Glucocorticoids have pleiotropic effects on small intestinal crypt cells

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Quaroni, Andrea, Jean Q. Tian, Michael Göke, and Daniel K. Podolsky. Glucocorticoids have pleiotropic effects on small intestinal crypt cells. Am. J. Physiol. 277 (Gastrointest. Liver Physiol. 40): G1027–G1040, 1999.—Glucocorticoids have long been known to accelerate maturation of the intestinal tract, but the molecular mechanisms that account for their physiological function in the epithelium remain poorly characterized. Using rat intestinal epithelial cell lines (IEC-6, IEC-17, and IEC-18) as models, we have characterized glucocorticoid receptors in crypt cells and documented striking morphological, ultrastructural, and functional alterations induced by these hormones in intestinal cells. They include arrest of growth, formation of tight junctions, appearance of long, slender microvilli, reorganization of the endoplasmic reticulum and trans-Golgi network, and downregulation of the cell cycle regulatory proteins cyclin-dependent kinase 6 and p27Kip1. These effects are consistent with the activation or modulation of multiple genes important in the physiological function of absorptive villous cells but are probably not directly involved in the induction of cell differentiation.

MATERIALS AND METHODS

Materials

DMEM, without or with 25 mM HEPES buffer, fetal bovine serum (irradiated), penicillin-streptomycin mixture, and trypsin (2.5% in Hanks’ balanced salt solution without calcium and magnesium) were obtained from M.A. Bioproducts (Walkersville, MD). Insulin (Iletin II, 100 U/ml) was from Eli Lilly (Indianapolis, IN). Hydrocortisone (Δ4-pregnen-11β,17α,21-triol-3,20-dione), dexamethasone (9α-fluoro-16α-methylpred-
nisolone), 17α-methyltestosterone (17α-methyl Δ4-androsten-
17β-ol-3-one), progesterone (Δ4-pregnen-3,20-dione), cortisone
(Δ4-pregnene-17α,21-diol-3,11,20-trione), β-estradiol
[Δ1,3,5(10)], estradiene-3,17β-diol), androsterone
(5α-androstan-3α-ol-
17-one), testosterone (Δ4-androsten-17β-ol-3-one), tetra-
hydrocortisol (5β-pregnane-3α,11β,17α,21-tetrol-20-one), and
Δ-d-alosterone (Δ4-pregnene-18-al-3β,21-diol-3,20-dione) were
obtained from Sigma Chemical (St. Louis, MO), [methyl-
3H]thymidine (20 Ci/mmol; sterile aqueous solution), [1,2,6,7-
3H(N)]cortisol (80.6 Ci/mmol), [1,2,4-3H(N)]dexamethasone,
[17α-methyl]-methyltestosterone (17α-G1028 EFFECTS OF GLUCOCORTICOIDS ON INTESTINAL CELLS

Antibodies

The antibodies used for Western blotting analysis of cyclins, cyclin-dependent kinases (Cdks), and cyclin-dependent kinase inhibitors (CKIs) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); anti-p21 (sc-397), anti-p27 (sc-528), anti-Cdk2 (sc-163), anti-Cdk4 (sc-601), anti-Cdk6 (sc-177), anti-cyclin D1 (sc-6281), anti-cyclin D3 (sc-182), and anti-p38 (sc-50). The secondary antibodies against mouse and rabbit Ig, conjugated with horseradish peroxidase, were purchased from Amersham Life Science (Arlington Heights, IL). The antigen-antibody complexes were visualized by enhanced chemiluminescence (ECL) and autoradiography, with ECL Plus Detection Kit (Amersham Life Science). The primary antibodies used for Western blotting analysis of cyc-

Cell Culture and General Methods

The intestinal epithelial cell lines used in this study, all
established from newborn rats, and their standard culture
conditions were described previously. IEC-6 cells were
derived from the entire small intestine (50), and IEC-17 cells
were from the proximal small intestine (50), and IEC-18 cells
were from the ileum (50). In this study, these cells were used
between the 8th and 15th passage. The complete medium
used to culture these cells was composed of DMEM
Bioactive Transforming growth factor-
Latent and active forms of TGF-
radioactivity was counted with a Beckman gamma counter.
The cell layer from each dish was dissolved in 2.0 ml of 0.1 N
NaOH, and aliquots from each sample were used for determi-
ation of the 32Cr remaining in the cell layer. 2) The second
toxicity test was clonal assay. Cells were seeded in 100-mm
dishes at 2 × 105 cells/dishes, allowed to recover for 24 h,
and then treated with standard complete medium (controls) or
test medium for 24 h. Subsequently, the medium was re-
moved and the dishes were washed three times with complete
medium. Culture was continued in standard complete me-
dium for 2 wk, at which time the cells were fixed with
methanol and stained with Giemsa. Colonies of at least 10
cells were counted. The survival fraction of cells treated with
standard complete medium (controls) or test medium was
calculated relative to the plating efficiency of control cultures.

Radioimmunobinding assay. Binding of MABs to monolay-
ers of IEC cells was performed as previously described for
caco-2 cells (6).

