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EPIDERMAL GROWTH FACTOR INDUCES REDISTRIBUTION OF ACTIN AND α-ACTININ IN HUMAN EPIDERMAL CARCINOMA CELLS

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SUMMARY

We have examined the possibility that epidermal growth factor (EGF)-induced morphological changes in human epidermoid carcinoma (A-431) cells are related to a reorganization of specific cytoskeletal elements affected by the hormone. It was found that EGF induced striking changes in the distribution of actin and α-actinin within these cells. After 30–45 min of exposure to EGF there was a marked decrease in the degree of organization of the microfilament bundles and appearance of diffuse and punctuate labeling of actin and α-actinin. These effects were transient and upon prolonged incubation for 8 h or more in the presence of EGF, the normal, well organized patterns of actin and α-actinin were restored.

Epidermal growth factor (EGF) is a low molecular weight polypeptide hormone (6045 D) which enhances the proliferation of various cell types both in vivo and in vitro [5]. EGF binds to specific receptors in the plasma membrane and subsequently initiates diverse physiological processes. These include the activation of several membranous transport systems and certain cytoplasmic enzymes, as well as long-term effects on cell proliferation and differentiation [5]. Many of the recent studies on the mechanism of EGF activity were performed with human epidermoid carcinoma, A-431 [12, 13]. These cells have an unusually high number of EGF receptors on their surface ($2–3\times10^6$ receptors/cell) [10] and exhibit both short and long-term responses to the hormone.

One of the earliest events induced in A-431 by EGF are rapid morphological changes. Within less than 5 min following the addition of EGF to the cells extensive ruffling of the cell membrane and extension of filopodia were observed [6]. After longer incubation (additional 15 min) the ruffling activity decreases and the dorsal cell surface appears smooth in scanning electron micrographs. The cells retract from the surface of the tissue culture substrate, become more rounded and apparently pile up to form multilayered colonies [6]. The molecular mechanisms responsible for these dramatic effects of EGF are, however, still unclear.

In view of the notion that cell morphology and dynamics are largely maintained by an integrated action of the different cytoskeletal systems [20] we have examined the possibility that EGF induces rapid changes in the organization of cytoskeletal elements within responsive cells.

We have found that EGF induced striking changes in the distribution of actin and α-

actinin within A-431 cells. This was manifested by a transient decrease in organized microfilament bundles and the appearance of excessive diffuse labeling for actin and α-actinin at the cell periphery.

MATERIALS AND METHODS

Cell culture

Human epidermoid carcinoma cells (A-431) were kindly provided by Dr G. Todaro. These cells were grown on glass coverslips in 35 mm culture dishes in Dulbecco's modified Eagle's Medium (DMEM) containing 10% fetal calf serum (FCS) under an atmosphere of 10% CO_2 and 90% air.

Materials

EGF was purified from the submaxillary glands of adult male mice by the method of Savage & Cohen [21]. 7-Nitrobenz-2oxa-1,3-diazole-phallacidin (NBD-phallacidin) was used for the specific fluorescent labeling of actin [2]. The fluorescent phallacidin was a generous gift from L. Barak.

Rabbit antibodies to chicken gizzard α-actinin were prepared as described [11]. Rhodamine–lissamine sulfonyl chloride was coupled to goat antibodies against rabbit IgG according to Brandtzaeg [4], and the fraction containing 3–4 fluorophores per molecule was selected by DEAE cellulose chromatography [4]. All antibodies used here were purified on an immunoadsorbent of the proper antigen coupled to glutaraldehyde-activated Ultrogel AcA 22 [24].

Fluorescent labeling of cells

Cells were prepared for fluorescent labeling by two alternative procedures: (*a*) Cells were fixed with 3% paraformaldehyde and permeabilized with 0.1% Triton X-100 as previously described [11]. (*b*) Unfixed cells were exposed to detergent solution (50 mM NaCl, 200 mM sucrose, 3 mM $MgCl_2$, 10 mM Hepes, 0.5% Triton X-100, pH 7.4) for 2 min and then fixed as above.

