Characterization of a glucocorticoid receptor and the direct effect of dexamethasone on herpes simplex virus infection of rabbit corneal cells in culture

Bernard I. Weinstein, Jerome Schwartz, Gary G. Gordon, Marcia Ochoa Dominguez, Santosh Varma, Michael W. Dunn, and A. Louis Southren

Homogenates prepared from a previously established cell line derived from rabbit cornea contain a macromolecule with many properties of a glucocorticoid receptor, namely high affinity ($K_D = 6 \times 10^{-9} M$) and saturable capacity (135 femtomoles/mg protein) for dexamethasone, extreme heat lability, and a pattern of competition similar to that found in other glucocorticoid target cells. Intact cells specifically bind dexamethasone with an affinity similar to that found in homogenates, and the amount of steroid bound at saturation is approximately 60,000 molecules of dexamethasone per cell. Specific dexamethasone binding was found to be localized to the cell nucleus. The corneal cells were susceptible to infection with herpes simplex virus (HSV). Dexamethasone increased cell susceptibility to the virus and facilitated the spread of the infection throughout the corneal cell culture. This effect was observed at concentrations of dexamethasone as low as $10^{-9} M$. Tetrahydrocortisol, an inactive glucocorticoid metabolite that does not compete with dexamethasone binding to the receptor, did not enhance HSV infection at a high concentration ($10^{-7} M$). This study demonstrates a direct effect of dexamethasone on corneal cell–HSV interaction in the absence of exogenous immunologic factors. This effect of dexamethasone may be mediated by the glucocorticoid receptor. (INVEST OPHTHALMOL VIS SCI 23:651-659, 1982.)

Key words: glucocorticoids, herpes simplex virus, corneal cells, SIRC, receptor characterization

Glucocorticoids are widely used in the management of a variety of ocular disorders, including the severe inflammatory phase of herpes simplex keratitis. The use of topical glucocorticoids in the early stages of an unrecognized herpes keratitis, however, often leads to a marked worsening of the disease process. In rabbits experimentally infected with herpes simplex virus (HSV), small amounts of virus have been demonstrated in the tear film in the absence of clinical disease. In the human, although fewer data are available, infectious virus particles have also been demonstrated in the tear film in symptom-free periods. The factors that prevent the virus from producing clinically damaging
corneal disease during these intervals is not clear. It is known, however, that the use of topical or systemic glucocorticoids can result in the appearance of clinically active herpes keratitis, although these steroids do not induce a latent HSV infection to an acute phase in the rabbit. During herpes keratitis, virus particles infect the epithelial cells, leading to virus replication and cell death, which can be seen as classic dendritic figures in the epithelium. Frequently, the lesion is self-limited but may progress to involve the corneal stroma and result in scarring and even perforation of the globe.

Glucocorticoids may play a role in the infectious process by altering the immunologic response in the cornea. In addition, we hypothesized that these steroids also have a direct effect on the replication and maturation of virus particles and/or on the spread of infection from cell to cell in this tissue. The finding of a glucocorticoid receptor in rabbit corneal epithelium and keratocytes in situ suggests a mechanism by which these steroids may alter the biochemical nature of these cells and lead to an increased spread of infection.

For the study of the direct effects of glucocorticoids on corneal cell–HSV interaction, a cell culture system was used, since it permits investigation of this interaction in the absence of exogenous immunologic factors. This cell culture system maintains two important properties present in corneal cells, i.e., sensitivity to HSV infection and the presence of a functional glucocorticoid receptor.

Using this system we have demonstrated a direct effect of dexamethasone on HSV infection of corneal cells.

