Influence of Glucocorticoid, Estrogen, and Androgen Hormones on Transformation of Human Cells in Vitro by Feline Sarcoma Virus

Joseph P. Schaller, George E. Milo, James R. Blakeslee, Jr., Richard G. Olsen, and David S. Yohn
Department of Veterinary Pathobiology, College of Veterinary Medicine, The Ohio State University, Columbus, Ohio 43210

SUMMARY

Infection of human foreskin cells (D-550) by the Snyder-Theilen strain of feline sarcoma virus produced small but countable foci and demonstrated "single-hit" dose-response kinetics. Significant quantitative and qualitative enhancement of focus formation was observed when the glucocorticoid hormones, dexamethasone, hydrocortisone, cortisol acetate, and prednisolone were added to cell cultures (1.0 μg/ml) 24 hr postinfection. However, aldosterone, while inducing qualitatively larger foci, did not bring about a quantitative enhancement in total foci number. By contrast, 17β-estradiol, progesterone, cortisone acetate, methyltestosterone, and estrone elicited little or no effect on focus induction by Snyder-Theilen feline sarcoma virus. Evidence is suggestive of a posttranscriptional effect possibly modulating viral genome expression resulting in an increased efficiency of viral transformation, and an increased proliferation of transformed cells.

INTRODUCTION

Several recent reports have documented the stimulation of MuLV synthesis in cell cultures exposed to optimal concentrations of glucocorticoid adrenocorticoestrogen hormones (8, 14, 19). For example, using hydrocortisone, prednisolone, and dexamethasone Paran et al. (14) demonstrated a 5- to 25-fold stimulation of virus production in BALB/c 3T3 fibroblasts after ldUrd treatment (2). This stimulation was inhibited by cordycepin, an inhibitor of poly(A) synthesis, and therefore suggested that hormonal effects may be related to the posttranscriptional processing of viral mRNA. These results were confirmed and extended by Wu et al. (19) who demonstrated that the period of maximum hormone sensitivity is 24 to 48 hr after ldUrd induction and that hormone treatment did not result in an increase in intracellular viral-specific RNA. Enhanced virus production in the presence of corticosteroid hormones also was observed in AKR cells chronically infected with MuLV, as well as in ldUrd-induced AKR cells (8).

Varying effects of hormones on the incidence and morphology of plaque and focus formation in different viral systems have been observed (10, 12, 13, 17). Dexamethasone enhanced the plaque formation by polyoma virus on mouse embryo fibroblasts (10). By contrast, however, dexamethasone did not stimulate focus formation by Yaba tumor poxvirus (13) in monkey kidney cells. Further, transformation by adenovirus type 12 (Ad-12) was enhanced in male and inhibited in female hamster embryo cells by dexamethasone (12, 17).

The susceptibility of human cells to infection by the FeSV and feline leukemia virus has been well established (3, 4, 6, 9, 15). The influence of steroid hormones on this virus-host cell system is not known. The objectives of this study, therefore, were to determine the effects of various corticosteroid, androgen, and estrogen hormones on the qualitative and quantitative transformation of human cells by FeSV.

MATERIALS AND METHODS

Cell Culture. Human foreskin cultures (Detroit 550:CCL 109 ATCC, Rockville, Md.), at passage levels 10 to 12, were serially subpassaged in MEM-E (Flow Laboratories, Rockville, Md.), supplemented with 1 mM sodium pyruvate, 2 mM glutamine, 50 μg gentamicin per ml (Schering Diagnostics, Port Reading, N. J.), 0.11% sodium bicarbonate, and 10% fetal bovine serum (Reheis Chemical Co., Kankakee, Ill.). Cultures were split every 3 to 4 days at a 1:2 ratio and incubated at 37° in 5% CO2 atmosphere.

Virus. ST-FeSV (18) was prepared as 10% cell free tumor homogenates in Leibovitz medium (L-15) supplemented with 5% fetal bovine serum. Tumor suspensions were homogenized in a blender and then clarified by centrifugation at 2,300 × g for 20 min followed by an 18,000 × g clarification for 1 min. Supernatants were stored at −70° in 1-ml volumes.

