Keratin Filaments of Mouse Epithelial Cells Are Rapidly Affected by Epidermal Growth Factor

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ABSTRACT The effects of epidermal growth factor (EGF) on the cytokeratin filaments of cultured murine epithelial cells were studied by the indirect immunofluorescence technique with affinity-purified antibodies. Mouse epithelial cells (MMC-E), grown on glass cover slips, and viewed by immunofluorescence microscopy, showed keratin-specific fluorescence as typical bright perinuclear aggregates corresponding to dense paracrystalline granules seen in electron microscopy. Within minutes after an exposure to EGF, the keratin granules in the MMC-E cells decreased. After 10 min of incubation, the cells had spread fibrillar keratin. Such an effect could not be found after a similar exposure to insulin, dexamethasone, dibutyryl cyclic AMP, or antimitotic drugs. EGF, therefore, has a relatively direct effect on the cytoskeletal organization of cultured epithelial cells. These rapid effects on the keratin filaments may explain the simultaneous EGF-induced ultrastructural surface changes of the cells. EGF may thus function as a regulatory factor in the migration of epithelial cells and in the mobility of their cell membranes. The epithelial cell line, MMC-E, should prove a useful model for studies on the action of EGF on nontransformed epithelial cells in vitro.

The interaction of a polypeptide hormone with its cell surface receptor induces a cascade of events that leads to DNA synthesis and cell division. Epidermal growth factor (EGF) has been widely used to characterize the biochemical and molecular mechanisms involved in the stimulation of growth (7, 10). Cell lines that have high numbers of EGF receptors have proved to be good models for such studies (9, 22).

The exposure of cells possessing a large number of receptors for the corresponding growth factor often results in rapid alterations in the cell membrane followed by certain long-term effects (cf. 3, 14, 17). Rapid membrane rufflings have been described in pheochromocytoma cells (8), in cultured human epidermoid carcinoma cells (6, 14), and in cultured mouse epithelial cells in association with increased pinocytosis (17; footnote 1). EGF in physiologic concentrations induced long-term membrane ruffling and pinocytotic activity in glial cells (3). However, to demonstrate rapid effects in vitro, it is usually necessary to use concentrations higher than those found physiologically (6, 14, 17). The initial event in EGF-mediated stimulation of cultured cells involves an interaction between EGF and its membrane receptors (4, 10, 15). The biochemical events that follow the binding of EGF to its receptors include proteolytic cleavage of the hormone-receptor complex and rapid phosphorylation of the receptor (1, 5, 20). This is followed by the internalization of the receptor, as shown by increased pinocytic rate within 15 min (6, 14). The mechanism of the simultaneous cytokinetic events found in the cells is not understood.

In this study we have characterized the organization of cytokeratin filaments of mouse epithelial (MMC-E) cells (22) upon an exposure of the cells to EGF. MMC-E cells contained large perinuclear keratin granules, but not cytokeratin fibers as most of the epithelial cells studied (cf. 11, 25, 28). After an exposure to EGF, the distribution of keratin was rapidly affected. Fibrillar structures of keratin appeared within 10 min, and the earliest changes could be seen within minutes. The results suggest that the change in keratin is associated with the rapid morphological and ultrastructral changes previously described (17; footnote 1). EGF is thus the first known polypeptide growth factor that can rapidly and transiently effect intracellular keratin filaments in cultured cells.

MATERIALS AND METHODS

Cell Cultures and Media

Mouse embryo epithelial MMC-E cells were seeded at a density of 3×10^5 cells per 60-mm diameter plastic petri dish (Falcon Plastics, Div. Becton, Dickinson & Co., Oxnard, Calif.) and were grown in BME (basal medium, Eagle's, Grand Island Biological Co., Grand Island, N. Y. [Gibco]) or in Dulbecco's MEM (modified Eagle's medium) (Gibco) supplemented with 10% fetal calf

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serum and antibiotics. The cells were grown on glass cover slips for immunofluorescence. A potaroo kidney epithelial cell line (PtK_2) and HeLa cells were obtained from American Type Culture Collection (Rockville, Md.) and were cultured in Dulbecco's MEM supplemented with 10% fetal calf serum and antibiotics. Human amnion epithelial cells were isolated as described (27) and were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum and antibiotics.

