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# Scanning Electron Microscopy of Glucocorticoid-Treated Hepatocytes and Hepatoma Cells in Culture (38964)

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We have recently described a line of rat liver cells derived from a Morris hepatoma whose growth is powerfully inhibited in the presence of low concentrations of glucocorticoid hormone (1). Inhibition of radioactive thymidine incorporation is accompanied by a corresponding suppression of cell proliferation, is unattended by a comparable inhibition of RNA synthesis, and is rapidly reversible upon removal of hormone.

In the course of these experiments we have observed that the glucocorticoid-induced inhibition of cell proliferation is accompanied by a pronounced change in cell morphology as observed by phase microscopy. Addition of either hydrocortisone or dexamethasone at concentrations as low as $3 \times 10^{-7}$ *M* and $3 \times 10^{-8}$ *M*, respectively, results within 24 hr in a flattening of the cells, a rounding of their borders, and a tendency to form monolayers rather than to pile up. At the same time the cells become noticeably more resistant to removal from plastic Falcon dishes by treatment with trypsin.

The preceding observations, made on cells growing in Petri dishes, are qualitatively similar to those made earlier on hepatoma cells in suspension culture by Ballard and Tomkins (2, 3), who found that exposure to glucocorticoid hormone resulted in a striking increase in cell adhesiveness and resistance to mechanical dislodgement from glass surfaces. This increased adherance could be prevented by inhibitors of either RNA or protein synthesis, and the authors concluded on the basis of additional observations that glucocorticoid hormone induced the synthesis of a characteristic surface protein which was responsible for the property of increased adhesiveness.

On the basis of these observations, and in view of a possible relation between changes in cell-to-substratum adhesiveness and changes in cell proliferative rate, it was of interest to examine a line of hepatoma cells whose proliferation is known to be suppressible by glucocorticoid hormone for possible accompanying surface changes. In the present study we have employed scanning electron microscopy to examine both a line of diploid cells derived from normal adult rat liver and a line of markedly anaplastic aneuploid cells derived from a Morris hepatoma. While hydrocortisone is shown to induce surface changes in both cell lines, by far the most dramatic changes occur in the less differentiated line, where the presence of glucocorticoid results not only in a suppression of cell division but in a (phenotypic) reversion of the surface morphology to one closely resembling that of the normal diploid cells.

*Materials and Methods.* The cell lines employed were a line of adult rat liver epithelial cells and a line of epithelioid cells cloned from a Morris hepatoma (No. 5123) (4). Line 1, derived from an adult Buffalo rat, has a well-differentiated morphology and a diploid complement of chromosomes closely resembling those in cells of primary rat embryo cultures (5). Cells in line 2 are anaplastic and aneuploid, and are capable of producing solid tumors when transplanted into adult Buffalo rats (6). All components of the tissue culture medium were obtained from the Grand Island Biological Co. Hydrocortisone was purchased from the Mann Research Co., and [methyl-$^3$H]thymidine (20

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Ci/mmole) from the New England Nuclear Corp.

*Maintenance and harvesting of cells; addition of hormone and radioactive thymidine to cultures.* Cell cultures were maintained as previously described (1). At the start of each experiment 60-mm plastic Falcon Petri dishes, some of which contained 12-mm (diameter) flat circular glass cover slips (Kimble, Exax no. 1, previously dipped in 70% ethanol and flame-dried), were seeded with $3 \times 10^5$ cells in a volume of 5 ml of medium. Initial plating was performed at a lower cell density (approx 100 cells/mm$^2$) than in earlier studies (approx 450 cells/mm$^2$) (1) in order to avoid confluent cell growth. A period of 20 hr was allowed to elapse between seeding and the addition of hormone, at which time ($t = 0$) either hydrocortisone in ethanol (final concentration 50 $\mu M$) or ethanol alone was added as previously described (1). In order to assess incorporation of radioactive thymidine into DNA, 5 $\mu$Ci of [$^3$H]thymidine was added to individual plates in 50 $\mu$l of 0.15 $M$ NaCl, and the cells were harvested 3 hr later as previously described (1). Because of the low cell densities employed in these experiments the initial perchloric acid precipitation of cell proteins and polynucleotides was carried out in the presence of 2 mg of bovine serum albumin added as "carrier."

