Effects of Glucocorticoids on Deoxyribonucleic Acid (DNA) Synthesis Stimulated by Growth Factors in Cultured Rat Skin Fibroblasts

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The effects of glucocorticoids on deoxyribonucleic acid (DNA) synthesis were studied by using confluent cultured rat skin fibroblasts prepared by enzymatic dispersion and expanded up to passage 3. Dexamethasone caused the inhibition of the DNA synthesis stimulated by 10% fetal calf serum (FCS) in a dose dependent manner. Maximum inhibition (90%—100%) was achieved by the concentration of 10^{-7} M. A similar dose dependent inhibition was also obtained in the experiment using epidermal growth factor (EGF) (1 ng/ml) as a stimulant. Dexamethasone (10^{-7} M) also inhibited the DNA synthesis stimulated by somatomedin C (100 ng/ml) or platelet derived growth factor (1 half-maximum unit/ml) almost to control levels. Binding studies with ^{125}I-labeled EGF suggested that dexamethasone caused this inhibitory action without modulation of cell surface receptors for EGF.

Furthermore, the effects of a variety of glucocorticoids on the DNA synthesis were studied to clarify the structural requirement of glucocorticoids for the inhibition of the DNA synthesis. The results showed that 11β-hydroxyl and 21-hydroxyl groups on the steroid nucleus were necessary for the inhibition of the growth factor-stimulated DNA synthesis. Meanwhile, the inhibitory action on the DNA synthesis was markedly diminished by the replacement of a 16α-methyl group by a 16β-methyl group in the presence of a bulky group at C-17 (*e.g.* 17α-valerate). For further elucidation of mechanisms of action of glucocorticoids on the inhibition of the growth factor-stimulated DNA synthesis, the relationships between the structural features of glucocorticoids and their binding ability to the glucocorticoid receptor ([^{3}H]-dexamethasone-binding receptor) were studied. In most steroids (*e.g.* triamcinolone acetonide, betamethasone and hydrocortisone), the degree of the inhibition of DNA synthesis was almost correlated with the binding ability. But some steroids (*e.g.* progesterone, 11-deoxycorticosterone and betamethasone 17-valerate) which possessed no, or little, inhibitory action on the stimulated DNA synthesis showed a significant, or high binding, ability to glucocorticoid receptors. These results suggest that there are at least two kinds of structural requirements for the inhibitory action of glucocorticoids on DNA synthesis, namely at the stage of receptor binding and the stage of post-receptor binding events.

Keywords cultured rat skin fibroblast; DNA synthesis; glucocorticoid; glucocorticoid binding assay

It is well known that glucocorticolds show inhibitory effects on the proliferation of several kinds of fibroblasts.[1-4] However, the exact mechanism of the action of glucocorticoids is unknown. Although the structural requirements for the inhibition of deoxyribonucleic acid (DNA) synthesis have been partly studied,[1,2] it is not so obvious which structures are necessary for binding to the glucocorticoid receptros. Conversely, it was also reported that glucocorticoids stimulated the proliferation in some kinds of fibroblasts.[5-7] Thus, ideas on the actions of glucocorticoids are conflicting at present.

The great majority of these studies have been carried out using tumor or transformed cell lines. These cells are known to have lost many of the properties of normal fibroblasts.[8,9] It was suggested that a loss of normal cell properties might result in changes of the responses to glucocorticoids in these cells.

On the other hand, glucocorticoids exert catabolic effects on connective tissue. One of the known examples is skin atrophy, a side effect of anti-inflammatory action. It is thought that skin atrophy is partly caused by the suppression of the proliferative capacity of skin fibroblasts.[10,11]

These results suggest that the discrepancies of glucocorticoid action *in vitro* systems are due to the nature of the cellular models used *in vitro*. Therefore, it seems important for the study of glucocorticoid action to use the *in vitro* model systems having the same responses to glucocorticoids as in fibroblasts *in vivo*.

In this work, we prepared rat skin fibroblasts at a relatively low passage number by means of enzymatic dispersion of the skin of adult rats and used them as an *in vitro* model system for studying the mode of action of glucocorticoids on DNA synthesis stimulated by various growth factors. These studies using rat skin fibroblast seem to be also significant for understanding the diverse effect of glucocorticoids on various cell types. Although detailed studies are reported using several lymphoma cell lines,[12-14] the action of glucocorticoids seems to be somewhat different from those using rat skin fibroblasts. In lymphoma cell lines, glucocorticoids induce growth inhibition and eventually cause cell lysis. The lysis process is thought to be mediated through functional glucocorticoid receptors and closely related to the inhibition of growth.[15] However, the exact mechanism is unknown.