Exposure to EGF and Other Agents

Mouse EGF was purified as described (23). It was used for short-term exposures at a concentration of 100 ng/ml in the medium. The experiments were carried out at 37°C, and new fresh medium was added to the control dishes when the incubations were initiated. Pork insulin (25.8 IU/mg, Novo Industri, Copenhagen, Denmark), dexamethasone (Sigma Chemical Co., St. Louis, Mo.) and dibutyryl cyclic AMP (dbcAMP; Sigma Chemical Co., Poole, England) were used at the concentration of 5–10 μg/ml, 10^{-4}–10^{-5} M, and 1–5 mM respectively, in the medium. For Colcemid (demecolcine, CIBA, Milan, Italy) and cytochalasin B treatment (Sigma Chemical Co.), the cells were exposed to the drugs (10 μg/ml) for 2 h.

Indirect Immunofluorescence Technique for Keratin Filaments

The exposure of the cells to EGF and other agents was terminated by a rapid washing with phosphate-buffered saline (PBS) followed by immediate fixing of the cells with methanol at −20°C for 20 min. The cells were subsequently washed three times with PBS, and stained for keratin and vimentin as described in detail elsewhere (28). The antiserum against purified keratin polypeptides from human plantar callus was raised in rabbits (28), and prepared according to Sun and Green (24). The antiserum was affinity purified by applying the serum on a Sepharose Cl 4 B-column (Pharmacia, Uppsala, Sweden) into which the keratin polypeptides were coupled in the presence of 8 M urea and 2-mercaptoethanol using the method of Porath (21). Antibodies attached to the column were eluted with 0.2 M glycine buffer (pH 2.6).

The purified antibodies reacted both with human epidermal keratin polypeptides and with multiple keratin polypeptides from cultured human amnion epithelial cells (Fig. 1, lanes *a* and *b*) when studied using the immunoblotting technique of Towbin et al. (26) in accordance with the results of Franke et al. (11). These reactions indicated that the antibody was not raised against a minor contaminant in the keratin preparation. A polypeptide with a molecular weight of 37,000, possibly a degradation product of keratin, was also observed. However, when MMC-E cells were analyzed by immunoblotting, only two polypeptides with molecular weights of 57,000 and 54,000 were found (Fig. 1, lanes *c* and *d*). The purified keratin antibodies gave a typical network of fibrillar cytoplasmic fluorescence in indirect immunofluorescence of both PtK_2 and human amnion epithelial cells (Fig. 2 *a* and *b*).

Electron Microscopy

For transmission electron microscopy, the cells cultured on small plastic dishes were fixed in 2.5% glutaraldehyde in 0.2 M Na-cacodylate buffer (pH 7.2) for 60

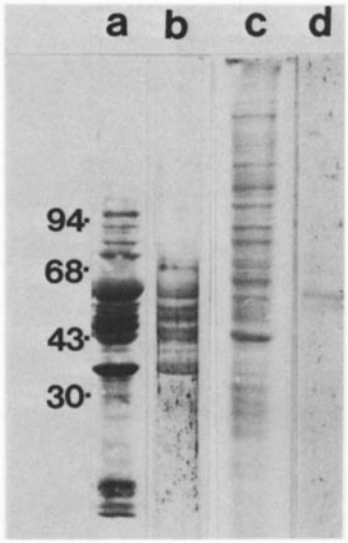

FIGURE 1 Specificity of keratin antibodies. Polypeptides of human amnion epithelial cells and of MMC-E cells were run in SDS PAGE and electrically transferred on a nitrocellulose sheet (*a*, *c*). After reaction of the nitrocellulose sheets with purified rabbit anti-keratin antibodies, followed by swine anti-rabbit IgG and peroxidase-anti-peroxidase complex, a distinct reaction is seen with five to six 37- to 67-kdalton polypeptides of human amnion epithelial cells (lane *b*). In MMC-E cells, only two (54- and 57-kdalton) immunoreactive polypeptides were seen (lane *d*). The molecular weight markers are indicated on the left ($\times 10^3$): 30, carbonic anhydrase; 43, ovalbumin; 68, human serum albumin; 94, phosphorylase B.

