Glucocorticoid Receptors in Cultured Human Skin Fibroblasts: Evidence for Down-regulation of Receptor by Glucocorticoid Hormone*

A. OIKARINEN,[1] H. OIKARINEN,[2] C. A. MEEKER,[2] E. M. L. TAN[2] and J. UITTO[2]

Department of [1]Dermatology, University of Oulu, Oulu, Finland, and [1,2]Department of Medicine, UCLA School of Medicine, Los Angeles, Division of Dermatology, Harbor-UCLA Medical Center, Torrance, California, USA

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In the present study, we have determined the specific glucocorticoid receptors in cultured human skin fibroblasts with [^{3}H]dexamethasone as the ligand. The whole-cell assay was employed for determination of glucocorticoid receptor densities and binding affinities in fibroblast cultures established either from 16 healthy control subjects, from 4 patients with active progressive systemic sclerosis (PSS), from 3 patients with keloids and 3 patients with diabetes mellitus. The receptor densities in PSS, keloid, diabetes and control fibroblasts were in the same range, the values being 6.3±4.9, 7.7±3.6, 5.3±1.3 and 7.9±6.2 fmol/μg DNA (mean ± SD), respectively. In further studies, the cells were incubated with 10^{-7} M dexamethasone for 4 or 9 days before the receptors were assayed. The specific binding of [^{3}H]dexamethasone in steroid treated cultures was 62 and 13% of that observed in controls, suggesting down-regulation. In contrast, incubation of fibroblasts with 10^{-5} M all-*trans*-retinoic acid did not alter the binding of [^{3}H]dexamethasone, suggesting lack of pharmacologic interference at the receptor level. (Received March 26, 1987.)

A. Oikarinen, Department of Dermatology, University of Oulu, Kajaanintie 50, SF-90220 Oulu, Finland.

Glucocorticosteroids are known to modulate various aspects of cell metabolism both in vivo and in vitro (2–5). Glucocorticoids mediate their effects on the cellular metabolism by binding to specific cytoplasmic receptors. The steroid receptor complexes are then translocated to the nucleus, where they influence the transcription of the genetic information into the corresponding messenger RNA precursor molecules.

Human skin fibroblasts in culture have provided a useful model to study the effects of glucocorticoids on various aspects of cell metabolism such as collagen production (6, 7). A careful analysis of these studies reveals, however, that the results are conflicting; in case of collagen production, increased, unaltered and decreased values have all been reported, even though apparently similar culture conditions and steroid concentrations have been employed (for summary of these studies see ref. no. 7). It has been shown that skin fibroblasts and dermis from humans and other animal species contain specific glucocorticoid receptors (8–12). A plausible explanation for the conflicting results observed in previous in vitro studies would be that the number and the binding capacity of the

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Abbreviations: FCS = fetal calf serum; DMEM = Dulbecco's modified Eagle's medium; HBSS = Hank's balanced salt solution; PSS = progressive systemic sclerosis; K_d = dissociation constant; NEM = N-ethylmaleimide; RA = retinoic acid.

glucocorticoid receptors might be variable in cultured cells and such variation would then correlate with the changes in the collagen gene expression.

In the present study, we have established assay conditions for determination of glucocorticoid receptors in cultured human skin fibroblasts employing [^{3}H]dexamethasone as the ligand.

MATERIALS AND METHODS

Human skin fibroblast cultures

Fibroblast cultures from 16 healthy controls, four progressive systemic sclerosis (PSS) patients, three keloid patients and three diabetes mellitus patients were studied. The skin biopsies from PSS patients were taken from the clinically active lesions, and the keloid fibroblast cultures were initiated from tissue removed surgically prior to therapy.