Transepithelial electrical resistance measurements. Transepithelial electrical resistance measurements were performed with cells cultured on membranes without addition of exogenous extracellular matrix as described (18).

Transforming growth factor-β determination. Latent and
bioactive transforming growth factor-β (TGF-β) concentra-
tions were determined in 2-day-old conditioned medium as
described previously (19).

DNA synthesis. DNA synthesis was determined by measur-
ing the incorporation of [methyl-3H]thymidine into cellular
DNA. Cells grown in 60-mm dishes were treated as required
by each individual experiment; the medium was then re-
moved, the cells were washed twice with standard complete
medium, and then fresh medium containing 0.5 µCi/ml
[methyl-3H]thymidine was added. After an incubation (at
37°C in an incubator), the duration of which depended on the
purpose of the experiment (usually 1–4 h), the medium was
aspirated and each dish was sequentially washed twice with
PBS and treated twice with cold 5% TCA (10 min each time).
The cells were then dissolved in 1 ml of 0.1 N NaOH,
and aliquots of the solution were used for determination of

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radioactivity incorporated (by scintillation counting) and of the protein, by the method of Lowry et al. (37). Autoradiography of labeled cells was performed as follows: after incubation with [3H]thymidine, cell monolayers were washed twice with cold PBS, treated twice with cold 10% formalin in saline, re-washed twice with cold PBS, and then treated twice with cold 5% TCA. Finally, the cells were washed twice with cold water, treated twice with absolute ethanol, air dried for 24 h, and then overlayed with NBT2 nuclear track emulsion (Eastman Kodak). They were then left 3–4 h at room temperature in photographic dark boxes containing calcium sulfate and then stored at 4°C. Dishes were developed after a total of 8–72 h of exposure (depending on the purpose of the experiment) in Kodak D-19 developer.

Western blotting. Cells were washed twice in cold PBS, scraped into PBS containing 1 mM phenylmethylsulfonyl fluoride, and centrifuged at 2,500 rpm (1,600 g) at 4°C for 15 min. The supernatant was removed, and the pellet was resuspended in 1× SDS-sample buffer (46). The DNA concentration was determined in these cell lysates using the Hoechst 33258 DNA assay and a minifluorometer from Hoefer (Pharmacia Biotech, Piscataway, NJ). Samples applied to each gel were normalized to the same amount of DNA. For SDS-PAGE and Western blotting (46) of cyclins, CdkS and CKIs, lysates obtained from 2×10⁶ cells/sample were subjected to SDS-PAGE on a 12% acrylamide gel. The proteins were transferred onto a nitrocellulose membrane (Hybond nitrocellulose, Amersham Life Science) using a transblot system (Bio-Rad, Hercules, CA) at 100 V, 5°C for 90 min. The membranes were then blocked in 80 mM Na₂HPO₄, 20 mM NaH₂PO₄, 2H₂O, 100 mM NaCl, and 0.1% Tween 20 containing 3% BSA at 4°C overnight. The enhanced chemiluminescence Western blotting protocol from Amersham Life Science was used for performing incubation with antibodies, washing, protein detection, antibody stripping, and reprobing.

Immunofluorescence staining. Cultured cells were washed three times with PBS, fixed with 3% formaldehyde, and then either directly processed for immunofluorescence staining (nonpermeabilized samples) or permeabilized by one of two methods: 1) incubation with acetone-methanol (1:1) at −20°C for 10 min or 2) lysis with 0.2% Triton X-100 in PBS for 2 min at room temperature. Staining for tubulin with MAB KMX-1 and further processing of the samples were as described previously (46). When appropriate, cells were counterstained with 0.01% Evans blue for 2 min. After antibodies were incubated and washed, the cells were mounted in glycerol-PBS (9:1) plus 2.5% 1,4-diazabicyclo-[2.2.2]-octane and covered with coverslips. Staining for actin was achieved using rhodamine-conjugated phalloidin (Molecular Probes, Eugene, OR) as previously described (45). Cells were examined with a Zeiss Axiosvert 10 microscope coupled with a Bio-Rad MRC-600 confocal laser scanning attachment: fluorescent images were saved to an optical disk and subsequently transferred to Kodak Tmax 100 Pro black and white film.

Steroid binding assays. Steroid binding assays were performed following published techniques with minor modifications (35, 32, 42, 59) as outlined below.

