Antagonistic Effect of Glucocorticoids on Retinoic Acid Induced Growth Inhibition and Morphological Alterations of a Human Cell Line

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ABSTRACT

When the human cell line NHIK 3025 was exposed to retinoic acid (1 nM; 10 μM), the cell cycle time was prolonged. Experiments using cells synchronized by mitotic selection showed that the retinoic acid induced growth delay was barely seen within the first cell cycle after exposure to 10 μM retinoic acid, whereas the next cell cycle durations were increased 30–60%. The effect was reversible as normal growth rate was restored after removal of the drug. DNA histograms indicated a prolongation of G1 of the cell cycle. We have shown earlier that glucocorticoid steroids also induce a prolongation of the cell cycle, located within G1. When the cells were exposed to the synthetic glucocorticoid, dexamethasone, in addition to retinoic acid, no additive effect was found; on the contrary, growth inhibition was less than that with retinoic acid alone. Dexamethasone from 1 nM upwards antagonized the growth inhibitory effect of retinoic acid. This glucocorticoid mediated effect seemed to be mediated via the glucocorticoid receptor, as no effect was seen when the receptor was blocked by the antagonist 17β hydroxy-11β, 4-dimethylaminophenyl-17α-propynyl estra 4,9 diene-3-one [RU 38486]. The growth inhibition studies were supported by morphological observations showing that dexamethasone induced cytoskeletal alterations dominated when the cells were exposed to both drugs simultaneously. These findings might be of importance in cancer therapy where both drugs are used.

INTRODUCTION

Retinoic acid (vitamin A) and several of its analogues play a significant part in the differentiation and response behavior of numerous normal and malignant cells both in vivo and in vitro (for review, see Refs. 1 and 2). In addition to their antineoplastic activity in vivo (3, 4), the retinoids inhibit the proliferation of a considerable number of cultured tumor cells (1). The growth inhibition is accompanied by a reduction in ornithine decarboxylase (5, 6) and an accumulation of cells in G1 of the cell cycle (7, 8). Other effects of the retinoids on cell lines include alterations of cell morphology accompanied by alterations of the cytoskeleton (8–11).

The glucocorticoids produce numerous anabolic or catabolic effects on various cell types, both in vivo and in vitro (for review, see Refs. 12 and 13). This group of steroids is used in cancer therapy (3, 21), it is of significance to elucidate the mechanisms of growth retardation induced by glucocorticoids (14–18). The “restriction point” at which the cells are blocked by both glucocorticoids (14) and retinoids (5) seems to be located in mid-G1. The mechanisms of growth retardation induced by glucocorticoids or retinoids, however, are not known and consequently it is not clear whether there are any common mechanistic pathways for these compounds.

Growth inhibition of the mammalian cell line NHIK 3025 by glucocorticoids is specific for this class of steroids (19), and the effect is probably mediated via the glucocorticoid receptor in these cells (20). Glucocorticoids inhibit growth of the NHIK 3025 cells by prolonging G1. In the current report we present data showing that retinoic acid induced growth inhibition of this cell line also leads to an accumulation of cells in G1. Moreover, I have studied the combined effect of the synthetic glucocorticoid, dexamethasone, and retinoic acid on growth and morphology of the cell line NHIK 3025. As both glucocorticoids and retinoids are used in cancer therapy (3, 21), it is of significance to elucidate the combined effect of these drugs and search for any possible synergistic/additive/antagonistic effects.

MATERIALS AND METHODS

Cell Culture and Synchronization. NHIK 3025 is an established cell line derived from hyperplastic human cervix tissue (22, 23). The cells are of epithelial morphology and are capable of anchorage-independent growth in soft agar (0.3%) or methocel (1.3%) but do not form tumors in nude mice. The cells were cultured as monolayers in Eagle’s minimal essential medium (Grand Island Biological Co., Paisley, Scotland) supplemented with 10% fetal calf serum (Grand Island Biological Co.)

Populations of synchronized cells were obtained from exponentially growing cells by collecting detached mitotic cells after a shaking procedure described previously (24). To maintain constant stable temperatures, the experiments with synchronized cells were performed in an incubator room at 37°C. Stable pH was assured by gently flushing 5% CO2 in air into the flasks whenever opened.

Cell Numbers and Cell Division. The relative increase in cell numbers was measured as follows. Ten thousand cells were seeded per 12-mm plastic petri dish whenever opened.