Glucocorticoid receptor assays

Two different methods were utilized for receptor assay. First, a whole-cell binding assay which has been extensively utilized for steroid receptor determinations in other cell systems (13–18), was adapted for glucocorticoid receptor determinations in fibroblasts. Confluent skin fibroblasts were harvested by brief trypsinization in HBSS, washed, counted and suspended in HBSS. The samples were incubated in the presence of [^{3}H]dexamethasone (0.5–100 nM) for 45 min at 37°C, and washed twice. For the estimation of the nonspecific binding of [^{3}H]dexamethasone, a parallel set of tubes containing an additional 200-fold excess of unlabelled dexamethasone was incubated and washed as above. Aliquots were taken for liquid scintillation counting of the radioactivity, and the remainder of the cells was used for assay of DNA (19) or protein (20), or for cell count by hemocytometer.

Secondly, a cytosol binding assay was utilized. Fibroblasts, after harvesting by brief trypsinization, were centrifuged at 1 000 × g and the pellet was homogenized on ice with a Teflon-glass homogenizer in 20 mM Tris-HCl, pH 7.5, containing 2 mM $CaCl_2$, 2 mM $MgCl_2$, 2 mM DTT, 10 mM sodium molybdate and 10% glycerol. The homogenates were centrifuged at 100 000 × g for 60 min at 4°C. The supernatants were then used for receptor binding assay and for protein determination (20). For receptor assay, cytosol preparations were incubated with 33 nM [^{3}H]dexamethasone for 4–20 h at 4°C. After incubation, the unbound [^{3}H]dexamethasone was removed either by treatment with charcoal-dextran or by gel filtration on Sephadex G-100. For charcoal-dextran treatment, an equal volume of solution containing 1% charcoal, 0.05% dextran, 30% glycerol and 10 mM Tris-HCl, pH 7.5, was added. The samples were incubated for 5 min at 4°C and centrifuged at 10 000 × g. Aliquots of the supernatant were taken for assay of ^{3}H-radioactivity. For removal of unbound radioactivity by gel filtration, the cytosol preparations following incubation with [^{3}H]dexamethasone were chromatographed on a Sephadex G-100 column (5.0×0.5 cm), equilibrated and eluted with 20 mM Tris-HCl, pH 7.5, containing 2 mM $CaCl^2$, and 2 mM $MgCl_2$. Fractions of 0.2 ml were collected and counted for ^{3}H-radioactivity.

RESULTS

Optimization of glucocorticoid receptor assays

In order to reliably assay glucocorticoid receptors in human skin fibroblasts, several techniques were tested. First, cytosol preparations were incubated with [^{3}H]dexamethasone at 4°C, and the unbound ^{3}H-radioactivity was removed by charcoal-dextran treatment. This method clearly demonstrated specific binding of ^{3}H-radioactivity. However, the results were variable especially when the number of cells was low in the original sample. Further studies indicated that reliability of the charcoal-dextran method was highly dependent on the amount of protein in the cytosol sample. In the presence of low protein content in the samples charcoal treatment removed also some of the specifically bound dexamethasone (not shown). Secondly after incubation of the cytosol preparations with [^{3}H]dexamethasone unbound and bound dexamethasone were separated by gel filtration on Sephadex G-100. A distinct peak of ^{3}H-radioactivity was noted in the void volume of the column and this peak was not present in samples incubated with a 100-fold

excess of unlabelled dexamethasone (not shown). Thus, this peak represents specific binding of [^{3}H]dexamethasone. Although this methodology was reliable for assay of glucocorticoid receptors, it is time-consuming since a chromatographic step is required for each data point.