**Materials and methods**

**Cell line.** SIRC cells were purchased from the American Type Culture Collection (ATCC CCL 60). This cell line was developed in 1957 from rabbit cornea (Oryctolagus cuniculus) by M. Volkert and has been in culture for over 400 passages. The cells grow in tissue culture with a generation time of about 24 hr. The line is susceptible to rubella and vaccinia viruses as well as to HSV, but not to polio virus 2 or Coxsackie A9 and B5, nor does it contain detectable reverse transcriptase. The species designation has been confirmed by an immunofluorescent test by the ATCC. Cells were grown in Dulbecco's modified Eagle medium supplemented with 10% bovine serum, either
Table I. Competition of various steroids with the binding of \(^3\)H-dexamethasone to the receptor

<table>
<thead>
<tr>
<th>Nonlabeled steroid</th>
<th>Percent of (^3)H-dexamethasone bound* in the presence of nonlabeled steroid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(2 \times 10^{-7}\text{M} ) (20-fold molar excess)</td>
</tr>
<tr>
<td>Tetrahydrocortisol</td>
<td>93</td>
</tr>
<tr>
<td>Dihydrotestosterone</td>
<td>99</td>
</tr>
<tr>
<td>Testosterone</td>
<td>90</td>
</tr>
<tr>
<td>Estradiol</td>
<td>100</td>
</tr>
<tr>
<td>Progesterone</td>
<td>81</td>
</tr>
<tr>
<td>Cortisol</td>
<td>20</td>
</tr>
<tr>
<td>Triamcinolone acetonide</td>
<td>4</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>15</td>
</tr>
</tbody>
</table>

*\(^3\)H-dexamethasone concentration was \(10^{-8}\text{M} \) and incubation was at 0° for 3 to 5 hr.

Dexamethasone and HSV infection in corneal cells

fetal or newborn, and antibiotics at 37° in a humidified 10% CO\(_2\) environment as previously described.\(^{12}\)

Preparation of cell-free extract and dexamethasone binding. Large quantities of cells were grown in 850 cm\(^2\) roller bottles to confluence at 37°. The cells were mechanically detached, pelleted at 600 \(\times\) g, and washed twice in Dulbecco’s phosphate-buffered saline (PBS). The pellet was frozen at −80° until used. Glucocorticoid receptor activity was found to be stable for at least 6 months during storage. Frozen pelleted cells were defrosted and disrupted by homogenizing in Tricine buffer (0.02M Tricine, 0.002M CaCl\(_2\), 0.001M MgCl\(_2\), 0.0005M dithiothreitol, pH 7.9) in a glass homogenizer with a motor-driven Teflon pestle. The homogenate was centrifuged at 140,000 \(\times\) g for 50 min and the supernatant was assayed immediately for dexamethasone binding and for protein concentration using bovine serum albumin as a standard.\(^{13}\)

Aliquots of the cell-free homogenate were incubated at 0° with various concentrations of \(^3\)H-dexamethasone (New England Nuclear; specific activity 20 to 50 Ci/mmol) with and without a variety of nonlabeled steroids.* All steroids were stored in absolute ethanol at −20°. Appropriate aliquots were dispensed into borosilicate test tubes and evaporated to dryness at room temperature. The test tubes were placed in an ice bath and aliquots of cytosol (about 5 mg protein/ml) were added and vortexed gently. At the indicated times, the bound hormone was separated from the free hormone by gel filtration and counted as described previously.\(^{14}\) Specifically bound dexamethasone is determined by subtracting the nonspecifically bound steroid from the total. The nonspecifically bound dexamethasone is the amount of radioactivity bound in the presence of a 200-fold excess of the nonlabeled steroid.

Dexamethasone binding to intact cells. Cells were grown to 80% to 90% confluence, detached with trypsin-EDTA, and washed twice with Hanks’ Balanced Salt (HBS) containing 0.025M HEPES buffer, pH 7.3, by centrifugation. The cells were resuspended in HEPES-buffered HBS, and the cell titer was determined with a hemacytometer. One-milliliter aliquots of the cell suspension (about \(2 \times 10^6\) cells) were pipetted into dry centrifuge tubes (15 ml Corex) containing various amounts of labeled and nonlabeled dexamethasone (evaporated from stock solutions in ethanol). The suspension was vortexed gently and incubated in a water bath at 36° for 1 hr. The tubes were then transferred to an ice bath for 10 min and centrifuged at 4° for 5 min at 2500 \(\times\) g. The cellular pellet was washed twice with 5 ml of PBS at 0°. The pellets were then extracted with 1 ml methanol at room temperature overnight, transferred to vials containing 10 ml Econofluor, and counted in a liquid scintillation counter.