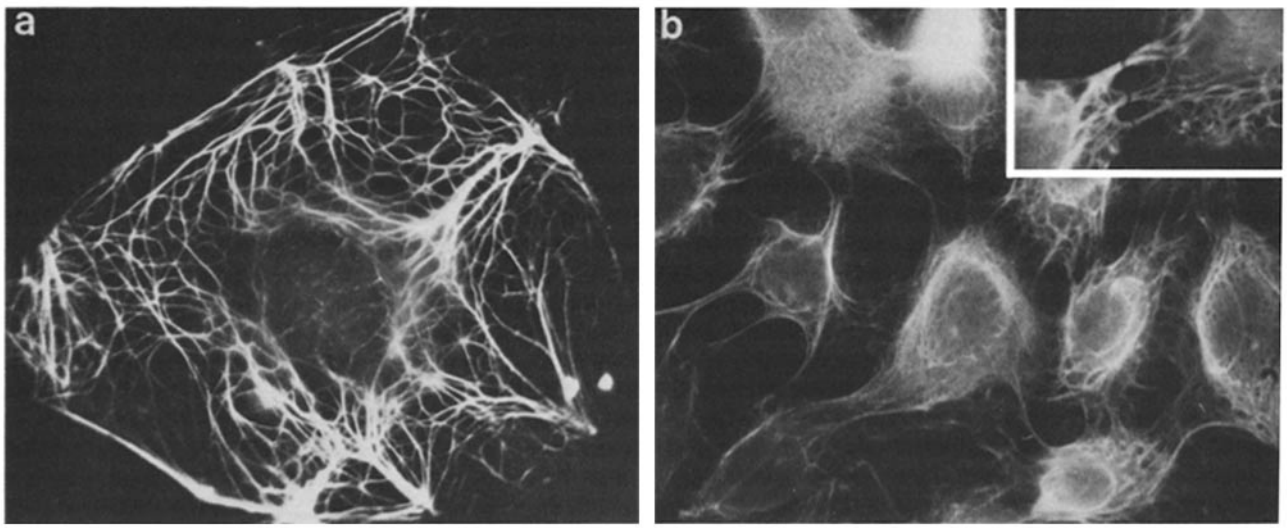

FIGURE 2 Staining of PtK_2 and human amnion epithelial cells with anti-keratin antibodies. In indirect immunofluorescence microscopy with rabbit anti-keratin antibodies, a bright fibrillar cytoplasmic staining is seen both in cultured PtK_2 cells (*a*) and in cultured human amnion epithelial cells (*b*). Note the typical gap of fluorescence seen in the middle of keratin fibers in human amnion epithelial cells, located apparently at the desmosomes (*b*, *inset*). × 700.

min. Thereafter the specimens were postfixed in 1% osmium tetroxide 0.1 M phosphate buffer for 60 min, dehydrated, and embedded in Epon 812. Thin sections were poststained with uranyl acetate and lead citrate and were examined in a JEOL 100B electron microscope.

Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis was carried out in the presence of SDS as described by Laemmli (18). For the immunoblotting technique, the method of Towbin et al. (26) was used.

RESULTS

The Expression of Cytokeratin Filaments in MMC-E Cells

Confluent and subconfluent cultures of MMC-E cells were studied for the presence of cytokeratin filaments in the cells by use of affinity-purified antibodies. In indirect immunofluorescence microscopy, keratin-specific fluorescence could be seen