Infectivity Assay. Actively growing D-550 cells (2 × 104/dish) between passage levels 18 and 30 were seeded onto 50-mm plastic Petri dishes in 4.5 ml of 1 × MEM-E medium. After 24 hr the growth medium was removed and the cells were treated with 1 ml of DEAE-dextran (40 μg/ml) in medium without fetal bovine serum. After 20 min the DEAE-containing medium was removed and the plates rinsed with 5 ml of MEM-E medium with 5% fetal bovine serum. Monolayers were subsequently infected with 0.2 ml for each of five 2-fold virus dilutions and allowed to adsorb for 2 hr. Four 50-mm Petri plates were used per dilution. Plates were
rocked at 10 to 15-min intervals to maintain an even distribution of inoculum. After virus adsorption the inoculum was removed and replaced with 5 ml of growth medium. Hormone-containing medium was added after an additional 24 hr. Monolayers were fed with fresh medium containing hormone on the 6th day after infection and were fixed with buffered formalin and stained with Giemsa 3 to 4 days later. Foci were counted at × 25 to 40 with a dissecting microscope.

Cloning Procedures. The effects of hormones on cell growth potentials were evaluated according to their ability to proliferate into clones when seeded at low density. Confluent monolayers were trypsinized and resuspended in cloning medium, and the cell concentration was adjusted to 10³ cells/ml and seeded in 28-sq cm plastic Petridishes (Linbro Chemical Co., Inc., New Haven, Conn.). Cloning medium, with or without added hormone, consisted of 1 × Eagle’s minimal medium Hank’s salts supplemented with 1 mM sodium pyruvate, 2 mM glutamine, 2 × vitamins, 20% fetal bovine serum, and 0.11% sodium bicarbonate. Cultures were incubated at 37° in a 5% CO₂ atmosphere, refed after 7 days, and fixed in 2% buffered formalin after 11 days. Fixed cells were stained with Ehrlich’s hematoxylin and eosin and enumerated. Cloning efficiency was determined as the proportion of inoculated cells per plate that formed proliferating clones.

Hormones. Fetal bovine sera were screened for their content of endogenous hormones and only those batches containing low levels were used in these experiments (12). Hormones were solubilized or suspended in acetone, stored at −20° at 500 µg/ml, and incorporated into media at 1 µg/ml. This level was 5-fold greater than the endogenous content of 17β-estradiol and from 10 to 20-fold greater for the endogenous serum levels of estrone and cortisol.

RESULTS

In Vitro Transformation of D-550 Cells by ST-FeSV. Titration of viral infectivity was easily performed and resulted in typical linear "1-hit" dose response patterns observed over a 4 to 8-fold range of virus concentration (Chart 1). In addition, no significant differences in focus-forming titers were observed when duplicate sets of assay plates were fixed at 7, 9, 14, and 21 days following infection. Infection of D-550 cells by ST-FeSV resulted in either diffuse or distinct areas of highly refractile transformed cells within 3 to 5 days postinfection, depending upon the virus concentration. At high virus concentrations, cellular transformation was uniformly detected throughout the cell monolayer. Under these conditions both cell lysis and cellular degeneration were observed. At lower virus concentration, discrete focal areas of transformed cells were observed consisting of networks of 5 to 10 or as many as 100 or more hyperrefractile, round and enlarged fibroblast cells (Figs. 1 to 4) thereby facilitating focus quantitation. Foci were clearly delineated against an intact monolayer of contact inhibited cells.

Variation in cell susceptibility to transformation appeared to depend upon the stage of cell growth in that actively dividing cells from preconfluent cultures uniformly were more susceptible than were contact-inhibited cells.

Quantitative Effects of Hormones on Transformation of D-550 Cells by ST-FeSV. Enhanced focus formation was observed when the glucocorticoid hormones were added to cell cultures 24 hr after virus infection (Chart 1). Dexamethasone or hydrocortisone, 1 µg/ml, incorporated into infected cell culture media resulted in 2- and 3-fold increases in observed foci, respectively, and were consistent over 3 or more 2-fold virus dilutions.

The influence of other hormones on the efficiency of ST-FeSV transformation of D-550 cells are presented in Table 1. Four of 10 hormones were found to stimulate focus formation in both experiments. Of the 5 hormones with glucocorticoid activity evaluated in the study, 4 significantly enhanced the transforming capacity of ST-FeSV (Table 1). Hydrocortisone, cortisol acetate, prednisolone, and dexamethasone resulted in enhancement ratios of 2.3, 2.2, 2.1, and 3.0 respectively, for 2 replicate experiments combined. Further, these hormones also caused cellular proliferation rather than degeneration in cultures infected at high virus concentrations. By contrast, progesterone, cortisone acetate, and methyltestosterone did not enhance the transformation frequency. Significant enhancement ratios were ob-
served with aldosterone and 17β-estradiol but were not observed in the subsequent experiment. Significant inhibition of focus induction was observed in 1 of 2 experiments using estrone.

