Photoelectron microscopy and immunofluorescence microscopy of cytoskeletal elements in the same cells

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ABSTRACT

Pt K2 rat kangaroo epithelial cells and Rat-1 fibroblasts were grown on conductive glass dishes, fixed, and permeabilized, and the cytoskeletal elements actin, keratin, and vimentin were visualized by indirect immunofluorescence. After the fluorescence microscopy, the cells were postfixed and dehydrated for photoelectron microscopy. The contrast in these photoelectron micrographs is primarily topographical in origin, and the presence of fluorescent dyes at low density does not contribute significantly to the material contrast. By comparison with fluorescence micrographs obtained on the same individual cells, actin-containing stress fibers, keratin filaments, and vimentin filaments were identified in the photoelectron micrographs. The apparent volume occupied by the cytoskeletal network in the cells as judged from the photoelectron micrographs is much less than it appears to be from the fluorescence micrographs because the higher resolution of photoelectron microscopy shows the filaments closer to their true dimensions. Photoelectron microscopy is a surface technique, and the images highlight the exposed cytoskeletal structures and suppress those extending along the substrate below the cells. The results reported here show marked improvement in image quality of photoelectron micrographs and that this technique has the potential of contributing to higher resolution studies of cytoskeletal structures.

Photoelectron microscopy (photoemission electron microscopy or PEM) has recently been introduced into the study of whole cells (1, 2) although the origins of this technique are old, predating both transmission electron microscopy and scanning electron microscopy (for review, see ref. 3 for physics and refs. 4 and 5 for biomedical applications). Photoelectron microscopy differs significantly from the established techniques of transmission and scanning electron microscopy even though the image is formed by electrons. The photoelectron microscope can be considered to be the electron optics analogue of the fluorescence microscope. UV light from a short arc lamp is focused on the specimen as in fluorescence microscopy but, instead of imaging the emitted fluorescent light with a light optics system, emitted electrons are accelerated and imaged with an electron lens system. Photoelectron microscopy has several advantages, including high sensitivity to topographic detail (2, 6), a new source of contrast based on the photoelectric effect (7-9), and an unusually short depth of information (10). The increase in image quality during the development of the photoelectron microscope over the past few years has been substantial. Although the basic mechanisms by which the photoelectron microscopy images arise are understood, the interpretation of photoelectron microscopy images of biological specimens is the focus of current research. Here we report the comparison of photoelectron micrographs with fluorescence micrographs of the same cells that have been labeled by indirect immunofluorescence techniques with antibodies specific for actin, keratin, or vimentin. The fluorescence microscopy images were obtained first and the photoelectron micrographs were obtained second so that the cytoskeletal structures observed by immunofluorescence would be directly comparable with those identified previously in Pt K2 and Rat-1 cells or similar epithelial and fibroblastic cells (11-14).

MATERIALS AND METHODS

Antibodies and Cell Lines. Mouse monoclonal antibodies (15) and rabbit antibodies (16) antibodies were provided by J. Lin (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) and by K. Burridge (University of North Carolina), respectively. L. C. Summerhayes prepared and characterized the rabbit antikeratin antiserum by the method of Sun and Green (17). The preparation and characterization of the mouse monoclonal antibodies recognizing vimentin filaments will be described elsewhere (unpublished data). The Pt K2 cell line (CCL 56) is from the American Type Culture Collection and has been described (18). Rat-1 is a normal rat fibroblast cell line (19).

Cover slip treatment. Conductive substrates, required for photoelectron microscopy, were prepared as follows. To 5.3 ml of ice-cold methanol (100%), 1.7 ml of NaCl (0.9%) was added slowly, and then 0.1 ml of NEF/AH (1 g/ml in water) was added. This solution was filtered through a Unipore disc filter (BioRad). Clean 5-mm glass coverslips (Bello Glass) were heated to about 40°C on an aluminum slab and then mounted for 30 sec on each side with the above solution. The tin oxide-coated coverslips were sterilized by exposure to a germicidal lamp and then incubated with unlabeled colu serum at 37°C for 10-60 min. The coverslips were rinsed in sterile distilled water, air dried, and stored under sterile conditions. Cells were grown on the coated coverslips in Dulbecco's modified Eagle's medium (GIBCO)/10% fetal calf serum (GIBCO) in a 10% CO2/90% air incubator at 37°C.

Immunofluorescent labeling. Localization of cytoskeletal elements was carried out by indirect immunofluorescence. For actin-containing stress fibers, cells were fixed in 3% formaldehyde in phosphate-buffered saline (PBS) for 30 min, washed in PBS, and permeabilized with either acetone at -20°C for 2 min or 0.1% Triton X-100 in PBS at room temperature for 3 min (for Pt K2 cells, the latter appeared to be preferable and was used in this work). Cells were then stained with rabbit antibodies (for Pt K2) or mouse monoclonal antibodies (for Rat-1) and then with rhodamine-conjugated goat anti-rabbit IgG (Meloy, Springfield, VA) or rhodamine-conjugated goat anti-mouse IgG (Cappel Laboratories, West Chester, PA). For keratin filaments in Pt K2 and vimentin filaments in Rat-1 cells that have been labeled by indirect immunofluorescence techniques with antibodies specific for actin, keratin, or vimentin. The fluorescence microscopy images were obtained first and the photoelectron micrographs were obtained second so that the cytoskeletal structures observed by immunofluorescence would be directly comparable with those identified previously in Pt K2 and Rat-1 cells or similar epithelial and fibroblastic cells (11-14).