The fraction of undivided cells was recorded at various times after mitotic selection by examining cell division among at least 100 cells within a delineated area in duplicate culture flasks by means of an inverted microscope located in a 37°C incubator room. The median cell cycle duration was defined as the time period starting 0.5 h after mitotic selection and ending when 50% of the cells had divided (24).

Cellular DNA Content. Cells were stained for DNA measurements with a combination of ethidium bromide (Sigma Chemical Co., St. Louis, MO) and mithramycin (Mitracon; Pfizer) (25). DNA histograms were recorded on a laboratory-built, microscope-based multiparameter flow

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1 Fellow of the Norwegian Cancer Society.

2 O. W. Ranning and E. O. Pettersen, unpublished observations.

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cytometer similar to that of Steen (26). The histograms were recorded in
256 channels and the fraction of cells in the different phases was
determined by a graphical method (14).

**Immunofluorescence Microscopy.** NHIK 3025 cells were cultivated
on 14-mm glass coverslips in 16-mm wells (Costar 3424) for four days.
The cultures were then washed briefly at room temperature in a stabiliz-
ing buffer consisting of 1 mm ethyleneglycol bis(β-aminoethyl ether)-N,
N′,N′-tetraacetic acid, 4% polyethylene glycol (M, 6000), and 0.1 m
piperazine-N,N′-bis(2-ethanesulfonic acid), adjusted to pH 6.9 with KOH.
The cells were extracted subsequently with 0.1% Triton X-100 in sta-
bilizing buffer for 30 s at room temperature. After washing in stabilizing
buffer, the cells were fixed in methanol at −10°C for 6 min, washed in
PBS, and incubated for 30 min at 37°C with monoclonal anti-α-tubulin
(Amersham) diluted 1/100 in PBS. The cells were then washed exten-
sively in PBS, incubated 30 min at 37°C with fluorescein isothiocyanate-
linked anti-mouse immunoglobulin (Amersham) diluted 1/10 in PBS,
washed thoroughly, and mounted on glass slides.

**Chemicals.** Dexamethasone, purchased from Steraloids, Inc. (Pauling,
NY) and RU 38486, a generous gift from Centre de Recherches, Roussel-
UCLAF, Romainville, France, were dissolved in ethanol to a concentra-
tion of 10 μM and diluted further with 0.9% NaCl. Retinoic acid (Sigma) was
also diluted in ethanol to a concentration of 10 μM and further diluted in
medium. The retinoic acid solution was prepared from powder before
every experiment to minimize degradation of this unstable compound.
Ethanol concentrations below 1% have been found not to inhibit cell
growth of NHIK 3025 cells (27), and the concentration of ethanol during
experiments was kept well under this limit. Equal concentrations of
ethanol were added to exposed cells and control cultures.

**RESULTS**

**Growth Response of NHIK 3025 Cells Exposed to Retinoic Acid.** When the NHIK 3025 cells were exposed to retinoic acid for 4 days, the cell numbers were reduced compared to control values (Fig. 1). The minimum concentration showing a significant growth inhibitory effect was 1 nm and this effect increased up to 10 μM, which was the highest concentration used.

Table 1 shows the median cell cycle duration for NHIK 3025 cells synchronized by mitotic selection and exposed to 10 μM retinoic acid at various times before mitotic selection. A small (1.0 h) cell cycle prolongation was seen within the first cell cycle after exposure. Exposure to retinoic acid 24 h before mitotic selection increased the cell cycle prolongation to 6.8 h, and an additional 1 or 2 days of exposure increased the prolongation to approximately 12 h. These results show that retinoic acid induces very little prolongation within the first cell cycle after exposure compared to the second, third, and fourth cell cycles. The median cell cycle durations did not continue to increase as the cells could be cultivated continuously for at least 3 mo in 10 μM retinoic acid (data not shown). Furthermore, the growth inhibition was reversible since the control growth rate was restored 3 days after removal of drug from cells which had been exposed to 10 μM retinoic acid for up to 3 mo (data not shown).

DNA histograms of control NHIK 3025 cells and cells exposed to 10 μM retinoic acid for 4 days are shown in Fig. 2. The exposed cells accumulated in G1 of the cell cycle. These results, together with the growth response shown in Table 1, indicate that the retinoic acid-induced prolongation of the cell cycle time is primarily due to a prolongation of G1.