BINDING TO INTACT CELLS. Because steroid binding proteins are known to be present in serum and because a small fraction of these proteins could remain bound to washed IEC cells and produce artifactual results when specific hydrocortisone binding is measured, binding of labeled dexamethasone, a synthetic steroid that does not bind to transcortin (35), was also studied. Monolayer cultures of IEC cells (in standard complete medium or preincubated for 24 h in serum-free medium) in 60-mm dishes, at different cell densities, were rinsed twice with serum-free DMEM containing 25 mM HEPES (pH 7.4) and then incubated with 1 ml of the same medium containing radioactive steroid ([3H]hydrocortisone or [3H]dexamethasone) at different concentrations for 1 h at 37°C. A set of cultures was incubated in parallel with medium containing the same amounts of labeled steroid plus a 100-fold concentration of the same, unlabeled steroid to correct for nonspecific binding. At the end of the incubation, the medium was aspirated, and the cell layers were washed three times with ice-cold PBS and then dissolved in 1 ml of 0.1 N NaOH in 50% ethanol (vol/vol). The solutions were quantitatively transferred to liquid scintillation vials, neutralized, and counted with 10 ml of Acquasol (NEN). Aliquots of the original solutions of labeled steroid in DMEM were also counted to determine the exact concentrations of steroid used. Specifically bound steroid was defined as the difference between cell-associated radioactivity in the dishes incubated with radioactive steroid alone and those incubated with an equal concentration of radioactive steroid plus excess unlabeled steroid. Parallel cultures were used for determining cell number per dish and total cellular protein. Binding sites per cell and equilibrium dissociation constants were obtained from Scatchard analyses (61) of binding data. Least-square regression was used to draw the best straight line to fit the data.

CYTOPLASMIC BINDING ASSAY. Monolayer cultures of IEC cells (in standard complete medium or preincubated for 24 h in serum-free medium) in 100-mm dishes at different cell densities were rinsed twice with serum-free DMEM and then suspended into 5 ml of ice-cold Tris-EDTA buffer (0.1 M Tris-0.0015 M EDTA, pH 8.0) and spun (at 200 g for 5 min). The supernatant was removed, and 1.5 ml of Tris-EDTA buffer were added to the pellets, which were then homogenized with three 5-s bursts from a Polytron (Brinkman Instruments) at setting 6 at 4°C. All subsequent procedures were performed at 4°C. The homogenates were centrifuged in an Eppendorf Microfuge (at 12,500 g) for 10 min, and the supernatants (cytosols) were divided into 100-µl aliquots in glass tubes. Labeled steroids at various final concentrations were added to the cytosols, followed by incubation for 2 h at 4°C. Preliminary experiments had shown that this time was sufficient to obtain near-maximum hydrocortisone and dexamethasone binding to IEC-6 cell cytosol. After this incubation, 200 µl of dextran-coated charcoal (1% Norit A and 0.1% dextran, molecular weight of 500,000 (Sigma), in 10 mM Tris and 1.5 mM MgCl₂, pH 7.6) were added to each sample. The tubes were vortexed, left to stand for 5 min, vortexed again, and then spun at 12,500 g for 3 min. Aliquots of the supernatants were counted in 10 ml of Aquasol. Triplicate samples of the original labeled steroid solutions used for incubation with cytosol were also counted to determine the exact steroid concentrations. Binding sites per milligram of cytosol protein and equilibrium dissociation constants were obtained from Scatchard analyses (61) of binding data.

DISTRIBUTION OF STEROID BINDING IN CYTOSOL AND NUCLEAR FRACTIONS OF IEC CELLS. Monolayer cultures (in standard complete medium or preincubated for 24 h in serum-free medium) in 100-mm dishes were rinsed twice with serum-free DMEM containing 25 mM HEPES. Next, they were incubated with the same medium containing 200 nM [3H]hydrocortisone in the absence or presence of 20 µM unlabeled steroid at 37°C for different periods of time (5–120 min). At the end of each incubation, the medium was removed, and the cells were washed three times with ice-cold PBS, suspended in 2 ml of hypotonic buffer (10 mM Tris-HCl, pH 8.0, 1 mM MgCl₂), and lysed by freeze-thawing. The lysates were separated into crude nuclear pellets and crude cytosol supernatants by centrifugation (200 g for 10 min at 4°C). Aliquots of the supernatants were used to determine specific [3H]hydrocorti-
for determination of specifically bound steroid (by scintillation counting) and protein content in the nuclear fractions.

RESULTS

Glucocorticoid Receptors in IEC Cells

Cellular binding sites for hydrocortisone and dexamethasone had essentially identical characteristics in all IEC cell lines studied (IEC-6, IEC-17, IEC-18); thus only the results obtained with IEC-6 cells will be documented here in detail. A representative binding curve, shown in Fig. 1a, illustrates the presence of high-affinity, limited capacity, hydrocortisone binding sites in IEC-6 cells that are ~50% confluent. Scatchard analysis of these data (Fig. 1b) is consistent with hydrocortisone binding to a single class of high-affinity receptors. Binding sites with characteristics similar or identical to those demonstrated in intact cells were also observed in cytosolic cellular fractions obtained from subconfluent or confluent IEC-6 cells (data not shown). Table 1 presents cumulative data obtained from several similar experiments in which the specific binding of [3H]hydrocortisone or [3H]dexamethasone was determined in confluent, about half-confluent, or low-density cultures. The number of receptor sites per cell varied with cell density, being about four times greater in low-density cultures compared with confluent cultures, but, in all cases, the Scatchard plots were linear, implying the presence of a single class of high-affinity binding sites. The dissociation constants were also very similar or identical at different cell densities. As expected, dexamethasone showed greater affinity than hydrocortisone, but the number of binding sites per cell for the two hormones (at each cell density) was not significantly different.