*Microscopy.* The cover slips supporting the various control and experimental cell cultures were rinsed briefly with isotonic NaCl solutions at 37° and then immersed in a 3% solution of glutaraldehyde adjusted to pH 7.0 with a cacodylate buffer. After fixing for 1.5 hr the specimens were washed in distilled water and postfixed for 30 min in 1% $OsO_4$ also buffered with cacodylate. The specimens were then rinsed in distilled water and placed in a helical cover slip holder while immersed in 10% ethanol. Dehydration was carried out in increasing concentrations of ethanol, the last being three changes of absolute ethanol. The specimen holder containing the cover slips was then rapidly transferred into the chamber of a Sorvall critical-point dryer which had been filled with absolute ethanol. Liquid $CO_2$ was used as the final solvent-replacement step. The specimens, after drying, were coated with a thin layer of gold–palladium (60:40) in a Denton evaporator equipped with a rotating, wobbling stage. Heat artifacts were produced during this step in some early experiments; these were subsequently avoided by increasing the filament-to-stage distance to 12 cm or more. After coating, the specimens were examined and photographed in a Cambridge Mark II scanning electron microscope using an accelerating voltage of 20 kV.

*Results. Morris hepatoma: A. Control cultures.* Twenty hours after initial plating (*i.e.*, at $t = 0$) the majority of the hepatoma cells were observed to be well spread and well separated, and they exhibited stubby microvilli most numerous over the nuclear hump and less so along the periphery away from the nucleus. The shape of the cells was in general irregular, and the number of spherical cells was small. *Twenty-four hours later* (at $t = 24$) the density of the cells had approximately doubled, and although the cells continued to be well separated, their morphology had changed. Spherical and cylindrical forms were now considerably more numerous than at $t = 0$, and the cells continued to exhibit prominent microvilli. *By $t = 48$* the cell density had undergone another approximate doubling, and by this time rounded and cylindrical cells showed a marked predominance over flattened cells. Cell pairs were common, and the spherical and cylindrical forms were covered with numerous long microvilli and demonstrated extended filopodia. Microvilli numerously dotted the entire surface of the cells, and were present even along the cytoplasmic processes (Figs. 1a and 2a).

*B. Hydrocortisone-treated cultures.* The addition of hydrocortisone at $t = 0$ resulted in striking morphologic changes which were evident within 24 hr and which were accompanied by a 44% suppression of cell proliferation (Table I). At $t = 24$, in the presence of hormone, the cells had assumed a much more flattened form, and rounded forms such as were seen in the control cultures at this time were rare. Most of the cells were in fact so closely applied to the glass surface that their marginal cytoplasmic "skirt" was