With this background, in further studies we attempted to develop a more convenient, yet reliable, method for glucocorticoid receptor assay. For this purpose, we adapted a whole cell binding assay, which has been previously utilized for steroid receptor determinations in other cell systems (13–18). For optimization of the methodology, it was critical to study several parameters of the assay. First, the time course of the [^{3}H]dexamethasone binding to the cells was tested at three different temperatures. The specific binding of radioactivity at 4, 22 and 37°C was maximal within 30 min. However, the maximum binding at 4°C was only about 20% of that observed at 37°C, and the binding at 22°C was 57% of that at 37°C. Thus an incubation of 45 min at 37°C was chosen for standard assay. Secondly, the effects of the washing conditions on the [^{3}H]dexamethasone binding were examined. The results indicated that two consecutive washings employing 10 min incubations at 22°C resulted in low non-specific binding while allowing maximum specific binding. Thirdly, since several washings were required for removal of the unbound radioactivity, we checked the recovery of the cells by assaying DNA or protein before and after the washing procedure. The recovery of the cells varied from 58% to 70%. In order to correct for this variation, cell DNA or protein was determined in each sample and the specific binding was expressed as fmol either per μg DNA or cell protein. Fourthly, the effect of FCS on the [^{3}H]dexamethasone binding was studied. The presence of 10% non-dialyzed or dialyzed FCS had no effect on the specific binding of [^{3}H]dexamethasone. However, incubation of the cells with 10 mM NEM completely abolished the specific binding of [^{3}H]dexamethasone while the non-specific binding was not affected. The latter observation suggests that free sulfhydryl groups are needed for the receptor binding (21). Fifth, the effect of cell density in the receptor assay on the specific binding of [^{3}H]dexamethasone was tested. Incubation of cells in three different concentrations, 0.1, 0.5, and 1.0×10^6 cells/ml, did not change the glucocorticoid binding when expressed as cpm per μg cell DNA.

Specificity of the glucocorticoid receptors

The steroid specificity of the receptors, determined by the whole cell binding assay, was tested in competition studies in which several other steroids, in varying concentrations, were incubated with 22 nM [^{3}H]dexamethasone. As expected, the addition of 22 nM unlabelled dexamethasone reduced the binding of radioactivity by 50% (not shown). Addition of 22 nM triamcinolone acetonide reduced the [^{3}H]dexamethasone binding by 62% and the addition of 220 nM concentration of the same steroid reduced the binding by more than 80%. Approximately 50% inhibition of the [^{3}H]dexamethasone binding was achieved with 220 nM hydrocortisone, 2200 nM progesterone, and 2200 nM deoxycortisone. Estradiol in 2200 nM concentration reduced the [^{3}H]dexamethasone binding by less than 25%.

Assay of glucocorticoid receptors in control human skin fibroblast cultures

A typical binding curve of [^{3}H]dexamethasone to human skin fibroblasts is shown in Fig. 1 A. The specific binding of the glucocorticoid was calculated by deducting the nonspecific binding from the total binding; the maximum binding and the dissociation constant (K_d) were then calculated from Scatchard plots (22) (Fig. 1 B). Assay of the glucocorticoid receptors in fibroblast lines established from the skin of 16 control subjets indicated that the maximum binding varied from 3.2 to 25.4 fmol/μg DNA. The highest specific binding