Fractionation of IEC-6 cells into cytosolic and nuclear fractions at different times after administration of labeled hydrocortisone provided evidence for nuclear binding of activated cytosolic receptors. Maximum total cellular binding was observed 20–30 min after addition of the hormone to the culture medium. The fraction of [3H]hydrocortisone present in the cytosol reached a peak at ~20 min, rapidly declining at later times. In contrast, total radioactivity in the nuclear fraction was constant.

Table 1. Characteristics of cytosolic glucocorticoid receptors in IEC-6 cells

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Cell Density, 10^4 cells/cm^2</th>
<th>Receptors/Cell</th>
<th>K_d, M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrocortisone</td>
<td>6.11 ± 1.1</td>
<td>2.8 ± 0.5 x 10^4</td>
<td>8.6 ± 0.1 x 10^{-8}</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>6.11 ± 1.1</td>
<td>3.62 ± 0.5</td>
<td>8.4 ± 0.2 x 10^{-8}</td>
</tr>
</tbody>
</table>

Values are means ± SE of 4 independent determinations. Specific hydrocortisone or dexamethasone binding to intact IEC-6 cells, cultured in 60-mm dishes and examined at the indicated cell density, was determined as described in MATERIALS AND METHODS. The number of receptors/cell and the dissociation constants (K_d) were derived from Scatchard plots of the binding data. Least-square regression was used to draw the best straight lines to fit the data.
negligible during the first 20 min but subsequently increased rapidly in correspondence to the decline observed in the cytosol.

Different steroids were evaluated for their ability to compete with [3H]hydrocortisone for binding to intact IEC-6 cells. In these experiments, a fixed concentration (150 nM) of [3H]hydrocortisone was added to the culture medium, alone or in combination with variable amounts of unlabeled test steroids. The results obtained with half-confluent IEC-6 cells are illustrated in Fig. 1c. The main implications of these results are as follows: 1) the OH at C-11, which is essential for glucocorticoid activity, proved important for binding inhibition because cortisone (with C=0 at C-11) had very limited activity, 2) substitution of —CH₂OH (in hydrocortisone) for —CH₃ (in progesterone) slightly increased inhibitory potency, 3) the presence of oxygen functions at C-21 but the absence of a hydroxyl group at C-17 (resulting in mineral-corticoid activity, e.g., D-aldosterone) resulted in very limited inhibitory activity, 4) reduction of the double bond at C-4 and of the cheto group at C-3 (e.g., tetrahydrocortisol) resulted in total lack of activity; and 5) the sex steroids (androstene, testosterone, 17-β-estradiol), which lack C-20 and C-21, were also completely inactive.

The relative affinity of the different steroids mentioned above for the hydrocortisone binding sites correlated well with their biological activity in IEC-6 cells, assessed as inhibition of [3H]thymidine incorporation into cellular DNA (Fig. 1d), with one exception. Proges-

![Graph of cell proliferation inhibition by hydrocortisone](http://apjg.physiology.org/)
terone, which was more active than hydrocortisone in inhibiting binding of [3H]hydrocortisone to the cells (Fig. 1c), produced only submaximal (30–40%) inhibition of DNA synthesis (Fig. 1d). This result suggests that progesterone acts in IEC cells as a suboptimal inducer (60), capable of generating a biological response (inhibition of DNA synthesis in this case) to a limited but characteristic level. Further evidence for this interpretation was obtained by comparing inhibition of DNA synthesis in IEC-6 cells after addition of different concentrations of 1) hydrocortisone alone (2 nM to 1 µM), 2) progesterone alone (2 nM to 1 µM), and 3) 200 nM hydrocortisone plus progesterone at different concentrations (2 nM to 1 µM). As illustrated in Fig. 1d, inset, an excess of progesterone reduced the inhibitory effect produced by 200 nM hydrocortisone (75% in this experiment) to a value corresponding to the maximum inhibition obtained with 1 µM progesterone alone (40%). As the concentration of progesterone was reduced (when given in combination with hydrocortisone), the same level of inhibition observed with hydrocortisone alone was progressively achieved.

Inhibition of Cell Growth

The two most readily noticeable effects of glucocorticoids on IEC cells were a striking change in morphology (see Effects of Glucocorticoids on Cell Morphology and Intracellular Organelles), evident already 1 day after addition of hydrocortisone to the culture medium, and inhibition of growth. However, growth curve analysis (Fig. 2a) revealed that inhibition of cell proliferation had complex characteristics. Depending on hydrocortisone concentration (50–500 nM), this became significant between 4 and 7 days after addition of the hormone to the culture medium, leading to different cell density plateaus. The lag period between hormone addition and onset of growth inhibition was also dependent on the initial density of the cells. Starting with half-confluent cultures, growth inhibition became significant 36–48 h after addition of 200 nM hydrocortisone to the medium. Growth inhibition required the continuous presence of hormone and was at least in part reversible (Fig. 2b). Dexamethasone produced the same growth-inhibitory effects as hydrocortisone, but in this case maximal inhibition was achieved at a 50 nM concentration. When IEC-6 cells were treated with glucocorticoids for only 6 or 12 h during day 1 from plating, no significant effect on the subsequent growth rates was noted. Treatment for 24–48 h produced a temporary slight but significant decrease in cell counts 2 days later, but cell proliferation subsequently continued at a normal rate. It is also important to note that, when the culture medium was changed in fully inhibited cultures, while a constant presence of 500 nM hydrocortisone was maintained, a temporary resumption of cell growth was observed (Fig. 2b). Both 51Cr release and plating efficiency assays failed to reveal any cytotoxic effect of hydrocortisone (50 nM to 1 µM) on IEC cells (not shown).

