Detection of glucocorticoid receptors in cultured human trabecular cells

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To evaluate potential direct effects of glucocorticoids on the aqueous outflow pathway, the cellular binding of steroids to cultured human trabecular cells was examined. After incubation of cells with 5 to 40 nM [3H]dexamethasone, specific binding (i.e., binding that could be blocked by an excess of nonlabeled steroid) was detected by measuring the total cell-associated labeled hormone. A binding affinity of 5 nM and 60,000 receptor sites/cell were demonstrated with labeled dexamethasone. Incubation of human trabecular cells with 40 nM [3H]dexamethasone for 60 min revealed that 62% ± 7 of the specific binding was found in the nuclear fraction and 38% ± 3 was in the cytoplasmic fraction. In competition studies, dexamethasone had a higher affinity for these sites than cortisol, which in turn had a higher affinity than progesterone. These studies suggest that functional glucocorticoid receptors are present in human trabecular cell cultures. Therefore it is possible that a direct action of glucocorticoids on trabecular cells could contribute to the decreased outflow facility observed in steroid glaucoma.

Key words: glucocorticoid receptors, trabecular meshwork cells, cell culture, glaucoma

After the introduction of corticosteroids for the treatment of ocular inflammatory conditions, it was observed that certain patients responded to glucocorticoid therapy with a sustained elevation of intraocular pressure (IOP) and glaucomatous visual field loss. The role of glucocorticoids in the regulation of IOP (especially in patients with glaucoma) has been a continuing source for study over the past three decades. 1, 2 Most evidence has demonstrated a significant reduction in outflow facility after long-term administration of steroids to susceptible individuals. This reduction in outflow facility suggests that glucocorticoids might exert a direct effect on the cells lining the aqueous outflow pathway. 3, 4 For this reason, we have begun to examine the action of steroids on the cells from the trabecular meshwork. Biochemical evaluation of glucocorticoid receptors in human trabecular meshwork is limited by the minute amounts of tissue available from dissected specimens. To overcome this difficulty, we have propagated human trabecular cells in vitro, 5 and we have been able to detect specific nuclear binding of [3H]dexamethasone, using these cells. 6 Also, in preliminary studies, we observed a reduced uptake of [3H]thymidine when cultured trabecular cells were exposed to moderate concentrations of glucocorticoids. 7 Concurrently, Southren's laboratory has reported the presence of specific glucocorti-
ticoid binding in dissected rabbit ocular tissues, which included the iris–ciliary body and adjacent corneoscleral areas. Recently, these investigators have developed methods to visualize nuclear binding of dexamethasone in human trabecular meshwork tissue, using autoradiography.

Here we report a more detailed evaluation of glucocorticoid receptors in cultured human trabecular cells and a quantitative determination of receptor binding parameters. Scatchard analyses of different trabecular lines revealed consistent values for the binding affinity and number of receptor sites. In addition, we were able to compare relative binding of dexamethasone, cortisol, and progesterone for glucocorticoid receptors in the trabecular cells.

**Methods**

Human trabecular cells were propagated in tissue culture by techniques we have previously described. For the present studies, fourth-passage cells were grown for 2 weeks in 10 cm dishes coated with 0.1% gelatin (type III; Sigma). Fig. 1 shows a confluent culture of human trabecular cells used for the present studies. These cells were observed to grow as a single layer of flattened cells with many cellular projections at confluency. This contrasts with the multiple-layered, fusiform appearance of confluent fibroblast cultures.

Glucocorticoid binding was evaluated by methods similar to those previously reported from our laboratory. The medium was changed 24 hr prior to study, and the cells were harvested by treatment with normal saline (pH 7.3), trypsin 0.05%, and versine 0.02% (STV) at 37°C, followed by two washes with Dulbecco's modified Eagle's medium (DME). Cells were counted in a hemacytometer, and 500 μl of 4 to 10 × 10⁶ cells/ml were placed in 10 by 75 mm polystyrene test tubes in a 10% CO₂ incubator. After 10 min of equilibration, varying concentrations of [³H]dexamethasone (34 Ci/mmole, New England Nuclear) were added to the samples, with or without nonradioactive steroid. Ethanol concentrations in all cases were less than 0.05%. Samples were incubated for 60 min at 37°C (by which time binding is maximal), placed on ice, and centrifuged at 1200 x g for 4 min at 4°C. Cell pellets were washed four times and were either assayed for radioactivity or used for preparation of nuclei. For the latter procedure, pellets were resuspended in 300 μl of buffer A (20 mM tricine, 2 mM CaCl₂, 3 mM dithiothreitol, pH 7.4). Tubes were then frozen over dry ice in acetone, thawed, refrozen, and thawed again. Samples were homogenized by 7 strokes with a Teflon homogenizer (setting 4, TRI-R homogenizer) and nuclei were sedimented at 1200 × g for 6 min.

Nuclei were identified with a Nikon phase-contrast microscope and a Zeiss microscope; no intact cells were present in the "nuclear" fraction. The supernatant medium ("cytoplasmic fraction") was assayed for radioactivity after Sephadex G-25 filtration. Specific binding for the cell fractions and for whole cells was calculated by subtracting the values obtained in samples that contained 10 nM nonradioactive dexamethasone (nonspecific binding) from the total bound [³H]dexamethasone. Nonspecific binding was approximately 50% in the whole cell studies and approximately 30% for the nuclei and cytoplasmic fractions.