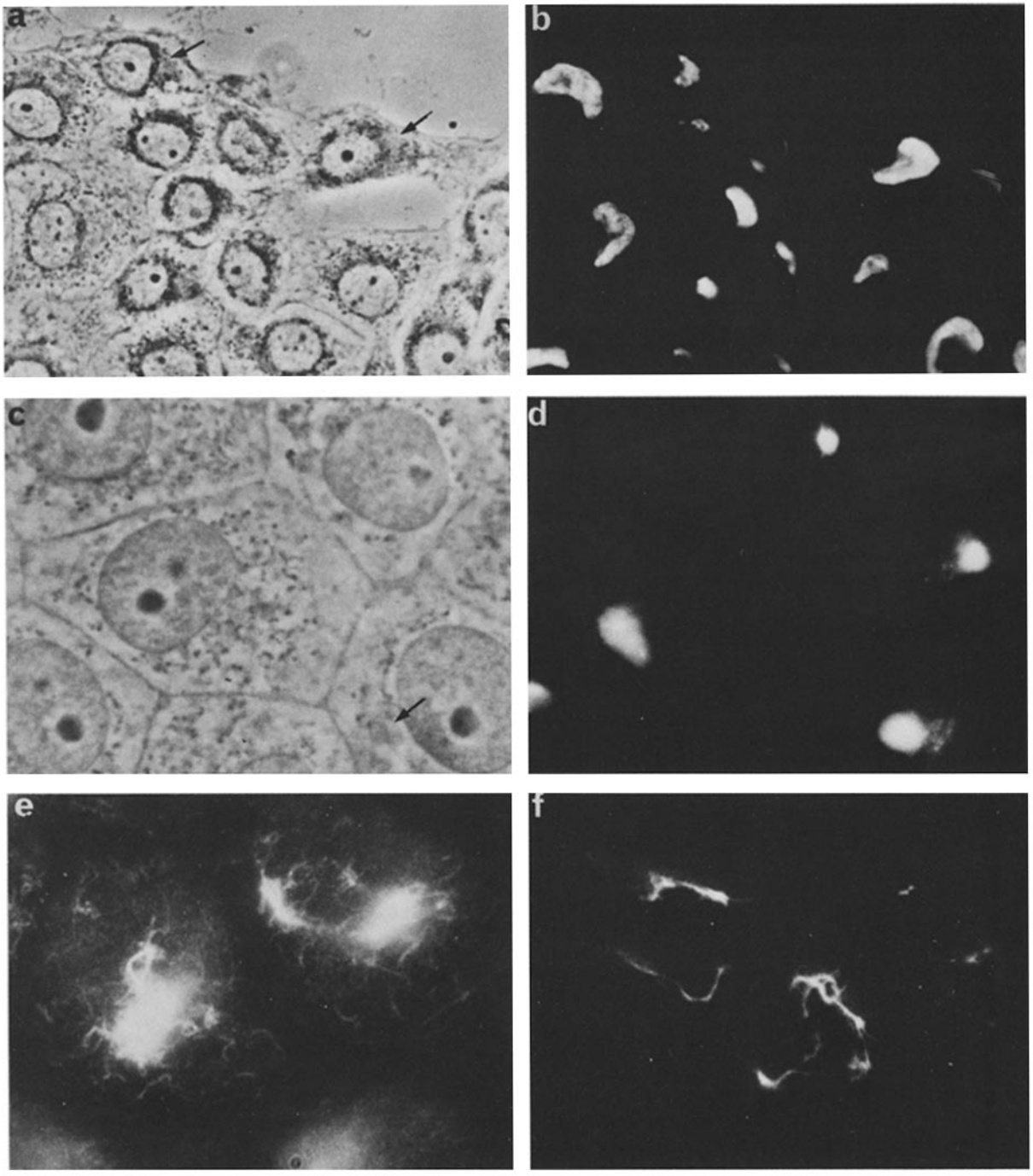

FIGURE 3 Distribution of cytokeratin in MMC-E cells and the effect of EGF. Subconfluent cultures of MMC-E cells were exposed to EGF (100 ng/ml) for short periods of time and the exposure was terminated by rapid washing with phosphate-buffered saline and immediate fixation (see Materials and Methods). The distribution of keratin was then demonstrated by indirect immunofluorescence. Phase-contrast photomicrographs were taken of some of the corresponding fields. (*a–d*) control cells. The perinuclear keratin granules can be seen also by light microscopy (arrrows in *a* and *c*) when their location is first demonstrated by immunofluorescence (*b* and *d*). (*e* and *f*) Cells exposed to EGF for 10 to 20 min, respectively. Note the appearance of fibrillar forms of keratin in these cells. (*a* and *b*), × 300; (*c–f*), × 700.

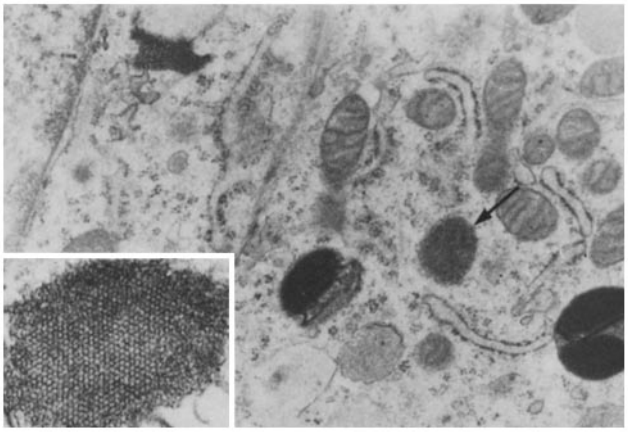

FIGURE 4 Electron micrograph of MMC-E cells showing the paracrystalline cytoplasmic granules seen in these cells (arrow). At higher magnification, such paracrystalline inclusions showed a tubular internal organization (*inset*). × 24,000; *inset*, × 78,000.

only as perinuclear granule–like phase-dense particles (Figs. 3 and 4). MMC-E cells usually contained one or two perinuclearly located keratin granules. The cells also showed vimentin as a faint, diffuse, fibrillar pattern. On the contrary, vimentin, but not keratin, was found in the syngeneic fibroblastic cell line, MMC-F (results not shown). Interestingly, in electron microscopy, dense paracrystalline perinuclear granules were seen in the MMC-E cells (Fig. 4), apparently corresponding to the granules seen in indirect immunofluorescence microscopy with keratin antibodies.

