Rearrangement of the Keratin Cytoskeleton after Combined Treatment with Microtubule and Microfilament Inhibitors

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ABSTRACT In addition to containing microtubule and microfilament systems, vertebrate epithelial cells contain an elaborate keratin intermediate-filament cytoskeleton. Little is known about its structural organization or function. Using indirect immunofluorescence microscopy with an antikeratin antiserum probe, we found that destabilization of microtubules and microfilaments with cytostatic drugs induces significant alterations in the cytoskeletal organization of keratin filaments in HeLa and fetal mouse epidermal cells. Keratin filament organization was observed to undergo a rapid (1–2 h) transition from a uniform distribution to an open lattice of keratin fibers stabilized by membrane-associated focal centers. Since addition of any one drug alone did not elicit significant organizational change in the keratin cytoskeleton, we suggest that microfilaments and microtubules have a combined role in maintaining the arrangement of keratin in these cells. Vimentin filaments, the only other intermediate-sized filaments found in HeLa cells, did not co-distribute with keratin in untreated or drug-treated cells. These findings offer a new way to approach the study of the dynamics and functional roles of the keratin cytoskeleton in epithelial cells.

The structure and function of the vertebrate cytoskeleton are dependent upon three major groups of polymers: microtubules, microfilaments, and intermediate filaments. Unlike microtubules and microfilaments, whose distributions are universal in vertebrate cells and whose functions are well characterized (1–4), intermediate filaments are generally restricted in distribution to specific cell types, and their functions remain unresolved (5, 6). Lazarides (5) has suggested that they participate in the organization and mechanical integration of the cytoplasm. Attention has recently focused on elucidating the structure, function, and organization of keratins in epithelial cells, particularly their utilization as elements of an intermediate-filament cytoskeleton where they are designated ‘cytokeratins’ (7–10).

Keratin filament organization has been shown to be remarkably stable in epithelial cells in vitro by immunological methods (10, 13, 14). Microinjection of anti-α-keratin antibodies into epithelial cells has been used to directly alter the organization of the keratin cytoskeleton (11, 12). However, no biochemical inhibitors analogous to cytostatic drugs used to destabilize microtubules or microfilaments have been found that specifically interfere with cytokeratin structure or function (6). Recent immunofluorescence studies of cytokeratins during mitosis in certain epithelial cell types, including HeLa cells, have demonstrated that the otherwise immutable organization of cytokeratins changes from an extended filamentous arrangement to a condensed, spheroidal form as the cells divide, with the re-establishment of extended filaments following division (15, 16). This transformation suggests that there are specific mechanisms for the alteration of cytokeratin organization that may be dependent on, or triggered by, changes in the organization of microfilaments and microtubules. Since microfilaments and microtubules are rearranged during cell division in order to complete karyokinesis and cytokinesis, we have investigated the possibility that the organization of cytokeratins in interphase HeLa cells can be altered experimentally by drug-induced changes in the structure and organization of both microfilaments and microtubules. Previous studies, testing individual cytostatic drugs, have shown them to be relatively ineffective in altering the distribution of cytokeratin. Disruption of microfilaments with cytochalasins (10, 17) or microtubules by colchicine derivatives (13, 17) does not significantly alter the inherent organization of cytokeratins. However, a dramatic reorganization of...
cytokeratin filaments occurs in HeLa cells and other cultured epithelial cell types (17) after combined treatment of cells with cytochalasins, colchicine, and related compounds. The induction of a number of novel structural rearrangements in the keratin cytoskeleton of HeLa and fetal mouse epidermal (FME) cells described in this report provides further evidence for possible interactions among cytoskeletal elements in the organization of keratins.