<table>
<thead>
<tr>
<th>Concentration of Retinoic Acid (μM)</th>
<th>Median cell cycle duration (h)</th>
<th>Prolongation (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>18.8 ± 0.2</td>
<td>0</td>
</tr>
<tr>
<td>Retinoic acid (10 μM) given</td>
<td>19.8 ± 0.3</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>At time of mitotic selection</td>
<td>25.6 ± 1.3</td>
<td>6.8 ± 1.3</td>
</tr>
<tr>
<td>One day before mitotic selection</td>
<td>31.7 ± 1.7</td>
<td>12.9 ± 1.8</td>
</tr>
<tr>
<td>Two days before mitotic selection</td>
<td>30.8 ± 2.1</td>
<td>12.0 ± 2.1</td>
</tr>
</tbody>
</table>

Fig. 1. Effects of retinoic acid on growth of NHIK 3025 cells. Mean cell numbers are relative to control after 4 days incubation in various concentrations of retinoic acid. O, △, □, Δ. Data from four independent experiments; each retinoid concentra-
tion was tested in quadruplicate. SE for each data point was less than 5%.

**Combined Effect of Retinoic Acid and Dexamethasone.** Our earlier data show that growth inhibition induced by dexamethasone is maximal at 0.1 μM (14), probably due to saturation of the receptors. When this concentration of dexamethasone was added to the NHIK 3025 cells together with 1 μM retinoid acid, the result was a reduced magnitude of growth inhibition com-
pared to that obtained with retinoic acid alone (Fig. 3). At 10 nm retinoid acid the effect of the two modulators together was also similar to that obtained by 0.1 μM dexamethasone alone. At this concentration the steroid alone caused a higher growth inhibition than the retinoid (Fig. 3). An antagonistic effect could be seen for dexamethasone concentrations as low as 1 nm and the natural human glucocorticoid, cortisol, could also antagonize the effect of retinoic acid (data not shown).

In the above experiments the steroid and retinoid acid were added simultaneously. When NHIK 3025 cells were exposed to 10 μM retinoic acid for 4 days, dexamethasone in the same concentration range as above could still antagonize the retinoid acid induced growth inhibition (data not shown).

**Blocking of the Dexamethasone Effect by Anti-inducer.** The synthetic glucocorticoid antagonist, RU 38486, completely inhibi-

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2 The abbreviations used are: PBS, phosphate buffered saline; RU 38468, 17β-
hydroxy-11α, 4-dimethylaminophenyl-17α-propynyl estr-4,9-diene-3-one.
GLUCOCORTICOIDS AND RETINOIC ACID GROWTH INHIBITION

Fig. 2. DNA histograms of NHIK 3025 cells exposed to retinoic acid. A, control cells. (G<sub>1</sub> = 57 ± 3%; S phase = 26 ± 2%; G<sub>2</sub> + M = 17 ± 2%); B, cells exposed to 10 µM retinoic acid for 4 days (G<sub>1</sub> = 74 ± 3%; S phase = 17 ± 2%; G<sub>2</sub> + M = 9 ± 1%). Ten thousand cells were recorded in 256 channels. The relative number of cells in the various cell cycle phases is based on three independent experiments and given as mean ± SE.

Fig. 3. Effects of 0.1 µM dexamethasone on growth inhibition of NHIK 3025 cells by retinoic acid. Mean cell numbers relative to control after 4 days' incubation in various concentrations of retinoic acid alone (C) and in combination with 0.1 µM dexamethasone (A). Bars, SE. Data are from the same experiment and each point was tested in quadruplicate. Similar results were obtained in three independent experiments.

Fig. 4. Growth inhibition of NHIK 3025 cells by retinoic acid and the influence of dexamethasone and glucocorticoid antagonists. The cells were exposed for 4 days to the various modulators and the cell numbers were recorded. The differences in cell number between control cultures and exposed cells are shown relative to the control values (% inhibition). Bars, SE. Similar results were obtained in three independent experiments.

retinoic acid alone. This indicates that the reduction of the retinoic acid effect was mediated via a specific steroid receptor.