In our system, it was found that glucocorticoids showed inhibitory effects on the growth factor enhanced DNA synthesis of the cells. Then, the effects of dexamethasone on epidermal growth factor (EGF) binding to its receptor and structural requirements of glucocorticoids for the inhibition of the DNA synthesis were investigated, along with the structural requirements of glucocorticoids for binding to the glucocorticoid receptors of the cells. From these results, the mechanism of action of glucocorticoids on the proliferation of the rat skin fibroblasts has been discussed.

Materials and Methods

Materials Dexamethasone and collagenase were obtained from Wako Pure Chemicals, Osaka, EGF from Takara Shuzo, Kyoto, Somatomedin C (Sm-C) from the radiochemical centre, Amersham, and human platelet derived growth factor (PDGF) was obtained from Collaborative Research. Other steroids were mainly obtained from Sigma Chemical Company. [Methyl-^{3}H]-thymidine (6.7 Ci/mmol) and [^{3}H]-dexamethasone (49.9 Ci/mmol) were obtained from New England Nuclear.

Cell Cultures Rat skin fibroblasts were prepared as follows. The skin of adult male Wistar strain rats (49-d-old) was dispersed with trypsin (Gibco) and collagenase in order to prepare the fibroblasts at a relatively

low passage number in quantities sufficient for the present work.[16] Dispersed cells were cultured in Dulbecco's Modified Eagle medium (DME) containing 50 unit/ml penicillin, 50 μg/ml streptomycin and supplemented with 10% fetal calf serum (FCS) (filtron) at 37 °C in a humidified atmosphere containing 5% CO_2 in air. They were subcultured using 0.25% trypsin/0.2% ethylene diaminetetraacetic acid (EDTA) solution at a split ratio of 1 : 4. Stock cultures of rat skin fibroblasts (passage 3) were plated in 35 × 10-mm plastic dishes (Falcon) for experiments.

Assay of DNA Synthesis The DNA synthesis of the cells was assayed by measuring the incorporation of [methyl-^{3}H]-thymidine into cellular DNA. Confluent cells were incubated in 2 ml of DME containing 0.1% bovine serum albumin (BSA) (Sigma) for 48 h. After this preincubation, the medium was changed to 1 ml of DME plus experimental additions (see Results). The cells were further cultured for 20 h and were then incubated with [methyl-^{3}H]-thymidine (0.99 μCi/ml). After 2 h, the cells were washed with phosphate-buffered saline (PBS) and then immersed in 2 ml of 10% of trichloroacetic acid (TCA). They were solubilized by incubation at 37 °C for 1 h in 1 ml of 0.5 N NaOH after removing 10% TCA. The cells were then precipitated and washed with cold 10% TCA. DNA was then hydrolyzed by heating at 90 °C for 15 min in 0.5 ml of the same solution. Radioactivity in the hot TCA-soluble fraction was measured.[17] Cellular proteins were determined by the method of Bradford.[18]

Binding of EGF EGF was iodinated using the chloramine-T method of Carpenter and Cohen.[19] Confluent cells were incubated in 2 ml of DME containing 0.1% BSA for 48 h. The medium was changed to 1 ml of DME containing 0.1% BSA. The cells were then cultured in the fresh medium containing EGF (10 ng/ml) or EGF (10 ng/ml) plus dexamethasone (10^{-7} M) at various intervals over a 20 h period and washed three times in PBS. After this pretreatment, the cells were bound to [^{125}I]-EGF. [^{125}I]-EGF binding to the cells was measured by the method of Carpenter.[20]

Glucocorticoids Binding Assay Competition of specific dexamethasone binding to glucocorticoid receptors in the cells with various glucocorticoids was measured according to the methods of Griffin[21] and Kondo[22] with a slight modification. Confluent cells were incubated in 2 ml of DME containing 0.1% BSA. After 16 h, the cells were washed twice in DME containing 25 mM HEPES (*N*-2-hydroxylethyl-piperazine-*N*-ethane-sulfonic acid) pH 7.4 and 1 ml of the same medium was added to each dish. The cells were incubated in a concentration of 5×10^{-9} M [^{3}H]-dexamethasone with 10- and 100-fold molar excess of various glucocorticoid derivatives except betamethasone 17-valerate. After 45 min at 37 °C, the cells were washed five times with cold Hanks' solution containing 0.2% BSA and 25 mM HEPES, pH 7.4, and twice with PBS. The cells were solubilized with 1.2 ml of 0.05 N NaOH at 37 °C for 1 h. After the samples were neutralized, the radioactivity in 1 ml of sample was determined in 5 ml of Aquasol-2 (New England Nuclear).