MATERIALS AND METHODS

Cell Culturing

HeLa-S3 cells were obtained from Dr. Clive L. Bunn (University of South Carolina, Columbia) and maintained in Eagle's minimum essential medium (Gibco Laboratories, Grand Island, NY) containing nonessential amino acids and supplemented with 10% fetal bovine serum (KC Biologicals, Lenexa, KN) plus antibiotics. Cells were subcultivated in Leibovitz's L-15 medium (L-15, Gibco Laboratories) supplemented with 10% FBS on 15-mm glass coverslips within individual wells of multi-well culture plates. Cultures were grown for a minimum of 24 h at 37°C and used at subconfluent densities.

FME cells were obtained from the back skin of 14-d-old DBA/2J mouse fetuses. The epidermis was separated manually from each dermis after treatment of pieces of whole skin with 3.3 mg/ml collagenase (Sigma Chemical Co., St. Louis, MO) for 1 h at 37°C on a gyratory shaker. Isolated epidermis was dissociated into a cell suspension after trypsin treatment (17). Cells were plated on 15-mm glass coverslips. FME cells were grown in L-15 medium supplemented with 10% fetal bovine serum under culture conditions identical to those used for HeLa cells.

IMMUNOLOGICAL PROCEDURES: Cytoskeleton-enriched polypeptide fractions were extracted using a modification of the Triton X-100-1.5 M KCl method (13) developed for HeLa cells (14). The resulting proteinaceous pellets were dissolved in sample buffer and separated on 10% polyacrylamide gels at a pH of 8.3 (18).

For immunoblotting (“Western blotting”), the separated polypeptides were electrophoretically transferred from the gel to nitrocellulose membranes (19). Blotting was evaluated by staining duplicate sections of nitrocellulose with 1% Amido black 10B and comparing them with an equivalent Coomassie Brilliant Blue-stained portion of the polyacrylamide gel. The unstained membranes were then reacted with a 1:1000 dilution of anti-a-keratin antiserum (20). Antibody localization of polypeptide bands was achieved by incubating the membrane in the presence of goat anti-rabbit IgG antibody conjugated with horseradish peroxidase (Bio-Rad Laboratories, Richmond, CA). Enzyme activity was detected by reaction with 4-chloro-1-naphthol (21).

Indirect Immunofluorescence Microscopy

Fixed and permeabilized HeLa or FME cells were rehydrated in Sorensen's phosphate buffer (pH 7.2-7.4) for 5 min. 100 µl of a 1:30 dilution of avian α-keratin antiserum (20) made in rabbits or a corresponding dilution of preadsorbed antiserum or preimmune serum was applied to cells on each coverslip. In addition to anti-α-keratin antiserum, antiserums against bovine prekeratin, actin, tubulin (Miles Laboratories, Inc., Elkhart, IN), and vimentin (Transformation Res, Framington, ME) were used to assess the effects of drug treatments on selected elements of the HeLa cytoskeleton. Cells on coverslips were then incubated at 37°C in a humidified chamber for 20-30 min. Cultures were rinsed extensively in phosphate buffer and then treated with fluorescein-conjugated goat anti-rabbit IgG antibody (Miles Laboratories Inc.) for 20-30 min at 37°C. They were again rinsed extensively in phosphate buffer, mounted cultures side down in 10% glycerol on glass slides, and examined by epifluorescence microscopy.

Drug Treatments

Combinations of different cytostatic drugs used at several concentrations were tested for effect(s) on the organization of the keratin cytoskeleton of HeLa and FME cells (Table 1). Stock solutions of cytochalasin B, cytochalasin D, and β-lumicolchicine were made up in DMSO. Colchicine, demecolcine (Colcemid) and vinblastine sulfate were dissolved in L-15. All drugs were obtained from Sigma Chemical Co. Colchicine, demecolcine, and lumicolchicine were tested at final concentrations ranging from 10⁻⁸ M to 10⁻² M (0.4-400 µg/ml). Vinblastine was used at the same molar concentrations. Cytochalasin D was utilized at concentrations ranging from 0.05 to 5 µg/ml. Cytochalasin B was tested over a concentration range of 0.5 to 25 µg/ml. Parallel cultures of untreated and DMSO-treated controls were maintained for each series of experiments. Cell viability during the testing period was determined in all cases to be >95% by trypan blue dye exclusion. Cells were fixed and permeabilized as previously described and processed for indirect immunofluorescence.