The immunolabeling for α-actinin was performed as described [11]. For the labeling of actin, NBD-phallacidin (2 ng/ml) was incubated with the permeabilized cells for 20 min at room temperature [2].

The fluorescent preparations were observed with a Zeiss inverted microscope IM-35 equipped with filter sets for selective observation of NBD or fluorescein and rhodamine. Photographs were taken with Kodak film plus-X or Tri-X.

RESULTS

Cultured A-431 cells adhere to glass or plastic surfaces and grow in groups of flat, closely associated cells (fig. 1*a*). The addition of EGF to these cells induces drastic alterations in their morphology. These morphological changes are depicted in fig. 1. After 2 h in the presence of EGF a fraction of the cells showed extensive membrane ruffling (arrowheads in fig. 1*b*) and appearance of retraction fibers (fig. 1*b*). Most cells retract from the substrate and form multilayered colonies (fig. 1*c*, *d*). The distribution of actin after fixation and Triton permeabilization as determined by labeling with NBD-phallacidin is depicted in fig. 2. Actin in A-431 cells which were not exposed to EGF is primarily associated with fiber bundles oriented in different directions. These bundles often span the entire length of the cells (fig. 2*a*) and apparently terminate in areas of cell-to-cell (or cell-to-substrate) contacts. At the cell margins actin was organized in a belt array of circular and peripheral fiber bundles (fig. 2*b*). Whereas these actin fibers were observed predominantly close to the ventral cell surface, in more central areas of the cells polygonal arrays of actin filaments were often noticed (fig. 2*c*) in a pattern similar to that previously described for other cell types [17, 18].

The distribution of α-actinin in these cells was closely related to that of actin. The anti α-actinin antibodies labeled both stress-fiber-like bundles (fig. 3*a*) and the peripheral belt (fig. 3*b*) in a typical striated pattern. α-Actinin labeling was especially intense at the termini of bundles close to areas of focal contact with the substrate (fig. 3*a*, arrowheads) as verified by interference-reflection microscopy of the same cells (data not shown) or near cell–cell contacts (fig. 3*c*, arrowhead). In the latter case, intense α-actinin labeling was found in the two adjacent cells (fig. 3*c*). Often, intensely labeled dots were noticed, which apparent-