**Effect of Steroid Hormones on Cloning Efficiency of D-550 Cells.** Stimulatory or inhibitory effects which steroid hormones may exert on cell growth could affect observation of *in vitro* transformation. To test this effect, cloning efficiencies for D-550 cell suspensions at various hormone concentrations were determined (Table 2). Both enhancement as well as marked inhibition of cloning efficiencies were observed for hormones that either stimulated or had no effect on transformation of D-550 by ST-FeSV. For hormones demonstrating enhanced focus formation, hydrocortisone- and prednisolone-treated cells demonstrated slight increases in cloning efficiency, while cortisol acetate and dexamethasone were inhibitory at similar concentrations. Of those hormones demonstrating no effect on transformation, 17β-estradiol and cortisone acetate appeared to enhance cloning efficiencies while progesterone and estrone demonstrated significant inhibition. Slight inhibition of cloning efficiency was observed with methyltestosterone.

**Hormonal Effects on Morphology of Focus Induction of D-550 Cells by ST-FeSV.** Hormones that enhanced focus formation also produced much larger, more compact foci (Figs. 5 to 16). This effect was observed as early as 3 to 7 days following infection. Foci from cultures treated with these hormones and observed at 7 to 10 days routinely

### Table 1

**The effects of steroid hormones on quantitation of FeSV infectivity**

D-550 cells (2 × 10⁶) were plated into 50-mm Petri dishes with 5 ml growth medium. After 24 hr cells were infected with ST-FeSV as described in "Materials and Methods." Following an additional 24 hr incubation, cells were fed with growth media containing the appropriate hormone at 1.0 μg/ml. Cultures were fed at 6 days with fresh media and subsequently fixed and stained 3 to 4 days later. Viral induced foci were counted in all plates and the virus titer for hormone-treated and non-hormone-treated cultures was determined. The fold increase or decrease in virus titer was determined by dividing the titer of hormone and virus by that of the non-hormone-treated cultures. Significance values were determined using Student's t test and a minimum of 8 focus counts from 2 or more virus dilutions.

<table>
<thead>
<tr>
<th>Hormone + virus</th>
<th>Virus alone</th>
<th>Fold increase or decrease</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrocortisone</td>
<td>10.70 ± 1.6</td>
<td>3.58 ± 0.78</td>
<td>2.99</td>
</tr>
<tr>
<td>Cortisol acetate</td>
<td>2.05 ± 0.48</td>
<td>1.38 ± 0.19</td>
<td>1.49</td>
</tr>
<tr>
<td>Prednisolone</td>
<td>12.34 ± 2.7</td>
<td>7.38 ± 1.2</td>
<td>1.69</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>0.57 ± 1.94</td>
<td>0.14 ± 0.43</td>
<td>3.99</td>
</tr>
<tr>
<td>Aldosterone</td>
<td>34.4 ± 4.4</td>
<td>17.3 ± 3.1</td>
<td>1.99</td>
</tr>
<tr>
<td>17β-Estradiol</td>
<td>15.9 ± 2.9</td>
<td>11.1 ± 2.0</td>
<td>1.43</td>
</tr>
<tr>
<td>Progesterone</td>
<td>7.56 ± 1.10</td>
<td>7.82 ± 2.4</td>
<td>0.97</td>
</tr>
<tr>
<td>Cortisone acetate</td>
<td>7.55 ± 0.96</td>
<td>7.82 ± 2.4</td>
<td>0.97</td>
</tr>
<tr>
<td>Methyltestosterone</td>
<td>15.0 ± 2.9</td>
<td>15.7 ± 2.8</td>
<td>0.98</td>
</tr>
<tr>
<td>Estrone</td>
<td>8.57 ± 2.3</td>
<td>7.38 ± 1.2</td>
<td>1.16</td>
</tr>
</tbody>
</table>

* Mean ± S.D.
* Demonstrated enhanced focal morphologies consisting of greater cell number per focus and higher cell density.
* NS, not significant.

### Table 2

**Hormonal effects on cloning efficiency of D-550 cells**

Uniform suspensions of 200 to 400 D-550 cells were seeded into plastic Petri dishes as described in "Materials and Methods." Appropriate acetone solubilized hormones were incorporated into cloning medium at either 1.0, 0.1, 0.01, or 0.001 μg/ml. After 7 days, cultures were fixed in 2% buffered formalin, stained and enumerated. Cloning efficiency is expressed as the proportion of inoculated cells giving rise to individual colonies of proliferating cells.