Together, these results suggested that inhibition of IEC cell growth may have been due to activation and/or release into the culture medium of an inhibitor whose steady-state concentration was dependent on both the presence of glucocorticoids and cell density. This hypothesis was tested by measuring DNA synthesis in half-confluent IEC-6 cell cultures at 3-h intervals after addition of 1) conditioned medium from confluent IEC-6 cells not treated with glucocorticoids (control), 2) the same conditioned medium supplemented with 500 nM hydrocortisone, 3) standard medium freshly supplemented with 500 nM hydrocortisone, and 4) conditioned medium obtained from IEC-6 cultures treated with 500 nM hydrocortisone for 2 days. As illustrated in Fig. 2c, addition of hormone immediately before use to fresh or conditioned medium produced a slight but
significant stimulation of DNA synthesis during the first 18–21 h, followed by a rapid subsequent inhibition. In contrast, addition of conditioned medium from hydrocortisone-treated cells resulted in immediate inhibition of DNA synthesis. The inhibitory activity present in this conditioned medium was stable under refrigeration for at least 1 wk but was lost after dialysis, suggesting a relatively small size.

IEC cells are known to release into the culture medium a variety of cytokines (31, 43, 64), and one in particular was of obvious interest: TGF-β has been implicated as a powerful mediator in growth regulation, cell differentiation, and carcinogenesis in the intestinal tract on the basis of in vivo and in vitro studies (4, 33, 47, 64) and is known to be a potent inhibitor of IEC-6 cell proliferation (5, 29, 30, 33, 40, 47). We have therefore determined both latent and bioactive TGF-β concentrations in the conditioned medium of hydrocortisone-treated and untreated cells. Presence of the hormone (500 nM) for 48 h reduced latent TGF-β concentrations to essentially negligible levels, without a significant effect on the bioactive form (Fig. 2d). This result indicates that the growth-inhibitory activity present in the conditioned medium of hydrocortisone-treated cells is not due to TGF-β.

Expression of Cell Cycle Regulatory Proteins

The growth-inhibitory effects of glucocorticoids have been correlated with reduced phosphorylation of the retinoblastoma gene product, the retinoblastoma protein (pRb), in a variety of cell types. Depending on the cells under investigation, this effect has been attributed to induction of the CKI p21WAF1/Cip1 (56), downregulation of D-type cyclins (58), or reduced activity of G-phase Cdks (58). Examination of the different rat intestinal epithelial cell lines established in our laboratory revealed that one of them, IEC-17, entirely lacked pRb (Fig. 3b). This likely resulted from a spontaneous growth-enhancing selection that took place during serial culture of this particular IEC cell line, since IEC-17 and IEC-18 cells were derived from the proximal and distal segments, respectively, of the small intestine of the same rat (50). On the basis of growth curves (Fig. 3a), IEC-17 cells were found to be as sensitive as IEC-6 to hydrocortisone, indicating that inhibition of crypt cell proliferation by glucocorticoids is not centered on pRb phosphorylation and its related pathways.

Investigation of the other cell cycle regulatory proteins found to be affected by glucocorticoids in different cell types produced conflicting results (Fig. 3c). In cells treated with hydrocortisone for 1 wk, p21WAF1/Cip1 levels were slightly reduced (25–35% in 3 experiments). Expression of p27 and Cdk6 was even more strongly suppressed, and slight declines were also observed in cyclin D1 and in Cdk2 (Fig. 3c). The reduced levels of G1 phase cyclins and Cdks are consistent with entry into a quiescent state, but inhibition of CKIs expression should, in principle, have produced opposite effects on cell growth.

Lack of Induction of Brush-Border Enzymes

Expression of intestinal cell proteins was evaluated in IEC-6 cells cultured in standard complete medium or medium supplemented with 500 nM hydrocortisone for 7 days using a very sensitive radioimmunoassay. The results, summarized in Table 2, clearly demonstrated that none of the brush-border enzymes known to be affected by glucocorticoids in newborn rat intestine (sucrase-isomaltase, alkaline phosphatase, maltase) and neither aminopeptidase N nor dipeptidyl-peptidase IV (both expressed at very low levels by IEC cells) were altered in their expression levels by treatment with hydrocortisone. Cells at different densities, from about half confluent to confluent, were evaluated, with essentially identical results.