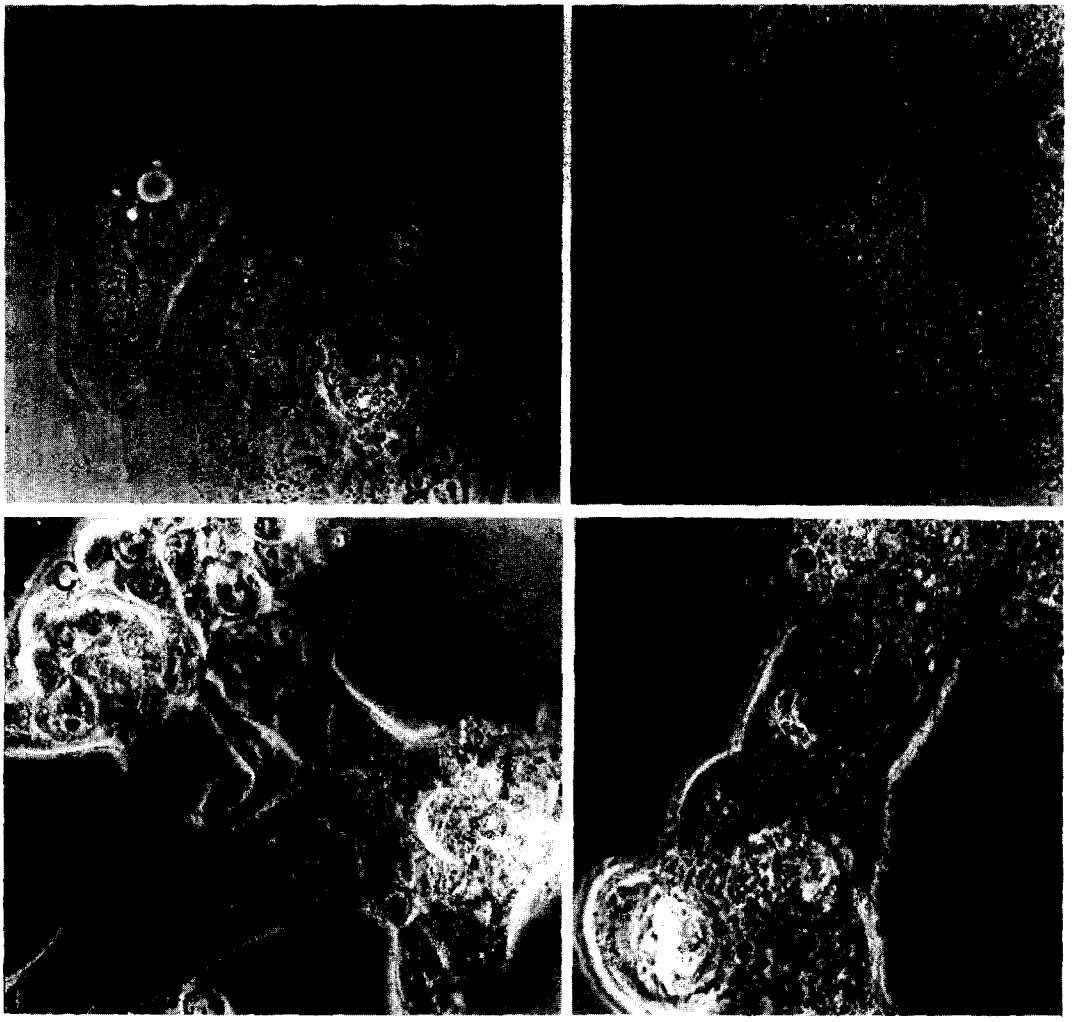

Fig. 1. Phase micrographs of cultured A-431 cells in the absence or presence of EGF. Cultured A-431 cells adhere to glass or plastic surfaces and grow in groups of flat, closely associated cells (*a*). Two hours after the addition of EGF a fraction of the cells show extensive membrane ruffling (see (*b*), arrowheads), while most of the cells retract from the substrate and form multilayered colonies (*c*, *d*). ×732.

ly corresponded to the vertices of the polygonal arrays of actin filaments [17, 18].

Upon addition of EGF to A-431 cells striking changes were observed in the organization of both actin and α-actinin. After 30–45 min of incubation with 100 ng/ml EGF most of the well organized actin bundles were no longer visible. Instead, intense diffuse labeling was found in the cytoplasm as well as in peripheral lamellipodium (fig. 4*a*) and ruffling membrane (fig. 4*b*). The disappearance of actin bundles was, however, not complete and some cables were still apparent close to areas of cell–cell contact (lower left area in fig. 4*a*). These changes were first apparent 30–45 min after addition of the hormone and the effect was maximal after 2 h incubation.

