Association of fodrin with brain microtubules. *Can. J. Biochem. Cell Biol.* 63; 372–381, 1985.
6. Fujimoto, T., Lee, K., Miwa, S. and Ogawa, K.: Immunocytochemical localization of fodrin and ankyrin in the bovine chromaffin cell *in vitro*. *J. Histochem. Cytochem.* 39; 1485–1493, 1991.
7. Fujimoto, T. and Ogawa, K.: Immunoelectron microscopy of fodrin in the rat uriniferous and collecting tubular epithelium. *J. Histochem. Cytochem.* 37; 1345–1352, 1989.
8. Fujimoto, T. and Ogawa, K.: Fodrin in the human polymorphonuclear leukocyte: redistribution induced by the chemotactic peptide. *J. Cell Sci.* 96; 477–486, 1990.
9. Fukui, Y., Lynch, T. J., Brzeska, H. and Korn, E. D.: Myosin I is located at the leading edges of locomoting Dictyostelium amoebae. *Nature* 341; 328–331, 1989.
10. Haston, W.: F-actin distribution in polymorphonuclear leucocytes. *J. Cell Sci.* 88; 495–501, 1987.
11. Langley, R. C. and Cohen, C.: Association of spectrin with desmin intermediate filaments. *J. Cell. Biochem.* 30; 101–109, 1986.
12. Maupin-Szamier, P. and Pollard, T. D.: Actin filament destruction by osmium tetroxide. *J. Cell Biol.* 77; 837–852, 1978.
13. Nicol, A., Nermut, M. V., Doeinck, A., Robenek, H., Wiegand, C. and Jockusch, B. M.: Labeling of structural elements at the ventral plasma membrane of fibroblasts with the immunogold technique. *J. Histochem. Cytochem.* 35; 499–506, 1987.
14. Oliver, J. M., Krawiec, J. A. and Becker, E. L.: The distribution of actin during chemotaxis in rabbit neutrophils. *J. Reticuloendothel. Soc.* 24; 697–704, 1978.

15. Roos, E., Spiele, H., Feltkamp, C. A., Huisman, H., Wiegant, F. A. C., Traas, J. and Mesland, D. A. M.: Localization of cell surface glycoproteins in membrane domains associated with the underlying filament network. *J. Cell Biol.* 101; 1817-1825, 1985.
16. Schliwa, M. and Blerkom, J.: Structural interaction of cytoskeletal components. *J. Cell Biol.* 90; 222-235, 1981.
17. Singer, S. J. and Kupfer, A.: The directed migration of eukaryotic cells. *Annu. Rev. Cell Biol.* 2; 337-365, 1986.
18. Valerius, N. H., Stendahl, O., Hartwig, J. H. and Stossel, T. P.: Distribution of actin-binding protein and myosin in polymorphonuclear leukocytes during locomotion and phagocytosis. *Cell* 24; 195-202, 1981.