RESULTS

Electrophoretic transfer of isolated HeLa cell polypeptides from polyacrylamide gels to nitrocellulose paper and subsequent reaction with α-keratin antiserum (Fig. 1) resulted in the demonstration of principal cross-reaction with one major human keratin polypeptide band. Indirect immunofluorescence microscopy utilizing this antiserum allowed visualization of a distinctive pattern of cytoskeletal filaments characteristic of cytkeratins in both HeLa and FME cells. Antiserum against mammalian α-keratin allowed visualization of the same distinctive organization of filaments demonstrated with antiserum against avian α-keratin.

FIGURE 1 Immunoblot of Triton X-100, 1.5 M KCl insoluble HeLa cell poly-peptides with antikeratin antisemur. (A) SDS PAGE pattern of cytoskeleton-enriched HeLa cell extracts stained with Coomassie Brilliant Blue. (B) Polypeptides electrophoretically transferred from a polyacrylamide gel to nitrocellulose and stained with amido black. (C) Reaction of antikeratin antisemur with polypeptides transferred to nitrocellulose and detected with peroxidase labeled second antibody. Upper arrowheads locate keratin, lower arrowheads locate vimentin. The position of the vimentin band was determined separately by immunoblotting procedures using antivimentin antisemur.

*Abbreviations used in this paper: FME, fetal mouse epidermal (cells).
The distribution and arrangement of cytokeratin in a control culture of HeLa cells that has been reacted with anti-keratin antiserum and visualized using indirect immunofluorescence is shown in Figure 2a. The uniform-sized, wavy-appearing keratin filaments are evenly distributed throughout the cytoplasm. This arrangement of evenly distributed, uniform-sized filaments is also characteristic of FME cells. The nucleus of each cell is a centrally located, nonstaining oblate structure (Fig. 2a) surrounded by keratin filaments. The filament arrangement observed is typical of the interphase organization inherent to most cultured epithelial cells in the unperurbed state (12). The distribution and arrangement of other elements of the HeLa cell cytoskeleton including microfilaments, microtubules, and nonkeratin intermediate-sized filaments is visualized by indirect immunofluorescence microscopy using antisera specific for actin, tubulin, or vimentin, respectively (Fig. 2, b–d).

The redistribution of cytokeratin filaments in HeLa cells following a 2-hour treatment with both 0.5 μg/ml cytochalasin D and 4 μg/ml colchicine (Fig. 2c) demonstrates the dramatic effect of the combination of these two cytostatic drugs. This treatment also results in reorganization of cytokeratin in FME cells (Fig. 3). The effects of these drugs on the other elements of the cytoskeleton are shown in Fig. 2, f–h.

Other cytostatic drugs and drug combinations were tested in HeLa cells for effects on the organization of the keratin filament cytoskeleton. Cytochalasin B, at a concentration of 10 μg/ml, in combination with colchicine was as effective as cytochalasin D in inducing keratin rearrangement. Both cytochalasin B and cytochalasin D were effective in combination with demecolcine or vinblastine sulfate (Table 1). Cytochalasin D in combination with β-lumicolchicine had a markedly reduced effect on the organization of cytokeratins (Table 1). This effect was qualitatively similar to the effect of cytochalasins alone, which consistently resulted in surface retraction at the cell perimeter and limited peripheral reorganization of the keratin cytoskeleton (17). β-lumicolchicine alone had no discernible morphological effect on keratin organization.

Cytochalasin D and colchicine were selected as representative microfilament and microtubule inhibitors and used as the principal combination of drugs for studies reported here. Optimal concentrations of cytochalasin D and colchicine were determined qualitatively from minimum dosages capable of inducing maximal observable effects on both individual cells and the number of cells effected in the population.