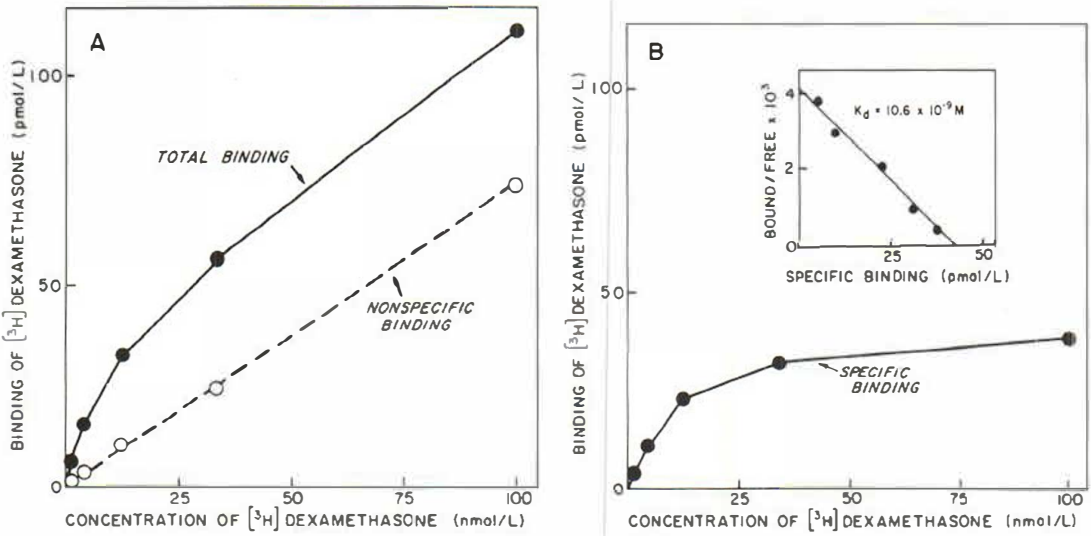

Fig. 1. Typical binding curve of [^{3}H]dexamethasone to glucocorticoid receptors in human skin fibroblasts, and determination of receptor densities and binding affinities by Scatchard plot. For determination of total binding, cells were incubated for 45 min at 37°C with various concentrations of [^{3}H]dexamethasone. For determination of non-specific binding, parallel set of tubes was incubated in the presence of a 200-fold excess of unlabelled dexamethasone. The specific receptor binding was calculated by deducting the non-specific binding from the total binding. (A) Total and non-specific binding of [^{3}H]dexamethasone. (B) Specific binding and the Scatchard plot of values, indicating a dissociation constant (K_d) of 10.6×10^{-9} M.

was noted in two cell lines derived from the breast skin of relatively young females, but otherwise neither the age of the donor nor the site of the biopsy correlated significantly with the receptor density. The receptor binding affinity (K_d) in the control cell lines varied from 3.5 to 24.2×10^{-9} M. Again, no correlation with respect to the age of the donor or the site of skin biopsy was noted.

Since the receptor assay with control cells were performed in passages varying from the

Table 1. *Glucocorticoid receptor binding and dissociation constants (K_d) in fibroblast cultures from the skin of patients with progressive systemic sclerosis (PSS) or keloids*[a]

Cell line	Age (years)/ sex of subjects	Site of biopsy	Passage no.	Dexamethasone binding (fmol/μg DNA)	k_d ($\times10^{-9}$ M)
PSS 1	32/F	Arm	8	5.3	24.0
PSS 2	54/M	Arm	3	4.3	18.1
PSS 3	58/M	Arm	14	13.3	9.7
PSS 4	60/F	Arm	6	2.2	9.6
PSS, mean ± SD				6.3±4.9	15.4±7.0
Keloid 1	15/F	Buttock	3	6.7	11.1
Keloid 2	23/F	Earlobe	3	11.7	7.3
Keloid 3	64/F	Earlobe	3	4.8	13.5
Keloids, mean ± SD				7.7±3.6	10.6±3.1
Control, mean ± SD (n=16)				7.9±6.2	11.8±7.0

[a] The maximum binding and the K_d were calculated from the specific binding of [^{3}H]dexamethasone by Scatchard plots, as described in Materials and Methods and in Fig. 1.

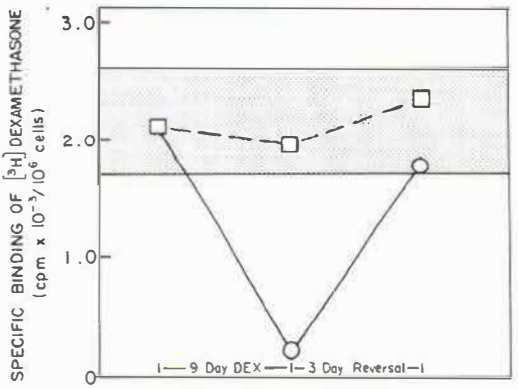

Fig. 2. Effect of dexamethasone pretreatment on the specific glucocorticoid receptor binding of [^{3}H]dexamethasone in human skin fibroblasts. Cells were precultured for 9 days in the presence of 10^{-7} M dexamethasone (9 day dex); the control cultures were incubated with the corresponding concentrations of ethanol. A parallel set of cultures, after the initial pretreatment with dexamethasone, was further incubated for 3 days in a medium without dexamethasone (3 day reversal). At the time points indicated, the specific receptor binding of [^{3}H]dexamethasone was determined in the cultures. The individual values indicated in the figure are means of two parallel flasks. The mean ± SD of all controls (n=6) is indicated by the hatched area. ○, cells precultured with unlabelled dexamethasone for 9 days and subjected to 3 day reversal in steroid-free medium; □, parallel control cultures analysed at the same points of time.