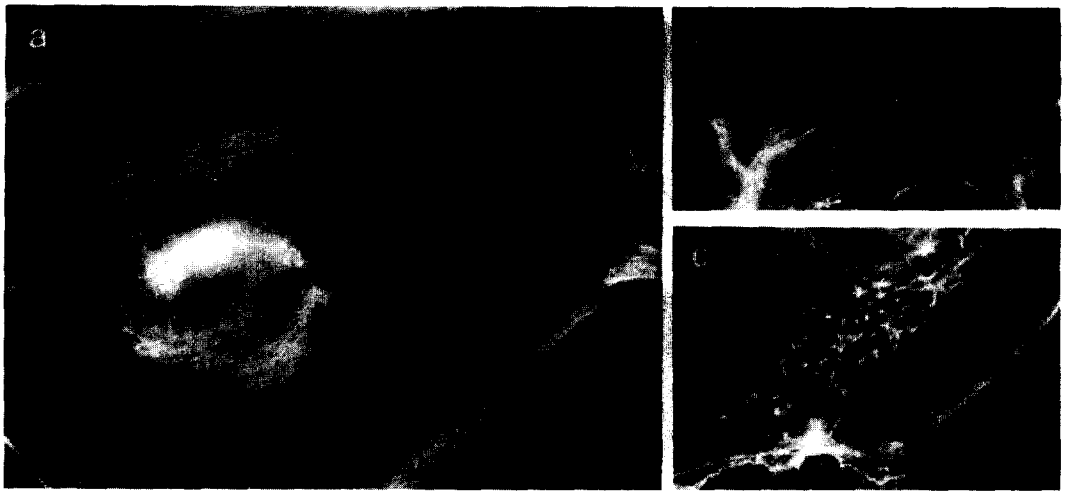

Fig. 2. Fluorescent labeling of cultured A-431 cells for F-actin with NBD-phallacidin. Actin in these cells is usually found in tightly organized fiber bundles. Those bundles, at the ventral cell portion are often long and span the entire length of the cells (*a*). At the cell margins actin bundles form a circular, peripheral belt (*b*), whereas in more central areas actin filaments form polygonal structures (*c*). ×732.

Similar changes were also observed in the distribution of α-actinin. Most of the labeling for this protein was no longer associated with well organized bundles but was rather diffuse or organized in the cells in numerous dots (fig. 4*c*, *e*) which did not form a defined pattern. Like actin, intense immunolabeling for α-actinin was associated with lamellipodium and with ruffling membranes (fig. 4*d*).

To evaluate the possibility that the apparent decrease in organization of actin- and α-actinin-containing structures result from excess diffuse label which obscures the filament bundles, we have extracted the cells briefly with detergent prior to fixation under conditions that were found to preserve cytoskeletal structures. The cells were then fixed and labeled for α-actinin or actin. In our experience such treatment resulted in a marked decrease in diffuse labeling for actin, α-actinin and vinculin.

Fig. 3. Indirect immunofluorescent labeling of A-431 cells for α-actinin. The labeling has a characteristic punctate appearance and is distributed along stress fibers (*a*). The immunolabeling is especially enriched at the termini of these fiber bundles, close to areas of cell-substrate contact (see (*a*), arrowheads). Similar labeling for α-actinin is associated with the peripheral fiber belt along the cell margins (*b*). α-Actinin labeling is also strong in sites of cell–cell contact, in dense cultures (see (*c*), arrowhead). ×732.