4 μg/ml colchicine proved to be the most effective dosage in combination with 0.5 μg/ml cytochalasin D, in terms of both the onset of keratin redistribution, which begins within 15–30 min of combined drug treatment, and the greater percentage of cells effected within the 2-h treatment time. The keratin filaments of treated cells are reorganized into an open lattice arrangement with filament focal centers both on the basal surface and along the cell periphery (Figs. 2e and 3). The focal centers appear to maintain the organization of the filaments after the depolymerization of microfilaments and microtubules. Microtubule and microfilament organization is

![Figure 2](image-url) Indirect immunofluorescence localization of HeLa cell cytoskeletal elements. Untreated control cells are represented in a–d and cells treated with colchicine and cytochalasin D for 2 h are represented in e–h. Characteristic differences in cytoskeletal organization are compared for keratin (a and e), actin (b and f), tubulin (c and g), and vimentin (d and h) in the absence and presence of colchicine and cytochalasin D. N, nucleus. Arrows in e indicate focal sites for keratin filaments. (a) Bar, 10 μm. × 1,500; (b–d and f–h) bar, 10 μm. × 685; (e) bar, 10 μm. × 1,800.
completely disrupted in HeLa cells following combined drug treatment (Fig. 2, f and g). Vimentin filaments show little discernible change in morphological appearance after combined drug treatment, an appearance which is distinctly different from that of cytokeratins, microtubules, and microfilaments (Fig. 2). Under the conditions of these experiments, they do not form the perinuclear structures observed after long-term treatment of cells with colchicine derivatives. Treatment of cells for 2 h at 37°C with 4.0 μg/ml colchicine or 0.5 μg/ml cytochalasin D alone results in little or no significant alteration in the inherent distribution of cytokeratins, though treatment with cytochalasins alone has a greater effect in general than treatment with any of the microtubule inhibitors. Cells reacted with preabsorbed anti-α-keratin antiserum or preimmune serum showed no fluorescence regardless of treatment.

The star-like configuration of cytokeratin observed in Figs. 2e and 3 is only one of several configurations displayed by cytochalasin- and colchicine-treated cells. These keratin filament patterns are also visualized by indirect immunofluorescence using anti-mammalian α-keratin antiserum. In some cases the keratins are organized in a pattern of filaments that reflects major alignment, with a few peripheral focal attachment sites located at the vertices of polygonal structures (Fig. 4a). The cytokeratins do not appear to be completely rearranged in the central, perinuclear region of the cytoplasm. In other cases the majority of cytokeratin is condensed in a single region of the cell. The filaments appear radially symmetrical, arising from a central core (Fig. 4b). The distribution of the cytokeratins in these cells shows a marked polarity relative to the position of the nucleus. The rest of the keratin cytoskeleton, including some membrane attachment sites, remains associated with the cytoplasm and cell membrane surrounding the nucleus. Fig. 5 demonstrates the reorganization of keratin in an untreated dividing HeLa cell. The spheroidal bodies (15, 16) stain intensely with antikeratin antiserum.