The Effect of EGF on the Distribution of Keratin

When the cells were exposed to EGF (100 ng/ml) in fresh or conditioned media, rapid changes in the keratin distribution were seen. The granular form of keratin-specific fluorescence decreased in amount within minutes and was replaced with a faint fluorescence that extended to the periphery of the cells and was often difficult to reproduce photographically (Fig. 3 *e* and *f*). The first changes were found 2–5 min after the onset of the incubation. The spreading of keratin was, however, a temporary event. Cells exposed to EGF for 2 h had, again, granular keratin instead of fibrillar and were much like the untreated control cells (figures not shown). The change was not brought about only by the change of medium. Lower concentrations of EGF caused similar changes in the distribution of keratin, but they were more difficult to observe. The cells were also studied by phase-contrast microscopy to demonstrate rapid effects in the morphology. Even though scanning electron microscopy revealed rapid changes, the morphology of these cells during the incubation did not vary much.[1]

EGF did not cause toxic effects on these cells. No floating cells were observed during or after the incubation and, if the cells were treated with EGF (100 ng/ml) for 1 h, trypsinized, and subcultured, the cells were comparable to untreated cells. On the other hand, when the MMC-E cells were similarly exposed to dexamethasone (10^{-4}–10^{-5} M), insulin (5–50 μg/ml), or dbcAMP (1–5 mM), similar changes in the keratin distribution could not be revealed (data not shown). The effect of EGF on keratin, therefore, appears to be specific. On the other hand, neither cytochalasin B nor Colcemid treatment of the MMC-E cells caused any reorganization of the keratin granules (Fig. 2). No reproducible changes in the distribution of the faint vimentin fibers by EGF could be seen. When we cultivated the cells with EGF (2 ng/ml) for longer periods of time (24–48 h) to observe the long-term effects as described (17) and then stained the cells for keratin, no clear, reproducible effects were demonstrated. The EGF-treated cells had granular forms of keratin and only trace amounts of keratin fibers as did the controls.

DISCUSSION

Cells of different origin express different types of intermediate filaments. Two types of these filaments have been reported in a number of cells (19). Thus, fibroblastoid, muscle, glial, neuronal, and epithelial cells seem to contain specific types of intermediate filaments (19). Cultured epithelioid cells are known to have both keratin-containing intermediate filaments, found only in epithelial cells, and fibroblast-type of intermediate filaments (vimentin) possibly acquired during adaptation into a cell culture (19, 28). Cells possess several genes capable of coding for keratin. During cell differentiation different genes are activated, and this results in the synthesis of new molecular forms of this protein (12). The observation that MMC-E cells express keratin-specific fluorescence further confirms their epithelial character, because endothelial cells, resulting in morphologically similar cultures, contain vimentin but not keratin (19). Unlike most known cultured epithelial cells (11, 12, 25), the MMC-E cells did not contain fibrillar cytokeratin but showed granular organization of keratin both in indirect immunofluorescence and in electron microscopy. The paracrystalline inclusions found in MMC-E cells were closely reminiscent of keratohyalin granules recently described in newborn rat epidermis (13).

We have used the mouse epithelial MMC-E cells for studies on the effects of EGF on nontransformed epithelial cells. The interaction of polypeptide growth factors with their receptors often results in morphological alterations that apparently derive from a relatively rapid sequence of events after the binding. MMC-E cells have a large number of receptors for EGF on their surfaces. Sarcoma growth factor, SGF, that binds to EGF receptors, can affect these epithelial cells in addition to fibroblastic cells (16). In these cells, EFG brings about long-term changes that are expressed by increased cell spreading and reduction in the amounts of surface microvilli (17). EGF, as certain other mitogenic polypeptides, can also stimulate proliferation of these cells. The short-term effects included membrane rufflings within 10 min after the exposure to the growth factor.[1]

The distribution of vimentin-type intermediate filaments, but not those of keratin-type, can be affected by Colcemid in cultured cells (11, 12, 25). Recently, it has been reported that the exposure of cultured rat hepatoma cells to butyrate results in long-term flattening of the cells accompanied by the disappearance of a juxtanuclear keratin and vimentin aggregates and appearance of keratin fibrils (2). However, a similar reorganization of vimentin fibrils was also seen in those cells. This differs from our present results indicating that EGF had no effect on the cellular vimentin organization.

The spreading of keratin fibrils after an exposure to EGF was surprisingly fast, and the binding of EGF to its receptors might thus cause a reorganization of the cytoskeleton before the internalization of the EGF receptor complex (cf. 10). It would be interesting to know whether a rapid phosphorylation of either keratin or some other cellular proteins could be responsible for this phenomenon. Thus far, no polypeptide growth factors have been known to affect the distribution of

keratin filaments in cultured cells. EGF, then, may function as a regulatory factor in the control of epithelial cell shape, locomotion, and, possibly, differentiation.

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