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Enhanced transformation</th>
<th>10⁻</th>
<th>10⁻¹</th>
<th>10⁻²</th>
<th>10⁻³</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrocortisone</td>
<td>+</td>
<td>22.0 ± 2.1</td>
<td>19.0 ± 1.7</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Cortisol acetate</td>
<td>+</td>
<td>1.2 ± 0.1</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Prednisolone</td>
<td>+</td>
<td>21.0 ± 0.9</td>
<td>19.0 ± 0.8</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>+</td>
<td>0.0</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>17β-Estradiol</td>
<td>+</td>
<td>26.0 ± 1.8</td>
<td>ND</td>
<td>18.0 ± 1.8</td>
<td>ND</td>
</tr>
<tr>
<td>Progesterone</td>
<td>+</td>
<td>0.0</td>
<td>9.0 ± 0.8</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Cortisone acetate</td>
<td>+</td>
<td>23.0 ± 1.5</td>
<td>20.0 ± 1.3</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Methyltestosterone</td>
<td>+</td>
<td>11.0 ± 0.8</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Estrone</td>
<td>-</td>
<td>0.0</td>
<td>ND</td>
<td>1.0 ± 0.3</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Mean ± S.D.
* ND, not determined.
* No significant differences were observed between acetone control and control without acetone.

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DISCUSSION

These experiments demonstrated that steroid hormones with glucocorticoid activity enhanced focus formation of human foreskin cells (D-550) by ST-FeSV, whereas those without this metabolic effect did not. Significant enhancement of both the frequency of transformation as well as the number of transformed cells per focus were noted for dexamethasone, hydrocortisone, cortisol acetate, and prednisolone. No enhancement was detected for cortisone acetate. Although an apparent increase in transformed focus size was observed for aldosterone, no significant increase in focus number occurred. By contrast, the androgens and estrogens, 17β-estradiol, progesterone, and methyltestosterone demonstrated little or no effect on focus formation.

Studies presented here illustrating the apparent hormonal mediated enhancement of viral transformation suggest confirmation and extension of the specific hormonal effects described by others (8, 14, 19). Specific enhancement of MuLV synthesis by corticosteroid hormones occurred in murine cells induced with IdUrd (14, 19) as well as by cells already producing MuLV (8). In the former studies, enhancement or viral genome expression was determined by the observation of increased levels (5 to 25 times) of viral specific extracellular reverse transcriptase in hormone-treated virus-producing cell cultures. The observations reported here of increased viral genome expression resulting in greater frequency of cellular transformation and subsequent enhancement of transformed cell proliferation may therefore be consistent with the results obtained in the murine system. However, the effects of steroids on growth of infected cells as well as the mechanism or mode of focus formation and development are important considerations in the interpretation of these data.

Cloning experiments to study the effects of hormones on cell growth were intended to provide information for possible correlation with hormonal-mediated effects on transformation of human cells by FeSV. Hormones that inhibited cell proliferation at 1 μg/ml (cortisol acetate, dexamethasone, progesterone, and estrone) either inhibited (progesterone and estrone) or enhanced (cortisol acetate and dexamethasone) cellular transformation. Similarly, hormones that increased cell proliferation or had no effect (hydrocortisone, prednisolone, 17β-estradiol, cortisol acetate, and methyltestosterone) either inhibited (17β-estradiol, cortisol acetate, and methyltestosterone) or enhanced (hydrocortisone and prednisolone) cellular transformation. Therefore, no apparent correlation was observed between hormonal effects on cellular transformation and their effects on cloning efficiency. However, it is possible that these effects may be linked to inhibition of cellular DNA synthesis reported for the glucocorticoids (7) but that they are concentration dependent for each hormone. This study also demonstrated that hydrocortisone failed to inhibit DNA synthesis in virus-transformed cells at much higher concentrations than those reported to produce significant inhibition in normal cells (7). Further experiments to analyze the inhibitory or enhancing effects on DNA synthesis and other parameters of cell growth in infected and noninfected hormone-treated cells will help in the further characterization of these apparently selective effects on infected and transformed cells.

Infection of D-550 cells by ST-FeSV at high virus concentrations resulted in a rather generalized degenerative change in infected cells as was described by Chan et al. (3). At low virus concentrations, however, small areas of cellular transformation were observed which could be counted at ×50 magnification. The addition of the glucocorticoid hormones apparently reversed the degenerative effect induced by high virus concentrations and resulted in cellular proliferation producing an abundance of transformed cells. Cellular proliferation within transformed foci was also observed at low virus concentration and permitted a more accurate quantitation of cellular transformation. In fact, it is possible that the apparent enhancement of focus formation as a result of corticosteroid hormone treatment may represent the expression of transformation events that without hormone would otherwise have gone undetected.