DISCUSSION

Extensive rearrangement of keratin filament organization is observed in HeLa and FME cells after combined drug treatment (Figs. 2e and 3). A lattice of filaments occurs throughout the cytoplasm, and filament-organizing sites are clearly observed at the cell perimeter and over the basal cell surface (Figs. 2e, 3, and 4). The spherical accumulation of keratin-positive material at these foci results from the overlap of these sites and/or an accumulation of filamentous keratin into a condensed form that remains associated with them. A less frequently observed arrangement of keratin filaments in treated cells, and one restricted to smaller cells, involves incomplete transformation of cytokeratins in the interior regions of the cytoplasm. The reorganization of cytokeratins in these cells results in the formation of simple polygonal arrangements with filaments or groups of filaments stretched
between the vertices (Fig. 4a). This configuration could result from a realignment of the filaments potentially involved in maintaining epithelial cell morphology. In the absence of microfilaments and microtubules, cytokeratins and their membrane attachment sites could provide a major stabilizing network in the cells. Nuclear membrane sites for association with or organization of keratin filaments have also been proposed to be involved in stabilizing the organization of cytokeratins (12). Displacement of keratin filaments, similar in extent to that seen in Figure 4b, has been induced by cytoplasmic microinjection of antikeratin antibodies into other epithelial cell types (11). In this context, there is a nearly complete polarization of keratin distribution in a small number of cells (Fig. 4b). Instability of membrane attachment sites and/or disruption of filament interactions with the nucleus may be involved. A small portion of the keratin filaments remain in the perinuclear cytoplasm with attachment sites at the cell membrane, but most of the keratin is displaced and condensed in a single core structure with filaments or loops of filaments extending radially from it (Fig. 4b). It appears that combined drug treatment can indirectly induce alterations in keratin-membrane and keratin-nucleus interactions similar to those observed after microinjection of selected antikeratin antibodies (11).

Horwitz et al. (15) and, more recently, Franke et al. (16) show that HeLa cells can regulate keratin organization from a filamentous form to condensed, multiple spheroidal arrangements during different phases of the mitotic cycle (Fig. 5). Cytokeratin organization in HeLa cells can also be modulated by a combination of the cytostatic drugs colchicine and cytochalasin D, as well as related cytostatic compounds, with similar physiological effects (Table 1). This cellular response to a diversity of related but not identically acting compounds reinforces the suggestion that organization of microfilaments and microfilaments influences the distribution of cytokeratins on epithelial cells. However, the drug-induced keratin filament reorganization differs somewhat in nature from that of mitotic rearrangements (Figs. 2e, 3, and 5). Mouse squamous cell carcinoma cells and primary cultures of FME cells also show this response to combined drug treatment (17; Fig. 3). It is of considerable importance to note that this reorganization of keratin filaments also takes place in primary cultures of FME cells, which lack vimentin filaments entirely (5, 17). This demonstrates the independence of the organization of keratin filaments under these conditions from other intermediate filaments (e.g. vimentin) present in HeLa cells. In addition, the immunological cross-reaction of antiserum to avian α-keratin with Triton X-100-KCl-insoluble extracts of HeLa cell polyepitopes in Western blots (Fig. 1) demonstrates that the primary reactivity is to a keratin polypeptide (M, ~56,000), with limited reactivity to several other keratin polypeptides. There is no detectable cross-reaction of vimentin with antikeratin antisera (Fig. 1).

The various organizational configurations of cytokeratins in HeLa cells induced by combined drug treatment (Figs. 2e and 4) appear different from the spherical bodies seen in normal, dividing cells (Fig. 5; 15, 16). The spherical keratin-containing bodies of dividing cells are observed in both control and drug-treated cultures. They are found in cells that are in late phases of mitosis. However, the normal and experimental modulation of cytokeratin organization in HeLa cells suggests that this rearrangement of keratin intermediate filaments is coordinated in some way with both microfilament and microtubule function and distribution. A dividing epithelial cell may be able to circumvent possible cytoplasmic constraints imposed by the interphase organization of cytokeratins by altering keratin filaments into a condensed form, and redistributing them throughout the cytoplasm, possibly at the cell periphery. In this sense drug treatment does not bring about the multiple site condensation of keratin filaments in interphase cells seen during cell mitosis. However, it may represent part of the physiological process of keratin filament reorganization that goes to completion only with appropriate cytoplasmic cues, including rearrangement of microtubules and microfilaments, available during cell division.

Cytokeratin organization is also responsive to exogenous biochemical cues. A transitory effect of epidermal growth factor on keratin distribution in mouse embryonic epithelial MMC-E cells causes a short-term keratin filament formation different from the pre-existing condensed keratin normally present in these cells (22). This suggests that the regulation of cytokeratin organization can function in both directions, filamentous → condensed → filamentous and condensed → filamentous, and can be influenced by external as well as internal triggering mechanisms. That a number of epithelial cell types, including HeLa cells, can modulate the organization of the keratin cytoskeleton is also indicated by the reversibility of the combined effects of cytochalasin D and colchicine after withdrawal of these drugs (data not presented).