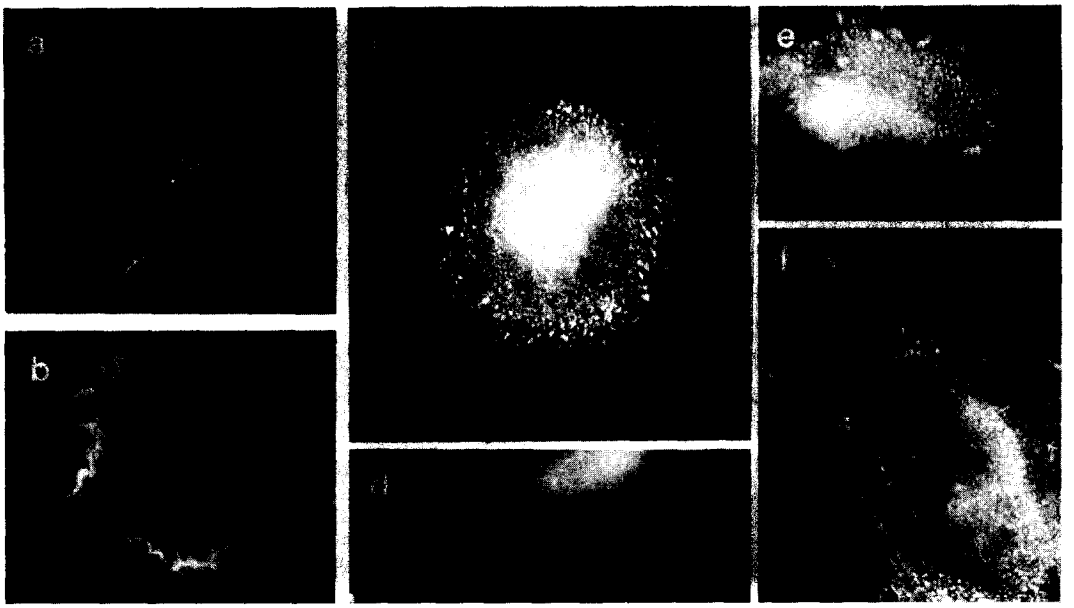

Fig. 4. The effect of EGF (100 ng/ml, 2 h of incubation) on the organization of actin (*a*, *b*) and α-actinin (*c*, *d*, *e*) in A-431 cells. Actin labeling becomes diffuse and fiber bundles are less apparent (*a*). Much label is associated with filopodia and ruffling membranes (*b*) at the cell periphery. α-Actinin in these EGF-treated cells is primarily associated with intensely labeled dots (*c*) and does not follow a defined pattern of organization. Considerable labeling is associated with ruffling membranes (*d*). The EGF-treated cells in (*e*) were first extracted with Triton X-100 before fixation (see Materials and Methods). The labeling patterns obtained by this technique are similar to those seen in prefixed cells (*c*). (*f*) Cells incubated for 8 h in the presence of EGF, immunolabeled for α-actinin. Note the restoration of α-actinin-containing filament bundles (compare with fig. 3). ×732.

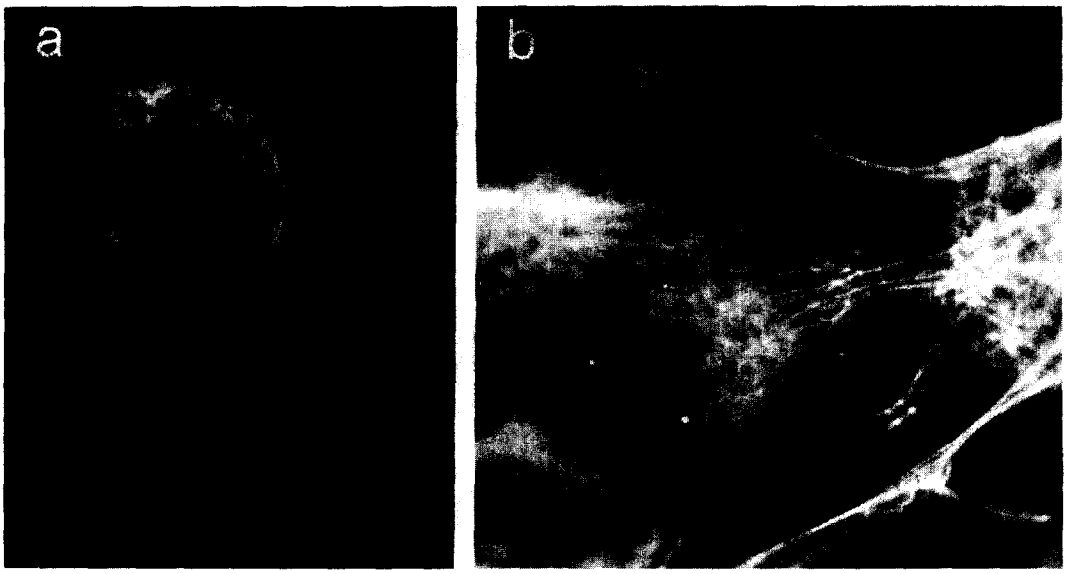

Fig. 5. Restored distribution of F-actin labeled with NBD-phallacidin after 8 h incubation with EGF. A-431 cells incubated for 3 h with EGF (*a*) or for 8 h with EGF (*b*). After 3 h most actin labeling is still diffuse. After 8 h in the presence of EGF, actin-containing filament bundles are observed. ×732.

The immunofluorescent labeling for α-actinin under those conditions was not altered, however (fig. 4*e*) and the well organized filament bundles found in cells in the absence of EGF were largely disaggregated after 2 h of exposure to the hormone, as described for fixed cells.