Cell fractionation after incubation of intact cells with dexamethasone. Cells grown on Petri dishes were mechanically detached with a rubber spatula and dispersed by vigorous pipetting. The

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*Generic names of steroids: dexamethasone = 1,4-pregnadien-9-fluro-16a-methyl-11β,17α,21-triol-3,20-dione; cortisol = 4-pregn-11β,17α,21-triol-3,20-dione; triamcinolone acetonide = 1,4-pregnadien-9-fluro-11β,16α,17α21-tetrol-3,20-dione; tetrahydrocortisol = 5β-pregnan-3α,11β,17α,21-tetrol-20-one; estradiol = 1,3,5(10)-estratrien-3,17β-diol; progesterone = 4-pregnen-3,20-dione; testosterone = 4-androsten-17β-ol-3-one; dihydrotestosterone = 5α-androstan-17β-ol-3-one.
Fig. 2. Binding of $^3$H-dexamethasone to intact SIRC cells. A, Total and nonspecific binding as a function of steroid concentration. B, Scatchard plot of the data after subtraction of nonspecific binding. The results indicate the presence of a high affinity ($K_D = 3 \times 10^{-9}\text{M}$) binding for dexamethasone with a saturable capacity (60,000 molecules bound per cell at saturation).

Table II. Localization of $^3$H-dexamethasone to various cell fractions*

<table>
<thead>
<tr>
<th>Cell fraction (cpm)</th>
<th>Nuclear (1000 $\times$ g pellet)</th>
<th>1000 to 140,000 $\times$ g pellet</th>
<th>Cytosol (140,000 $\times$ g supernatant)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^3$H-Dexamethasone (total bound)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. 1</td>
<td>8439</td>
<td>222</td>
<td>5790</td>
</tr>
<tr>
<td>No. 2</td>
<td>8720</td>
<td>294</td>
<td>6630</td>
</tr>
<tr>
<td>Average</td>
<td>8560</td>
<td>255</td>
<td>6210</td>
</tr>
<tr>
<td>$^3$H-Dexamethasone + excess nonlabeled steroid (nonspecific binding)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. 3</td>
<td>3390</td>
<td>179</td>
<td>5520</td>
</tr>
<tr>
<td>No. 4</td>
<td>3787</td>
<td>196</td>
<td>5890</td>
</tr>
<tr>
<td>Average</td>
<td>3589</td>
<td>188</td>
<td>5550</td>
</tr>
<tr>
<td>Specific binding (total less nonspecific binding)</td>
<td>4981</td>
<td>70</td>
<td>660</td>
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</table>

*Cells were incubated at 30° for 30 min with 5 nM labeled dexamethasone with and without a 200-fold excess of nonlabeled hormone (see Materials and methods for details).
Fig. 3. Electron micrographs of HSV-infected SIRC cells. A, Viral nucleocapsids accumulate in the cell nucleus (n). Arrows, Thickened areas of the cell surface that are not present in uninfected SIRC cells. c, Cytoplasm; e, extracellular space. (Bar = 120 μm). B, Viral nucleocapsids are visible in the cell nucleus (n) while mature virions accumulate within cytoplasmic compartments. (Bar = 280 μm.)