Abbreviation: PBS, phosphate-buffered saline.

[Further text and references not shown due to page limitation]
Dehydrated, and photographed in the photoelectron microscope. The arrows point to a few of the actin-containing stress fibers that can be identified.

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DISCUSSION

The fluorescence and photoelectron micrographs are remarkably similar in appearance considering that the origins of the images are so different and that the specimens have been dehydrated in proceeding from A to B in Figs. 1-4. The fluorescence image primarily relies on the contrast between labeled and unlabeled structures whereas the contrast in the photoelectron images here is primarily due to the topography of the sample. Two sources of contrast, material and topographic, can contribute to the imaging of cytoskeletal structures in photoelectron micrographs. Material contrast is provided by differences in photoelectron quantum yield (electrons produced per incident photon) just as contrast in fluorescence microscopy is due to differences in fluorescent quantum yields (7-9). It has been suggested that the photoelectron quantum yields of some dyes might be sufficiently larger than that of the background to permit these dyes to act as photoelectron labels (23-25). However, this type of material contrast was not observed under the experimental conditions used in this study. For example, Fig. 4b shows the characteristic pattern of the stress fibers (see the region below the white arrowhead) in addition to the labeled vimentin-containing intermediate filaments (arrows) seen in the fluorescence micrograph. Although the vimentin-containing fibers are decorated with antibodies having one to three rhodamine molecules attached per antibody molecule, they do not appear significantly brighter in the photoelectron micrographs than do the unlabeled stress fibers. The major source of contrast seen in these and previously published photoelectron micrographs is topographical contrast. Photoelectron microscopy is one of the most sensitive surface techniques for imaging fine topographical detail. For example, steps as small as 5 nm have been detected (26). This sensitivity occurs because the electrons emerging from the specimen have very low kinetic energies before acceleration and are easily deflected by small variations in the electric field, producing contrast. Photoelectron microscopy is due to differences in photoelectron quantum yields (7-9) are well within the range of this technique.

Photoelectron microscopy and fluorescence microscopy also differ in the depth of information. Photoelectron microscopy is a surface technique with very high depth resolution (short depth of information) because only those electrons that are photoionized at or very near the surface can escape from the specimen to form the image (10). In the cells used here, the majority of stress fibers occur on the cytoplasmic face of the lower cell surface, although they also occur throughout the cell. The fluorescence micrographs of Figs. 1 and 2 were taken in the substrate plane of focus. Therefore, of the stress fibers shown in the fluorescence micrographs, only those that are either exposed in the preparative procedures (for instance, the process of rendering the cells permeable to the antibodies) or lie directly under the cell surface and cause ridges are visualized in the photoelectron micrographs. In this aspect, the techniques are complementary. For the same reasons, differences between the fluorescence and photoelectron micrographs can also be seen in and around the nuclear regions. The photoelectron micrographs show a jumbled surface (presumably caused by partially collapsed and aggregated cellular components) surrounding sharply defined nuclei whereas, in the corresponding fluorescence micrographs, the nuclei are unlabeled and consequently appear darker.

Another difference between fluorescence and photoelectron microscopy is the resolution in the image. The resolution of the optical microscope is limited to about 200 nm by the wavelength of the emitted light whereas the resolution of the photoelectron microscope is determined by the wavelength of the emitted electrons plus aberrations in the electron optics system. The resolution of the photoelectron microscope in its present configuration is on the order of 10-20 nm and may reach the design goal of 5 nm when completed (15). The higher resolving power of photoelectron microscopy is evident in all four figures. Fluorescence micrographs give the overall impression that the cytoskeletal structures occupy a much larger fraction of the cytoplasmic space whereas photoelectron micrographs show these...
structures at more nearly their true dimensions. For example, in Fig. 3 the smallest keratin fibers that can be traced in both types of micrographs have diameters of ~30 nm in the photographs, but they are imaged at a minimum diameter of roughly 200 nm, the resolution limit of the optical microscope. Similarly, the smallest vimentin fibers measured in the photoelectron micrographs on a firmer basis, these results provide some insight into how photoelectron microscopy will be useful in future experiments in cell biology. The very high sensitivity to fine topo-

graphical detail makes exposed surface structures clearly visible in the photoelectron images without the need for staining the preparation. No metal or other conductive coating is required, eliminating one possible source of loss of resolution and con-

trast. Furthermore, as photoemission labels for photoelectron microscopy are developed and come into use (5), it should be possible to enhance contrast of specific cytoskeletal elements or cell surface components while still observing the remaining structures. For these reasons, photoelectron microscopy promises to provide useful and unique complementary information to that attainable by more established microscope techniques.

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