Effects of Glucocorticoids on Cell Morphology and Intracellular Organelles

IEC cells cultured under standard conditions on plastic substrata form “leaky” confluent monolayers, characterized by an epithelial morphology, a centrally

Table 2. Intestinal surface membrane proteins in IEC-6 cells

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Antibody</th>
<th>Subconfluent</th>
<th>Confluent</th>
</tr>
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<tbody>
<tr>
<td>Aminopeptidase N</td>
<td>BB4/33</td>
<td>3,512 ± 152</td>
<td>4,018 ± 77</td>
</tr>
<tr>
<td>Dipeptidylpeptidase IV</td>
<td>CLB/40</td>
<td>7,153 ± 354</td>
<td>6,965 ± 298</td>
</tr>
<tr>
<td>Na+K+-ATPase</td>
<td>IEC1/48</td>
<td>69,818 ± 2,221</td>
<td>71,745 ± 1,976</td>
</tr>
<tr>
<td>Sucrase-isomaltase</td>
<td>BB3/34</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Maltase-glucoamylase</td>
<td>BBC1/8S</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>BBC3/90</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Lactase</td>
<td>FBB3/24</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>BB5/16</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>YBB2/61</td>
<td></td>
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</tr>
</tbody>
</table>

Values are means ± SE of 3 independent determinations. Values are expressed as counts/min of 125I bound/mg of total cell protein (background, corresponding to negative controls incubated with nonimmune mouse Ig, was subtracted from all data). Binding of monoclonal antibodies to cells cultured in 60-mm diameter dishes was measured using a radioimmunobinding assay as previously described (6). Cells were either half-confluent or confluent for 4 days when used in this assay. They were either cultured in standard complete medium (−) or medium supplemented with 500 nM hydrocortisone for 7 days before assay (+).
located nucleus, relatively frequent but short and stubby microvilli (Fig. 4a), and intercellular spaces filled with extracellular matrix (51, 53). Addition of glucocorticoids to the culture medium produced marked changes in cell morphology and organization that were apparent at the light microscopic level already after 18–24 h. The thickness of the cells was reduced from a maximum of 3.5 µm to 1.6 µm, measured in the nuclear area, and to 0.35 µm in the widely extended peripheral regions (Fig. 5). Transmission electron microscopy examination revealed a region of membrane overlap 1–2 µm wide between adjacent cells. When examined by scanning electron microscopy (Fig. 4b), hydrocortisone-treated cells occupied a surface area two to three times larger than untreated cells, and their volume (estimated by Coulter counter sizing) was increased on average 1.5-fold. The cell borders overlapped completely, leaving no intercellular spaces, and the apical surface became covered with a dense layer of thinner, more elongated microvilli, morphologically distinct from the ones present in untreated cells (compare insets in Fig. 4). These morphological changes were consistent with the formation of cellular junctions and reorganization of the cytoskeletal network, as further demonstrated by immunofluorescence staining for ZO-1, actin, and tubulin (Fig. 6). In untreated cells, no significant staining was observed with an antibody specific for the tight junction-associated protein ZO-1. In contrast, after 2–3 days of treatment with hydrocortisone, a thin, continuous band of ZO-1 entirely surrounded the cells. The electrical

Fig. 4. Morphology of confluent IEC-6 cells cultured in standard growth medium (a) or in medium containing 500 nM hydrocortisone (b) for 1 wk. Cells were examined by scanning electron microscopy as described previously (50). Note the smaller cell surface area and presence of relatively large intercellular spaces in untreated cells. In contrast, hydrocortisone-treated cells are tightly opposed to each other, and their surface is covered with a layer of elongated microvilli, in addition to the relatively sparse short and stubby microvilli also present in untreated cells (illustrated in the insets at a higher magnification). Bars = 10 µm (in the insets, bars = 1 µm).
resistance across the monolayers, negligible in untreated cells, was 39 ± 6 Ω·cm² (n = 9) in hydrocortisone-treated cells. The actin microfilament network, poorly developed and limited primarily to the peripheral cell cortex in control cells (Fig. 6e), increased greatly in complexity, consistent with the formation of a large number of new microvilli (Fig. 6f). The microtubules, forming a well-developed but poorly organized network in control cells, became convergent to a single perinuclear area, presumably representing the centrosome.

Staining for organelle markers revealed a significant increase in the number of lysosomes, endocytotic pits, and vesicles (not shown), but the membrane-delimited components of the secretory pathway were most affected. Treatment with glucocorticoids caused a perinuclear compaction of the endoplasmic reticulum (Fig. 7, a–d). The trans-Golgi-network, highly fragmented and spread over most of the cytoplasm in control cells (Fig. 7g), became tightly organized in one to three perinuclear Golgi-like cisternae. These changes were selective, since the mid-Golgi (revealed by staining for mannosidase II) was not significantly altered in its morphology or cellular distribution by addition of hydrocortisone (Fig. 7, e and f).