HSV infection of corneal cells. SIRC cells were grown in Dulbecco's modified Eagle medium supplemented with antibiotics and 10% fetal or newborn bovine serum. In one experiment, cells were infected at a multiplicity of 100 infectious units per cell and were prepared for electron microscopy after 24 hr as previously described. In other experiments, cells were grown on glass coverslips in medium supplemented with 10% newborn bovine serum. Twenty-four hours prior to HSV infection the coverslips were placed in fresh medium containing various concentrations of dexamethasone (prepared without ethanol). Tetrahydrocortisol, an inactive cortisol metabolite, was used as a control. After the preincubation with and without steroids, the medium was removed from all of the coverslips and virus was added (1, 10, 100, or 1000 infectious units per cell) into 0.1 ml. The coverslips were then incubated for 1 to 2 hr at 37° for viral adsorption to take place. Two milliliters of fresh medium supplemented with 1% bovine serum (fetal or newborn), with and without the steroids, were added to the coverslips and incubated for an additional 48 hr. The coverslips were then fixed, stained, and examined by light microscopy to determine the number of infectious centers per coverslip.

Results and discussion

Specific binding of dexamethasone was found in the 140,000 × g supernatant prepared from frozen SIRC cells as described in Materials and methods. The specifically bound hormone increased during the first 3 hr of incubation at 0° and remained constant over the next 17 hr. In subsequent experiments with this supernatant fraction, incubation was carried out for 3 to 20 hr at 0°. The specific binding was completely obliterated by warming the extract to 40° for 30 min prior to the addition of the labeled hormone, indicating its
Table III. Susceptibility of SIRC cells to HSV infection in the presence and absence of dexamethasone

<table>
<thead>
<tr>
<th>Input of infectious HSV units/10^5 cells</th>
<th>Infectious centers produced per coverslip</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
</tr>
<tr>
<td>28</td>
<td>96</td>
</tr>
<tr>
<td>100</td>
<td>Confluent infection</td>
</tr>
<tr>
<td>1000</td>
<td>Confluent infection</td>
</tr>
</tbody>
</table>

*D* As determined in HEp-2 cells.

*Data represent average of three experiments.

Dexamethasone was added to the cell cultures 24 hr prior to infection with HSV. Each coverslip contained 10^5 SIRC cells; 48 hr after infection the coverslips were fixed and stained, and the number of infectious centers was determined by light microscopy.

extreme heat lability. Specifically bound hormone could be detected at concentrations of dexamethasone as low as 1 nM and approached saturation at about 10 nM (Fig. 1, A). A Scatchard analysis of these data is shown in Fig. 1, B. In this particular experiment the *K*<sub>D</sub> was 2 × 10<sup>-9</sup>M for dexamethasone, and the amount bound at saturation was 95 femtomoles of steroid/mg protein. Repeated analysis on different batches of cells gave similar results (average *K*<sub>D</sub> 6 × 10<sup>-9</sup>M with a range of 2 to 12 × 10<sup>-9</sup>M and the average amount bound at saturation of 135 femtomoles/mg protein with a range of 95 to 156 femtomoles/mg protein). Thus the binding parameters for dexamethasone, i.e., high affinity, finite capacity, and extreme heat lability, are characteristic of that found in glucocorticoid receptors in other glucocorticoid target tissues such as lens epithelium,17 iridociliary body,14 and liver.18, 19

The competition of various nonlabeled steroids for dexamethasone binding is seen in Table 1. The active glucocorticoids, dexamethasone, cortisol, and triamcinolone acetonide, are strong competitors of dexamethasone binding. The inactive glucocorticoid metabolite, tetrahydrocortisol, as well as the sex steroids, testosterone, dihydrotestosterone and estradiol, were largely inactive. Progesterone was inactive at 20-fold molar excess but could compete significantly at a higher concentration (200-fold molar excess). This pattern of competition is similar to that found with glucocorticoid receptors from other target tissues.14, 18