The effect of EGF on the actin-containing filament bundles was transient. As mentioned previously the effect was first observed 30–45 min after addition of EGF and reached maximum after about 2 h. Upon further incubation with EGF the cells gradually regained the normal pattern of actin and α-actinin organization. Fig. 4*f* shows the common distribution of α-actinin in A-431 cells and fig. 5*b* the distribution of actin in these cells after 8 h of incubation in the presence of EGF. Fully developed filament bundles are found within the cells in a pattern similar to that of untreated cells.

DISCUSSION

The addition of EGF to responsive cells induces a series of gross morphological changes in the cells. This includes membrane ruffling and extension of filopodia, increased macropinocytosis, retraction from the substrate and formation of multilayered colonies [6, 14]. It is not known at the present time whether the diverse effects of EGF on cell morphology result from a common primary process triggered by the hormone, nor is it known what the molecular mechanism of such triggering may be.

We have tested here the possibility that the effects of EGF on cell morphology are related to changes in the organization of the cytoskeleton, induced by the hormone. In a series of preliminary experiments we have screened the EGF effect on the organization of three major classes of cellular filaments, i.e. microtubules, intermediate filaments (prekeratin type) and microfilaments. The immunofluorescence labeling for microtubules and intermediate filaments, which is not shown here, did not indicate any significant change in the organization of these fiber types which could not be accounted for by the gross change in cell shape. However, the actin-associated microfilament system underwent striking changes, as visualized by labeling for both actin and α-actinin. These changes included the loss of tightly organized filament bundles and the simultaneous increase in diffuse labelling for these proteins, in the cytoplasm, in membrane ruffles and in retraction fibers. EGF effects on cell morphology and organization of the cytoskeleton are specific to this hormone. The addition of either 0.2 μg/ml insulin or 0.2 μg/ml nerve growth factor (NGF) to A-431 cells did not induce changes in cell morphology and in the organization of the microfilament system. Moreover, neither insulin nor EGF induces morphological or cytoskeletal changes in pheochromocytoma or in neuroblastoma cells.

The effect of EGF on the microfilament system is transient. It starts after incubation of the cells for 30–45 min in the presence of the hormone, reaches maximum effect after 2 h and then declines and after about 8 h of incubation the effect is no longer apparent. Thus, most of the changes in actin and α-actinin organization were seen after the initiation of extensive ruffling and macropinocytosis induced by the hormone. It is, however, still possible that even the most early effects on membrane activity result from rapid subtle changes in cytoskeletal interactions which cannot be resolved by the techniques used here.

We do not know yet the mechanism of recovery of the microfilament system from the EGF effect, which is found after pro-

longed incubation of A-431 cells with the hormone. At that time, however, more than half of the EGF receptors are lost from the cell surface due to EGF-induced down regulation [26]. This temporal relationship suggests that the effects described here require the occupation of all or most available receptors.

To date we do not know the biochemical nature of these EGF-induced structural changes in the cells. One possible mechanism is based on the observation that purified membranes from A-431 cells undergo EGF-induced autophosphorylation [16]. Therefore, it is possible that EGF induces phosphorylation of certain cytoskeletal elements which gives rise to the observed morphological changes.

In qualitative terms, the effect of EGF on the actin-containing system is somewhat similar to the effect of several oncogenic viruses on these structures. Avian sarcoma viruses, for example, induce rapid membrane ruffling and cell rounding, accompanied by a marked disorganization of actin filament bundles [1, 9, 19]. It was recently shown that the transforming gene product is a membrane-bound protein kinase [7, 15], which can phosphorylate membrane and possibly cytoskeletal components [8]. A protein kinase activity was recently reported also for Abelson Leukaemia Virus [25]. Interestingly, murine sarcoma and Abelson Leukaemia viruses cause a considerable decrease in the amount of available EGF receptors in responsive cells [3, 22, 23]. These findings raise the possibility that the EGF response and transformation-specific effects may operate via a related or even common mechanism.

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