**DISCUSSION**

In this study, we have demonstrated the presence in intestinal crypt cells of glucocorticoid binding sites that possess the characteristics of steroid receptors described in other systems. These are 1) a limited capacity, with saturation achieved for hydrocortisone at a concentration close to 400 nM, 2) high affinity, with dissociation constants of 85 nM for hydrocortisone and 10 nM for dexamethasone, and 3) specificity, with high affinity for dexamethasone and hydrocortisone, a moderate affinity for aldosterone, and essentially no affinity for sex steroids. The steroid specificity of the glucocorticoid receptor could be correlated with biological activity by demonstrating equivalent potency in inhibition of DNA synthesis in IEC-6 cells.

An important aspect of this work is that all the intestinal cell lines we used were derived from weaned (18–24 days old) rats, an age at which the intestinal mucosa appears to have lost responsiveness to glucocorticoids (8, 25). However, relatively little is known regarding the possible role of these hormones in the function of the intestinal epithelium beyond the early developmental stage. Administration of betamethasone 17-valerate to adult rats was found to cause hypoplasia in the jejunum (62), but other studies have reported both stimulation and inhibition of crypt cell proliferation (2, 67, 70). Studies in vitro have been equally limited in both number and scope. Dexamethasone was found to induce expression of heat shock protein 72 in IEC-18 cells, possibly affording protection against oxidant injury (69), and to modulate expression of E/EBP isoforms in IEC-6 cells (9). Although potentially impor-
tant for regulation of expression of a variety of genes, the increased levels of C/EBPβ and C/EBPδ demonstrated in the latter study were transient and only implicated in the acute phase response (9).

Thus the effects of glucocorticoids on IEC cells that we have documented here are novel and challenge the notion that regulation of important intestinal physiological functions becomes largely independent of glucocorticoids after weaning. They also focus our attention on a different set of cellular activities. Most studies conducted on newborn rodents and in humans have centered on brush-border enzymes, including sucrase-isomaltase, lactase, and alkaline phosphatase, whose expression was not affected by glucocorticoids in IEC cells (Table 2). Our failure to induce such enzymes may be due to a lack of glucocorticoid responsiveness of their genes or to the characteristics of the cells we have established in continuous culture. However, by using a coculture model system, Kedinger and co-workers (28, 63) have demonstrated that glucocorticoids influence such markers of intestinal cell differentiation primarily via the mesenchymal cell compartment and that extracellular matrix components such as laminin isoforms may play a key role in the process. A three-step mechanism accounting for the action of glucocorticoids on epithelial cell maturation has been proposed (36) based on these observations: 1) glucocorticoids directly interact with mesenchymal cells, altering their metabolism and leading to the production of a modified extracellular matrix microenvironment, 2) glucocorticoids accelerate the formation of the basement membrane, and 3) this basement membrane is directly responsible for changes in expression of brush-border enzymes. Thus it could be argued that it is the mesenchymal compartment that loses responsiveness to glucocorticoids just before or at the time of weaning. An alternate interpretation is that, once a basement membrane capable of supporting adult-type differentiation of the epithelium is formed, no further changes take place in older animals. Our results complement such a model by demonstrating that other important epithelial cell activities are directly affected by glucocorticoids without need for a mesenchymal compartment or basement membrane. However, the presence of low levels (5%) of FCS in the standard culture medium leaves open the possibility that some of the effects we have observed were influenced by hormones or other cytokines present in the serum. The major effects of glucocorticoids we have documented concern regulation of cell proliferation and cell morphology, including the formation of microvilli and organization of the endomembrane system.

Fig. 6. Formation of tight junctions and reorganization of the cytoskeleton in hydrocortisone-treated cells. Confluent IEC-6 cells, cultured in standard growth medium (−H) or in medium containing 500 nM hydrocortisone for 1 wk (±H), were processed for immunofluorescence staining with antibodies specific for the tight junction-associated protein zonula occludens-1 (ZO-1; a and b), phalloidin-rhodamine visualizing F actin (e and f), or a monoclonal antibody specific for β-tubulin (g and h). c and d: Evans blue (EB) counterstaining of the same cells shown in a and b. Bar = 25 μm.
Inhibition of proliferation by administration of glucocorticoids is a common finding in many types of cells in culture (14, 16, 22, 58) and is sometimes accompanied by loss of cell viability (23), but to date its role in crypt cell growth has not been investigated in detail. Results by Tutton (67) seem to suggest that glucocorticoids stimulate intestinal cell proliferation, but in other studies (62, 70) single injections of prednisolone tertiary butyl acetate resulted in depression in thymidine labeling and mitotic indices. Seven days after a single dose, a compensatory hyperplastic response was demonstrated, possibly representing an escape or rebound phenomenon (70). Much more is known about the mechanism of action of glucocorticoids in fibroblastic cell types, in which inhibition of cell proliferation has been attributed to a marked decline in pRb phosphorylation and consequent extension of the G1 phase of the cell cycle (22, 58). A key role for the CKI p21WAF1/Cip1 has been proposed in this process (14, 56). In human osteosarcoma cell lines, ectopic expression and activation of the glucocorticoid receptor have provided evidence for two distinct mechanisms causing cell cycle arrest (58): one involving transcriptional repression of G1 cyclins and Cdk5 and the other involving enhanced transcription of the CKIs p21WAF1/Cip1 and p27Kip1. By targeting multiple transcriptional elements within a steroid-responsive region of the p21WAF1/Cip1 promoter, stimulation of its expression was also demonstrated in rat hepatoma cells (14), again leading to inhibition of cell growth. On the basis of the strong resistance to dexamethasone-mediated inhibition of cell growth afforded by transfection with the adenoviral E1A gene (22), a mechanism centered on pRb-related pathways has also been proposed for established mouse Pam 212 keratinocytes.