Influence of Retinoic Acid and Dexamethasone on Morphology and Cytoskeleton of NHIK 3025 Cells. NHIK 3025 cells alter their morphology by becoming more elongated and growing partly on top of neighboring cells when exposed to dexamethasone (Fig. 5b). The microtubuli organizing center also became more elongated, with parallel microtubuli instead of the radiating microtubuli organizing center seen in control cells (Fig. 5a). In the presence of retinoic acid, however, the cells flattened out to a "starfish"-like morphology (Fig. 5c). When the NHIK 3025 cells were exposed to both retinoic acid (10 µM) and dexamethasone (0.1 µM) for 4 days, the dexamethasone morphology dominated (Fig. 5d), indicating that dexamethasone also inhibits the retinoic acid induced morphological changes. When the glucocorticoid antagonist RU 38486 (1 µM) was given in addition to dexamethasone and retinoic acid, the effect of the steroid was abolished (micrograph not shown), indicating that the dexamethasone induced morphological alterations are receptor mediated.

DISCUSSION

Retinoic acid caused a dose dependent inhibition of growth of the human cell line NHIK 3025 in the range 10 nM–10 µM. The retinoic acid induced growth delay was barely seen within the first cell cycle after exposure, whereas the next cell cycle durations were increased 30–60% as compared to control cells. The cells accumulated in G<sub>1</sub>, indicating a prolongation of this phase of the cell cycle.

Glucocorticoids also prolong the cell cycle duration of NHIK 3025 cells. This cell cycle prolongation is manifested within the first cell cycle after exposure and is located within G<sub>1</sub> (14, 15). When the NHIK 3025 cells were subjected to retinoic acid in addition to dexamethasone or cortisol, no additive growth inhibition was found. On the contrary, the growth inhibition was similar to that caused by the steroid alone, indicating that glucocorticoids antagonize retinoic acid induced growth inhibition.

We have shown earlier that the glucocorticoid antagonist cortisolone antagonizes glucocorticoid growth inhibition (19). Current data demonstrate that the synthetic competitive receptor antagonist RU 38486 (28, 29), is more efficient, abolishing the dexamethasone growth inhibition completely. RU 38486 also blocked glucocorticoid antagonism of retinoic acid. Together with
the observation that dexamethasone is active at concentrations known to saturate the glucocorticoid binding sites in other mammalian cells (13), this indicates that the antagonistic effect(s) of glucocorticoids are mediated via specific receptors.

That glucocorticoids dominate when added together with retinoic acid was further supported by morphological observations. Retinoic acid induces an altered cell morphology in many cell lines (8, 9) and this was also the case for the NHIK 3025 cells which became spread out on the growth surface. Dexamethasone also altered the morphology of the NHIK 3025 cells but in a different manner. When retinoic acid and dexamethasone were added together the organization of microtubules resembled that of cells exposed to dexamethasone alone.

Glucocorticoids modify and regulate many processes in a cell and their interaction with retinoic acid could be on various levels. The possibility that dexamethasone acts directly on retinoic acid is not probable since the effect is eliminated if the steroid is blocked at the receptor level. One difference between the effects of the two drugs on the cell cycle is that glucocorticoids induce maximal growth inhibition in the first cell cycle after exposure (14, 15), whereas I show here that retinoic acid needs more than one cell cycle for a full effect. This indicates that glucocorticoids influence the cell cycle control more directly than retinoic acid, which might work through slower, intermediate mechanisms.

Both glucocorticoids and retinoic acid inhibit growth of NHIK 3025 cells by prolonging the G1 of the cell cycle, suggesting that they may work via similar mechanisms within the cell cycle. The point of interaction of the two growth modulators could be at the gene level, for instance, since both retinoids and glucocorticoids modulate gene expression in mammalian cells (2, 30, 31). By analogy with the steroids it has been suggested that the effects of retinoids in controlling gene expression are mediated by specific intracellular proteins (32).

The activity of the enzyme alkaline phosphatase is increased by both glucocorticoids (33, 34) and retinoids (35, 36) whereas glucocorticoids decrease and retinoids increase the binding of...
epidermal growth factor to its receptor (37). Such results suggest that the point of interaction could be on regulation of enzyme activities and/or growth factor response.

The present investigation shows that glucocorticoids in vitro may antagonize and reverse the growth inhibition and suppress the morphological alterations induced by retinoic acid. This effect is most probably mediated via the glucocorticoid receptor. The growth effect is not unique for the NHIK 3025 cells, as human K562 cells, mouse 3T3 cells and rat HTC cells behave somewhat similarly when exposed to these mediators.4

Both glucocorticoids and retinoids are used in cancer therapy (3, 21). If the in vitro effects reported here also take place in human malignant tissue in vivo, they should be taken into account when using these drugs in cancer treatment.

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* Unpublished data.

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