A key organizational difference between cytokeratins and other intermediate filaments is the interaction of the keratins (tonofilaments) with desmosomes and related structures (5, 6, 11, 23-26). Our results show that cytokeratin organization in HeLa cells depends on interactions with cell membrane structures which provide a peripheral network of stabilizing attachment sites for keratin filaments, as well as with microtubules and microfilaments, which may provide a cytoplasmic framework. Other workers have reported the possibility that nuclear membrane sites may also be involved in cytokeratin organization (12). Sites for cell membrane-keratin association serve two major purposes in epithelial cells: one as intercellular or cell-substratum adhesive sites (macula adhaerens, hemidesmosomes) and the other as intracellular sites for attachment of tonofilaments in the cytoplasm, which establishes a network of filaments potentially capable of distributing stress within and between groups of cells. The lattice of keratin filaments in drug-treated cells appears to be connected to these focal points to form the star-like patterns observed (Figs. 2e and 3). It appears that the association of cytokeratins with membrane plaques may be crucial to filament organization and function in epithelial cells. No analogous organizational structures have been described for the remaining classes of intermediate filaments (5). With regard to interaction between the nucleus and keratins, it has been reported that keratin polypeptides are organized as filaments at a specific site or sites on or in the nuclear membrane in vitro (12). Both cell membrane and nuclear membrane sites may, therefore, function to organize and maintain the distribution of keratin filaments. Conversely, cytokeratin may function in positioning the nucleus in the cytoplasm (5, 6). Microinjection of α-keratin antibodies into the cell cytoplasm of cultured epithelial cells can result in the dissociation of keratin filaments from cell and nuclear membranes and the disorganization of the keratin cytoskeleton (2, 12). Microinjection of antibody provides an unique tool for directly and specifically effecting cytokeratin organization only, since no exogenously
applied inhibitors have yet been found to do so. This approach, in conjunction with specific inhibition of other elements of the cytoskeleton with which the keratins interact, adds greatly to our ability to experimentally manipulate structural and functional interactions among the cytoskeletal elements so far implicated in keratin filament organization. Thus, loss of microtubules and microfilaments results in an organization of cytokeratins that is dependent on the distribution of stable keratin filament-membrane attachment sites. These cell membrane sites are proposed to be desmosomal structures. Antibodies to desmosomal proteins are localized at keratin filament-membrane attachment sites observed in drug-treated cells (unpublished observations). The nature of nuclear membrane association sites is not known. The suggestion that desmosomal-type structures are involved in stabilization of keratin organization in drug-treated cells is supported by previous reports of the distribution of antidesmosomal protein antiserum staining on the basal surface of cultured epithelial cells (27, 28), which is similar in distribution to focal centers in drug-treated cells (17), the pattern of keratin filaments in relation to the periodic distribution of intercellular connections between apposed epithelial cells (11, 17, 28), and the redistribution of desmosomal structures in epithelial cells injected with antikeratin antibodies (11). A further possibility that deserves exploration is that keratin filament-membrane attachment sites, like desmosomes or hemidesmosomes, may act not only as a system of organization and stabilization sites for the distribution of tonofilaments in interphase cells, but may potentially be structurally involved in cyclic redistribution of keratin into spherical bodies for use by daughter cells during and after cell division.

The organization of keratin filaments is no longer conserved static in epithelial cells. The study of the coordinated interactions of cytokeratins with microtubules/microfilaments and membrane attachment sites not only provides a new experimental approach for establishing the role(s) of keratin filaments in epithelial cells, but also focuses attention on understanding the role(s) of all classes of intermediate filaments in the structure and function of the vertebrate cytoskeleton.

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