Dose-response patterns for ST-FeSV on human cells were linear with virus dilution, indicative of "single-hit," 1st-order kinetics. Further, the morphological pattern of developing foci was strongly suggestive of the proliferative type rather than one involving recruitment of surrounding cells by released virus. Transformed cells appeared as small round cells restricted to specific areas of an intact monolayer consisting of contact-inhibited cells. For these reasons it is unlikely that secondary focus formation is a significant factor in this system. In addition, no increase in focus formation was observed between 1 and 3 weeks in experiments designed to determine whether sequential patterns of infectivity (2nd order to 1st order) were occurring in this system, as has been demonstrated with rat tropic sarcoma virus (M-MSV-O) in normal rat kidney cells (1). Therefore, it appears that focus development is not dependent upon local virus spread through culture media to neighboring cells. Similar findings were observed for ST-FeSV in feline cells (16).

Assuming that proliferation of the initially transformed cell is the primary mode of focus development, the mechanism for hormonal-mediated enhancement of transformed cell growth can therefore be attacked. It has been shown that transcription of viral mRNA occurs during the 1st 24 hr after oncornavirus infection (5). It would then be possible to determine whether or not posttranscriptional events are affected in this system as has been demonstrated previously (19). Although present experiments suggest a posttranscriptional mode of action because of hormone addition 24
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hr after infection, additional experiments are necessary to provide definitive answers to these questions.

From these results it is apparent that the presence of certain hormones can alter the cellular response after virus infection and subsequent transformation. It has been shown that hormonal concentrations in fetal bovine serum influence cellular growth, depending upon a given lot number (11). Thus, fluctuations in transformation efficiencies could be explained on this basis. It is, therefore, conceivable that transformation by viruses and possibly by chemical carcinogens may be influenced by the hormone content of serum.

Use of glucocorticoid hormones may permit increased yields of virus from cell cultures because of their apparently specific effect on viral genome expression. In addition, they may prove to be of value in standardizing and increasing the sensitivity of viral infectivity assays in general. Studies are currently in progress to evaluate these possibilities.

ACKNOWLEDGEMENTS

The authors wish to thank Ann M. Elliot for her skillful technical assistance.

REFERENCES

Fig. 1. Discrete ST-FeSV-induced focal area of hyperrefractile D-550 cells, 9 days postinfection, and in hydrocortisone-containing medium. Unfixed, × 37.

Fig. 2. Two discrete ST-FeSV-induced focal areas of transformed D-550 cells, 9 days postinfection, and in hydrocortisone-containing medium. Orientation according to monolayer growth pattern. Unfixed, × 37.

Fig. 3. Part of ST-FeSV-induced transformed D-550 cell focus at 15 days postinfection. No hormone treatment. Unfixed, × 72.

Fig. 4. ST-FeSV-induced transformed D-550 cell focus at 15 days postinfection. No hormone treatment. Unfixed, × 72.
Fig. 5. Altered cell focus induced in D-550 cells by ST-FeSV at 12 days postinfection. In cortisol acetate-containing medium. Giemsa, × 27.
Fig. 6. Altered cell focus induced in D-550 cells by ST-FeSV at 12 days postinfection. No hormone. Giemsa, × 27.
Fig. 7. As in Fig. 5 but in hydrocortisone-containing medium. Giemsa, × 27.
Fig. 8. As in Fig. 6 but as hydrocortisone control. Giemsa, × 27.
Fig. 9. As in Fig. 5 but in prednisolone-containing medium. Giemsa, × 27.
Fig. 10. As in Fig. 6 but as prednisolone control. Giemsa, × 27.
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Fig. 11. As in Fig. 5 but in dexamethasone-containing medium. Giemsa, × 27.
Fig. 12. As in Fig. 6 but as dexamethasone control. Giemsa, × 27.
Fig. 13. As in Fig. 5 but in aldosterone-containing medium. Giemsa, × 27.
Fig. 14. As in Fig. 6 but as aldosterone control. Giemsa, × 27.
Fig. 15. As in Fig. 5 but in estrone-containing medium. Giemsa, × 27.
Fig. 16. As in Fig. 6 but as estrone control. Giemsa, × 27.
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