3rd to the 11th passage, it was important to determine whether the passage number might affect the [^{3}H]dexamethasone binding. For this purpose, control cells were cultured so that the same fibroblast line could be assayed for the receptor density in passages 2, 6 and 10. The binding of [^{3}H]dexamethasone in these three passages was 14.2, 16.8 and 10.1 fmol/μg DNA, respectively. Thus, no clear correlation between the passage number and the binding capacity could be noted.

Since considerable variability was encountered in the receptor densities in different control fibroblast lines, it was important to examine the possibility that cell selection or differences in handling of the parallel flasks might give rise to such variation. For this purpose, cells from a single control line were subcultured into four separate flasks, and after having reached early visual confluency, their receptor densities were determined in a parallel fashion. The results indicated that the receptor densities in four parallel flasks were 4.7±0.8 fmol/μg DNA (mean ± SD); the range of the individual values was 4.1–5.9 fmol/μg DNA. Thus it appears that cell selection or differences in the handling of the parallel flasks do not explain the variability noted in control cell cultures.

Glucocorticoid receptor assay in skin fibroblast cultures from patients with progressive systemic sclerosis, keloids and diabetes mellitus

The applicability of the receptor assay established in the study was further tested by assaying glucocorticoid receptors in fibroblast cultures from four patients with active PSS and three patients with keloids; these patients are frequently subjected to systemic or local steroid treatment. Specific binding of [^{3}H]dexamethasone could be demonstrated in both PSS and keloid cell lines (Table I). The mean of the PSS and keloid cell lines, 6.3±4.9 and 7.7±3.6 fmol/μg DNA, respectively, were not significantly different from the corresponding control value, 7.9±6.2 (mean ± SD). Also, the K_d values between the 3 groups were not significantly different (Table I). In further experiments glucocorticoid receptors were assayed from cell lines established from three patients with juvenile type diabetes mellitus. The mean of the diabetes cell lines was 5.3±1.3 fmol/μg DNA.

Table II. *Effect of dexamethasone pretreatment on the binding of [^{3}H]dexamethasone to specific receptors in cultured human skin fibroblasts*[a]

Cells	Specific binding of [^{3}H]dexamethasone	
	CPM×10^{-3}/mg cell protein	% of control
Control	9.78±1.11	100
Dexamethasone treated	6.04±1.49[b]	61.8

[a] Human skin fibroblasts were incubated for 4 days in the presence of 10^{-7} M dexamethasone, washed three times with PBS, and incubated further in steroid-free medium for 4 h at 37°C. The flasks were washed twice with PBS, the cells were collected by trypsinization and used for receptor assay, as described in Materials and Methods. The values are the mean ± SD of six parallel determinations.

[b] $p<0.001$ compared to the control value.

Effect of dexamethasone pre-treatment on the glucocorticoid receptor density

In the first experiment the cells in monolayer culture were incubated for 9 days in the presence of 10^{-7} M dexamethasone and 5% charcoal treated FCS. Thereafter, the cells were first washed four times and then incubated for 1 h at 37°C in serum free medium without dexamethasone, followed by 1 h of incubation with [^{3}H]dexamethasone for receptor assay. Since the half-time of dexamethasone-receptor dissociation in intact cells at 37°C has been shown to be 78 min (18), this treatment should be sufficient to dissociate most of the unlabelled dexamethasone from steroid receptors. The specific binding of [^{3}H]dexamethasone in samples pretreated for 9 days with unlabelled steroid was only 13% of the corresponding binding in control cells preincubated in the medium containing ethanol (Fig. 2). Parallel flasks were then incubated for 3 additional days without dexamethasone before assaying the receptors. The specific binding of dexamethasone had now returned to the same level as was noted in the control flasks (Fig. 2). In the second experiment, fibroblasts were pretreated with 10^{-7} M dexamethasone for 4 days, washed three times with PBS, and incubated for 4 h in medium without steroid before receptor assay. The specific binding of [^{3}H]dexamethasone decreased by 38% in cultures incubated with 10^{-7} M dexamethasone (Table II).