**Results**

Human trabecular cells from nonglaucomatous subjects (ages 14, 21, and 67) were examined for glucocorticoid receptors. Our preliminary data showed specific nuclear binding for [³H]dexamethasone in cells from the 67-year-old patient. To begin the recent series of experiments, the distribution of [³H]dexamethasone receptor sites in cytosol and nuclear fractions was determined in cultured trabecular cells from a 21-year-old patient. These were propagated and examined in triplicate reactions for cytosol and nuclear binding using 40 nM...
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Fig. 2 A, Specific binding of [3H]dexamethasone to intact trabecular cells, as described in Methods. Each point represents data obtained with 2.4 × 10^5 cells per point and 70% of the sample assayed for radioactivity. B, Scatchard plot of data shown in A. The intercept corresponds to 24 fmol/sample or 60,000 dexamethasone molecules specifically bound per trabecular cell.

dexamethasone; 62% ± 7 of the specific receptor sites were localized to the nucleus, and 38% ± 3 were in the cytosol. Quantitative determinations for the affinity and number of receptors were performed in whole cell binding studies using increasing concentrations of labeled steroid as shown in Fig. 2. A saturation of binding was apparent for the 10 to 40 nM range of dexamethasone (Fig. 2, A); Scatchard analysis (Fig. 2, B) showed one class of high-affinity receptor sites with a dissociation constant (K_D) of 5 nM and 60,000 binding sites/cell (R_T). Additional Scatchard experiments were performed on trabecular cells from a 14-year-old patient. These studies revealed data similar to those obtained for the other cell lines: K_D range = 3 to 6 nM, R_T range = 40 to 80 × 10^3 binding sites/cell.

Fig. 3 presents a representative experiment in which the specificity of steroid binding was assessed in cells from the 21-year-old patient. A 30-fold excess of nonradioactive dexamethasone, cortisol, or progesterone was added immediately after addition of 10 nM [3H]dexamethasone, and competition for receptor binding determined by incubation at 37°C for 60 min. With this procedure it was apparent that the glucocorticoid receptors of trabecular cells can distinguish between these steroid analogues. Dexamethasone was the strongest competitor, followed by cortisol and then by progesterone. This same order of binding was obtained with cells from the 14-year-old patient, in which a 20-fold excess of the different unlabeled steroid hormones was employed.

Discussion

These studies demonstrate that it is possible to assess quantitatively the presence of specific glucocorticoid receptors in human trabecular cells. The direct biochemical evaluation of cultured cells thus complements qualitative evaluations of steroid receptor binding for trabecular tissue, using autoradiography or other methods.

Tissue culture provides the means to obtain a sufficient number of human trabecular cells to perform quantitative studies. Only cells at early passages, which had been extensively evaluated by light and electron microscopy, were employed; the cell lines used for these studies preserved the morphological features of noncultured trabecular cells. However, the amount of cellular material defined by these methods was limited, and it was necessary for us to reduce the number of cells used for binding studies to approximately 5 × 10^5 cells/point. This amount of cellular material was one-tenth that routinely
Fig. 3. Percent inhibition of specific dexamethasone binding to trabecular cells (with 10 nM [3H] dexamethasone) by unlabeled dexamethasone (DEX), cortisol (CORT), or progesterone (PROG), as described in Methods. CONT, control. Three data points per compound were tested; the range of values is indicated on the bar graphs.

employed in our laboratory to assay steroid receptors; nevertheless, we were able to obtain uniform and reproducible results using the procedures described in Methods.

[3H]dexamethasone was used to determine specific binding of glucocorticoid receptors, because of the known specificity of this hormone to selectively bind glucocorticoid receptors. In contrast to [3H]cortisol or [3H]corticosterone, the specific binding of [3H]dexamethasone is usually indicative of glucocorticoid receptors (for discussion see ref. 12). In the case of the other labeled hormones, specific binding may also include binding to nonglucocorticoid steroid receptors. The presence of specific [3H]dexamethasone binding in the cells and the demonstration of approximately 60% of the receptors in the nuclear fraction are characteristic of glucocorticoid receptors in model systems for glucocorticoid hormone action. In these systems, hormone action is dependent on active, steroid-stimulated translocation of the receptors to the cell nucleus and on association of hormone-receptor complex with chromatin. The affinity (approximately 5 nM) and number of receptor sites (approximately 60,000/cell) are similar to those observed in known target cells for glucocorticoid hormones, such as hepatoma cells. The specificity of binding (shown by the competitive binding studies) is likewise characteristic of glucocorticoid receptors, i.e., dexamethasone > cortisol > progesterone.

The ability of unlabeled dexamethasone to compete for glucocorticoid receptors may help to explain the effectiveness of this steroid to raise the IOP compared with other steroids. Although the binding of cortisol and progesterone appear to be appreciably less than that of dexamethasone, these compounds may nevertheless exert important effects through glucocorticoid receptors. Cortisol is the major circulating glucocorticoid normally present in humans; progesterone has been reported to lower the IOP when administered to patients. The effect of progesterone on IOP may be due to the ability of this steroid to act as a glucocorticoid antagonist when employed in high hormone concentrations.

Thus the data presented emphasize that cell culture provides a source of human trabecular cells for quantitative evaluation of specific glucocorticoid receptors. Continued studies of these receptors should allow for a more comprehensive understanding of the steroid binding characteristics of this important cell type, including the evaluation of steroid hormone analogues (agonists, partial agonists, and antagonists) that may have clinical significance. Also, it may be possible to measure steroid receptor characteristics in cells from patients with primary open-angle
glaucoma (or known ocular sensitivity to steroids) when these cells are successfully propagated in tissue culture.

REFERENCES