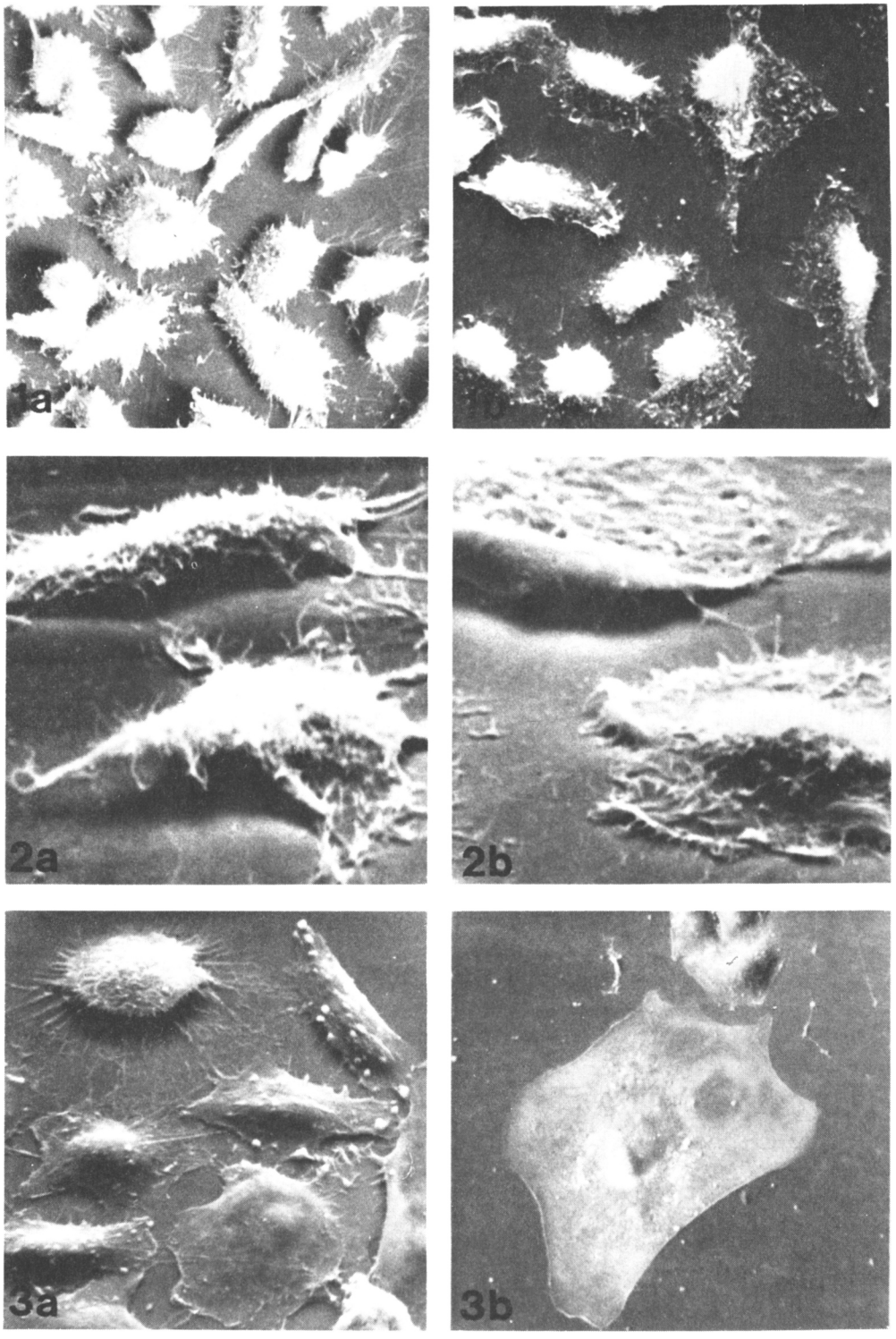


FIG. 1a. Control hepatoma cells ($t$ = 48) showing spherical and cylindrical forms densely covered with microvilli; some cells are in postmitotic pairs. 30° tilt. 680×. (Legend continued at foot of next page.)

TABLE I. [$^3$H]THYMIDINE INCORPORATION BY TWO CELL LINES AFTER EXPOSURE TO HYDROCORTISONE.

| Cell line | Time ($t$) (hr) | Incorporation of [$^3$H]thymidine into DNA (dpm/$\mu$g $\times$ $10^{-4}$; mean $\pm$ 1 SE) | | Percentage inhibition by hydrocortisone |
|---|---|---|---|---|
| | | Control | Hydrocortisone | |
| H5 | 24 | 9.9 $\pm$ 0.1 | 5.6 $\pm$ 0.6 | 44 |
| H5 | 48 | 16.0 $\pm$ 1.2 | 4.2 $\pm$ 0.4 | 74 |
| RLB | 24 | 18.0 $\pm$ 0.4 | 21.0 $\pm$ 4.4 | — |
| RLB | 48 | 20.0 $\pm$ 1.8 | 13.0 $\pm$ 0.6 | 35 |

much attenuated, and the nuclear hump as a consequence became prominent. There was a reduced number of microvilli over the marginal cytoplasm, while the nuclear hump remained densely covered. In addition, many cells had begun to exhibit marked ruffle formation (in some instances as many as four ruffles per cell); these ruffles frequently extended along more than one edge, and at times lined as much as 25% of the entire cell margin. At $t$ = 48 hr, by which time the inhibition of thymidine incorporation had increased to 74%, the morphological effects had become even more pronounced (Figs. 1b and 2b). In comparison with the "skirted" cells seen at $t$ = 24, the skirts present at $t$ = 48 were even larger, and the nuclear humps were so prominent that they resembled the yolks of fried eggs. The number of microvilli was further reduced, giving the cells a naked appearance, while the degree of ruffling remained about the same.

*RLB hepatocytes.* The RLB hepatocytes in control cultures grew in greater apposition to the underlying glass surface than the hepatoma cells, and even in the absence of hormone there was a relative preponderance of flattened forms showing well-developed cytoplasmic skirts and nuclear humps (Fig. 3a). In contrast to the striking effects of hydrocortisone on the hepatoma cells, addition of hormone produced only minimal changes in the surface morphology of the RLB cells, and its inhibitory effect on thymidine incorporation was considerably less impressive (see Table I). While prolonged exposure to hydrocortisone did result in some decrease in the number of rounded forms and in an increase in the diameter of the marginal cytoplasm associated with this flattening (Fig. 3b), the changes were not dramatic.

*Discussion.* The present report describes the effects of hydrocortisone on the surface morphology of two cell lines of liver origin as revealed by scanning electron microscopy. One of these cell lines, a diploid line derived from adult rat liver, showed only minimal morphologic changes in response to hydrocortisone, while the other, an aneuploid and markedly anaplastic line derived from a Morris hepatoma, exhibited striking changes. Addition of hydrocortisone to the growth medium caused the hepatoma cells, normally rounded and densely covered with surface microvilli, to become impressively flattened, to undergo a marked loss of surface microvilli, to exhibit prominent ruffle formation, and, by virtue of these changes, to assume a surface morphology closely resembling that of the (untreated) diploid cell line derived from normal liver. In addition to these effects on cell shape and surface morphology, observed in the present study at low

FIG. 1b. Hydrocortisone-treated hepatoma cells ($t$ = 48). Note the attenuation of the marginal cytoplasm and the decreased number of microvilli away from the nucleus. 30° tilt. 700×.