When intact cells were incubated at 36<sup>°</sup>C with various concentrations of labeled dexamethasone (Fig. 2), similar values for the *K*<sub>D</sub> were obtained (3 × 10<sup>-9</sup>M with range of 2 to 4 × 10<sup>-9</sup>M). The amount of steroid bound at saturation was found to average about 60,000 molecules of dexamethasone/cell with a range of 37,000 to 82,000. Similar values were recently reported for human trabecular meshwork cells in culture.20 Table II shows the subcellular localization of the specifically bound steroid by intact SIRC cells after incubation with labeled dexamethasone for 30 min; specific binding was present only in the cell nuclear fraction. Thus intact cells specifically bind dexamethasone with high affinity and finite capacity. The nuclear localization of the labeled hormone is probably the result of binding to the cytoplasmic receptor with subsequent translocation to the cell nucleus during incubation with the hormone (36<sup>°</sup>C for 30 min), as is seen in virtually all steroid hormone target cells.21

Fig. 3, A, is an electron micrograph of HSV-infected SIRC cells showing structural alterations of the cell surface (arrows) that were not observed in uninfected cells. Similar structures have been observed previously in HSV-infected HeLa,22 neuronal, and glial cells.23 Fig. 3, B, demonstrates intranuclear viral nucleocapsids and virions accumulating in the cell cytoplasm. Table III shows that in the presence of dexamethasone, these cells are 3 to 4 times more likely to become infected with the virus. The extent of the spread of the infection throughout the culture at various concentrations of dexamethasone is shown in Fig. 4. After 48 hr of exposure to the virus, the culture without the steroid shows small foci of infection characterized by areas of cell fusion (Fig. 4A). By contrast, the steroid-treated cultures show larger areas of viral cytopathic effect with marked enhancement of cell fusion and detachment, resulting in plaques in the
Fig. 4. Light micrograph of SIRC rabbit cornea cells infected with HSV for 48 hr. The holes in the layer of cells (plaques) represent viral cytopathic effect initiated at time 0 by one infectious unit of virus. With no dexamethasone added to the medium (A) the viral cytopathic effect is small. When the medium contains $10^{-4}$M dexamethasone (F), a large plaque is seen, indicating enhancement of the infection with more rapid spread of virus. Similarly, plaques appearing in cultures containing medium supplemented with dexamethasone at concentrations $10^{-5}$M (B), $10^{-6}$M (C), $10^{-7}$M (D), and $10^{-8}$M (E) all show larger plaques and enhanced virus infection. At higher magnification (G and H) viral cytopathologic changes are indicated by cell fusion and nuclear swelling. The culture shown in G contained no dexamethasone, while that in H contained $10^{-9}$M dexamethasone. (Hematoxylin and eosin stain; A to F, ×350; G and H, ×600.)
the glucocorticoid receptor in these cells (Table I), did not enhance the viral infection at a high concentration (10^{-5}M). This demonstrates a specificity of the dexamethasone effect. These data suggest that the dexamethasone-enhancing effect of the HSV infection is mediated by the glucocorticoid receptor in these cells.

A previous study of HSV-infected mouse fibroblasts (3T3) showed enhancement of infection with dexamethasone. HSV-infected rabbit kidney cells24 and human skin and corneal fibroblasts in culture,25 however, did not show any change in virus production when treated with glucocorticoids. These disparate observations may reflect differences in cell type and the presence or absence of a functional glucocorticoid receptor.

The effect of glucocorticoids on the interferon system is not clear. Although these hormones have been shown to increase interferon production (human lymphoid cell line27 and HSV-infected rabbit corneas25), high concentrations of cortisol have been found to partially antagonize the ability of interferon to protect cells in culture from HSV infection.28 Interferon inhibits HSV replication in cell cultures,25 28 although it has not yet been proven to be of value clinically either in the recovery from ocular HSV infection or in preventing recurrent infections.29 The ineffectiveness of interferon therapy may have been due to the lack of suitable quantities of pure human interferon for testing.

This study demonstrates that, in addition to the immunosuppressive effects of glucocorticoids,31 these hormones also have a direct effect on HSV-infected corneal cells, which may be mediated by the glucocorticoid receptor. The worsening of ocular herpetic infection that is seen after administration of glucocorticoids may be due in part to this direct effect.

REFERENCES