Demonstration that all-trans-retinoic acid does not affect the [^{3}H]dexamethasone binding

To explore the pharmacologic interactions of steroids and retinoids, the effects of all-*trans*-RA on the specific cellular binding of [^{3}H]dexamethasone were studied. First, skin fibroblasts in serum-free culture medium were incubated with 10^{-5} M all-*trans*-RA for 8 h. The receptor density, determined by the whole cell binding assay with [^{3}H]dexamethasone as ligand, was 22 100±3 680 sites per cell in control cultures (mean ± SD), while the corresponding value in all-*trans*-RA-treated cultures was 23 100±4 920. Furthermore, all-*trans*-RA added directly to the whole cell or cytosol assay in a final concentration of 10^{-5} M had no effect on the specific binding of [^{3}H]dexamethasone (results not shown).

DISCUSSION

The glucocorticoid receptor densities and the binding affinities were determined in 16 control fibroblast culture lines and also in skin fibroblast cultures established from patients with PSS or, keloids, two conditions which are frequently subjected to either systemic or

local treatment with glucocorticoid steroids. The presence of glucocorticoid steroid receptors could be clearly demonstrated in the cells established from these patients, but both the receptor densities and the binding affinities, on the average, did not differ from those noted in the control cell lines and were in good agreement with previous reports on determinations in human skin fibroblasts (8, 9) or human skin (10) utilizing different methodology.

Previous studies have demonstrated that steroids, such as progesterone, can decrease their own receptor densities in the cells (23–25); this phenomenon is known as down-regulation. To study the possibility that dexamethasone might also reduce the glucocorticoid receptor densities in human skin fibroblasts, the cells were preincubated in the presence of 10^{-7} M dexamethasone for 9 or 4 days. The results indicated that following the 9-day preincubation in the presence of unlabelled glucocorticoid, the specific binding of [^{3}H]dexamethasone was only about 13% of the binding found in the control cells. Following 4-day pretreatment with 10^{-7} M dexamethasone, the receptor density was 62% that in the control cells. The results from both experiments indicate that there is a significant reduction in the total number of receptors after treatment with dexamethasone (25). The mechanism for the reduction in receptor number in the dexamethasone-treated cells is not clear at this point. Two possible explanations could be offered, however: It is possible that dexamethasone inhibits the synthesis of the receptor protein in a manner of down-regulation. Recent studies employing glucocorticoid receptor cDNA have indicated that glucocorticoid treatment results in a marked decrease in the abundance of glucocorticoid receptor mRNA (26, 27). Alternatively, during the preincubation period with unlabelled dexamethasone the receptor-steroid complexes are translocated to the nucleus leading to their rapid turn-over (18). Nevertheless, the suppression of the receptor density in fibroblasts pretreated with dexamethasone may have relevance to tachyphylaxis clinically noted in patients subjected to prolonged treatment with steroids (28).

Since glucocorticoids and retinoids are often used in parallel or consecutive manner for treatment of various skin disorders, it was of interest to examine the effect of retinoids on dexamethasone binding to specific receptors. The results indicated that all-*trans*-RA in relatively high concentration did not affect the [^{3}H]dexamethasone binding either directly in whole cell or cytosol assay, or in fibroblast cultures incubated with all-*trans*-RA. Thus, retinoids appear not to interfere with glucocorticoid action at least at the level of specific steroid receptor binding.

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