FIG. 2a. Higher power of control hepatoma cells ($t$ = 48) showing cylindrical morphology and selective cell attachments to the substratum. 60° tilt. 3200×.

FIG. 2b. Higher power of hydrocortisone-treated hepatoma cells ($t$ = 48) showing again the attenuation of the marginal cytoplasm resulting in an increased surface area in apposition to the substratum; note the ruffles. 60° tilt. 2900×.

FIG. 3a. Control RLB hepatocytes ($t$ = 48). Even in the absence of hydrocortisone the cytoplasm of these cells is attenuated and microvilli are sparse. 25° tilt. 1300×.

FIG. 3b. Hydrocortisone-treated hepatocytes ($t$ = 48). Exposure of these cells to hydrocortisone results in few morphological changes except for a moderate increase in cell diameter and a possible further reduction in microvilli. 25° tilt. 630×.

cell density, we have found that addition of hydrocortisone to cultures of hepatoma cells plated at higher cell densities results in an increased resistance of these cells to detachment by trypsinization and a tendency of the cells to form flagstone-like colonies rather than to pile up. While the functional significance of these changes is at present unclear, it is of interest that all of these glucocorticoid-induced changes mimic a (phenotypic) reversion of both surface and growth characteristics toward those observed in the "normal" cell line in the absence of hormone.

The mechanisms underlying these hormone-induced changes are unclear, but it is of note that both the morphological modifications and the inhibition of cell proliferation described here resemble reversible changes known to be inducible by various other means. Thus the addition of cyclic AMP (cAMP) or its butyryl derivatives to cultures of sarcoma cells (7), to virally or chemically transformed fibroblasts (7, 8), or to Chinese hamster ovary cells (9–11) have been shown to result in extensive changes: in addition to modification of the surface morphology [including in the instance of L929 cells an early and extensive loss of surface microvilli after exposure in culture to either dibutyryl cAMP or methylisobutylxanthine (12)], these have variously included a flattening of the cells (8), increased adhesion to the substratum (11, 13), and either a fall in growth rate with a decreased tendency to pile up (7) or the establishment of actual contact inhibition (9, 10). More recently Tomkins and his co-workers have made similar observations on 3T3 cells under conditions of serum deprivation (14) and the associated rise in cAMP (15). While it is possible that the surface changes and the inhibition of proliferation in all three of these systems is mediated by a final common path, it is not yet known whether the glucocorticoid-induced inhibition of growth in the present system [similarly known to be rapidly and fully reversible (1)] is itself accompanied by a rise in intracellular cAMP.

The relation, if any, between glucocorticoid-induced changes in surface morphology and changes in growth rate requires further elucidation. The possibility that the hydrocortisone-induced flattening of the cells is itself secondary to the observed inhibition of proliferation can, however, be effectively excluded for two reasons: Extensive flattening in response to glucocorticoid occurs considerably earlier than the antiproliferative effects [the latter require a minimum of 12 hr to become manifested (1)], and, perhaps more importantly, glucocorticoid-induced flattening and increased adherance to the substratum are prominent effects of glucocorticoid exposure even in lines of hepatoma cells that respond minimally (16), if at all, to glucocorticoid with a suppression of cell division. Whether, conversely, glucocorticoid-induced changes in surface morphology mediate some of the antiproliferative effects of these hormones is considerably less clear, but even at the present time several observations permit at least a partial dissociation between the changes in growth characteristics and the changes in surface morphology. First, the surface changes reported here are already extensive at a time (24 hr) when proliferation is only partially inhibited; second, (as noted above) similar surface changes have very recently been reported in a line of transformed liver cells whose proliferation is considerably less susceptible to suppression by glucocorticoid than that of the hepatoma cells described here (16); finally, while the morphological effects on the cell surface are readily demonstrable at all cell densities, recent observations in our laboratory have shown that the suppression of cell proliferation, in contrast to the changes in surface morphology, increases markedly with increasing cell density (compare, for example, the degree of suppression in ref. (1) with that noted in the present study). While the present observations suggest a relation between glucocorticoid-induced surface changes and the resumption of more "normal" growth characteristics, it would seem appropriate to defer speculation about possible mechanisms until additional experiments have been completed.

*Summary.* The morphological effects of exposure to hydrocortisone have been examined in two cell lines of liver origin by scanning electon microscopy. In one of these, an aneuploid line derived from a Morris hepatoma, the presence of hormone results not

only in a suppression of cell proliferation, but in a marked flattening of the cells and loss of surface microvilli; in the other cell line, a diploid line derived from adult rat liver, the suppression of cell division is less marked, and the morphological effects of the hormone are far less striking. While the suppression of cell division in both of these cell lines is known to be rapidly reversible upon the removal of hormone, the presence of hormone causes the hepatoma cells to assume both monolayer growth characteristics and a morphology resembling those of cells derived from normal liver.

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