The results we have obtained indicate that quite a different mechanism is responsible for inhibition of proliferation in intestinal crypt cells. Most intracellular cell cycle regulators we have examined, including cyclins D1 and D3, Cdk2, and Cdk4, were unaffected by glucocorticoids, and expression of both p21WAF1/Cip1 and p27Kip1 was markedly suppressed rather than induced. Furthermore, the pRb-deficient IEC-17 cell line was as sensitive as IEC-6 and IEC-18 (both pRb positive) to growth inhibition, strongly suggesting that mechanisms independent of pRb must be considered. Other results also indicate an indirect effect of glucocorticoids, possibly mediated by an inhibitor of cell prolifera-
tion synthesized by IEC cells, secreted into the culture medium, and acting in a paracrine fashion. On the basis of growth curves, inhibition of cell proliferation was related to hormone concentration and required its continuous presence in the culture medium, but, in low-density cultures, a lag period of 2–4 days preceded any measurable effect on cell proliferation. The duration of the lag period was inversely correlated with cell density. In contrast, uptake of labeled steroid was rapid (maximal within 30–60 min) and produced distinct morphological changes in the cells within 24 h, independently of cell density. Glucocorticoid binding sites were present in both low- and high-density IEC-6 cells (Table 1) and displayed similar or identical characteristics. Release of a growth inhibitor into the culture medium was also supported by experiments in which spent conditioned medium from glucocorticoid-treated IEC cells was found to inhibit DNA synthesis without any measurable delay or lag period. Interestingly, in these experiments, addition of hydrocortisone to fresh medium initially appeared to stimulate DNA synthesis, although to a moderate extent. We hypothesize that synthesis and/or release of such inhibitor may be dependent not only on the presence of glucocorticoids but also on cell density. Alternatively, a critical concentration might have to be reached in the culture medium before its effects become apparent, and this would of course depend on the overall biosynthetic capacity of the cultures and thus on cell density.

The inhibitory activity present in the conditioned medium of hydrocortisone-treated IEC cells was lost to dialysis, indicating a mass lower than 6,000–8,000 Da. Because glucocorticoids actually drastically lowered the concentration of latent TGF-β in the culture medium and its bioactive form was present in negligible amounts with or without the hormone, this cytokine is not likely to represent the inhibitory factor. This conclusion should be taken with a note of caution: whereas the bioassay used should not have discriminated among different isoforms, we cannot entirely rule out the possibility that isoforms of TGF-β not detected by the bioassay might be involved in IEC growth inhibition. However, the inhibitory activity present in the conditioned medium was stable to prolonged incubation with the cells and when stored in the refrigerator, in contrast to the relative instability of all known TGF-β isoforms known under such conditions. This characteristic should also make the inhibitor amenable to purification by standard biochemical techniques. Its identification could shed light on the mechanisms responsible for intestinal cell renewal and possibly also the long proposed feedback inhibition of growth by the functional villous cell compartment (57).

The remarkable morphological changes induced by glucocorticoids in IEC cells can be likely attributed to stimulation of a complex set of genes controlling cell morphology, formation of microvilli, recruitment of tight junction components to the cell-cell borders, and compaction of the endomembrane system (specifically, the endoplasmic reticulum and trans-Golgi network). Overall, such changes are consistent with expression of a more differentiated phenotype, but the lack of induction of digestive brush-border enzymes indicates that they represent modulation rather than induction of cell differentiation. This conclusion is further supported by the downregulation of both CK1s p21WAF1/Cip1 and p27Kip1. Studies conducted on the conditionally immortalized human intestinal epithelial cell line tsFH1 (66) and more recently on a normal human crypt cell line induced to express p21WAF1/Cip1 or p27Kip1 genes by infection with recombinant adenovirus (Tian, unpublished results) strongly implicate these two CK1s (in particular p27Kip1) as key inducer(s) of intestinal cell differentiation. In tsFH1 cells, addition of glucocorticoids to the culture medium was found to be essential for prolonged attachment and/or survival of differentiated cells but did not directly influence expression of brush-border enzymes (48). Thus, on the basis of these combined studies, our working hypothesis is that glucocorticoids represent an important factor in the modulation of intestinal physiological functions, contributing to a more functional cell morphology and production of a potent regulator of crypt cell proliferation, but induction of cell differentiation is not directly dependent on these hormones.

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