Results

Effect of Dexamethasone on DNA Synthesis Figure 1 shows the effect of different concentrations of dexamethasone on the DNA synthesis stimulated by 10% FCS in cultured rat skin fibroblasts. Ten % FCS stimulated the DNA synthesis by about 70-fold compared with the control value (3×10^3 dpm/mg protein) shown in a closed circle. Dexamethasone markedly inhibited the stimulatory effect of 10% FCS in a dose dependent manner. Half maximum and maximum inhibition (90%) were obtained at about 10^{-8} and 10^{-7} M, respectively. Since FCS contains many nutrients and growth factors, it is too complex to elucidate the mechanisms of this growth inhibitory effect. To simplify and clarify this, the effects of dexamethasone on the DNA synthesis stimulated by various known growth factors were studied.

Figure 2 shows the effect of different concentrations of dexamethasone on the DNA synthesis stimulated by EGF (1 ng/ml). EGF stimulated DNA synthesis by about 7-fold compared with the control value shown in a closed circle and dexamethasone inhibited the DNA synthesis stimulated by EGF in a dose dependent manner. The dose response curves were almost the same as in the case of the 10% FCS

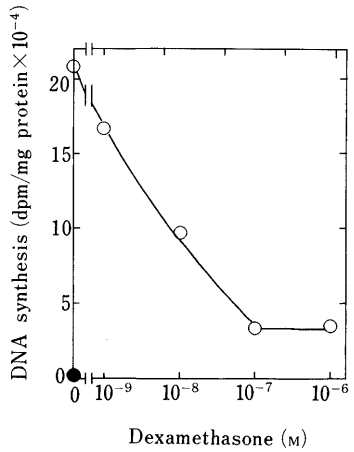

Fig. 1. Dose–Response Curves of the Effects of Dexamethasone on the Stimulation of DNA Synthesis by FCS in Cultured Rat Skin Fibroblasts

Cultured rat skin fibroblasts were stimulated by the addition of FCS (10%). Other experimental conditions are described in Materials and Methods. Values are means for duplicate dishes and expressed as dpm/mg protein. The closed circle represents the control value.

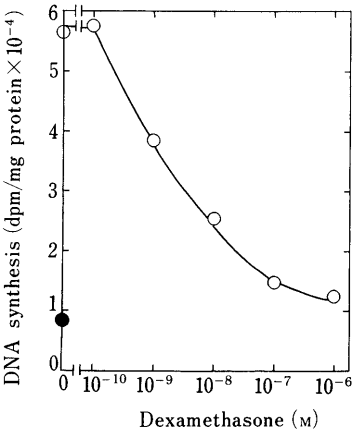

Fig. 2. Dose–Response Curves for the Effects of Dexamethasone on the Stimulation of DNA Synthesis by EGF in Cultured Rat Skin Fibroblasts

Cultured rat skin fibroblasts were stimulated by the addition of EGF (1 ng/ml) in the presence of FCS (1%). Other experimental conditions are described in Materials and Methods. Values are means for duplicate dishes and expressed as dpm/mg protein. The closed circle represents the control value.

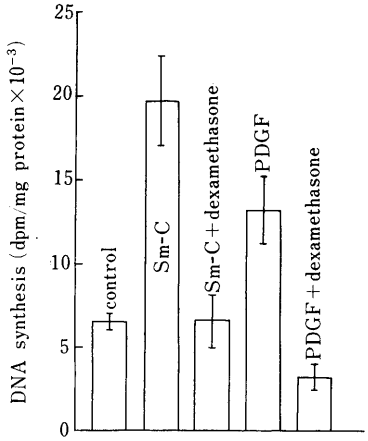

Fig. 3. Effects of Dexamethasone on the Stimulation of DNA Synthesis by Various Growth Factors in Cultured Rat Skin Fibroblasts

Cultured rat skin fibroblasts were stimulated by the addition of PDGF (1 half maximum unit/ml) or SmC (100 ng/ml) in the presence of FCS (1%). Dexamethasone (10^{-7} M) was added to the medium at the same time. Other experimental conditions are described in Materials and Methods. Values are means ± S.D. for two experiments of duplicate dishes and expressed as dpm/mg protein.

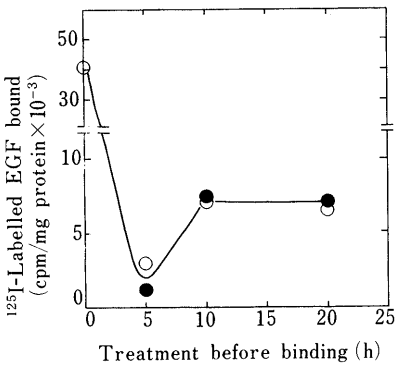

Fig. 4. Time Course of the Effect of Dexamethasone on the Binding of ^{125}I-EGF

Experimental conditions are described in Materials and Methods. Specific binding was calculated by the subtraction of nonspecific binding (in the presence of 20 μg/ml of unlabeled EGF) from total binding. Values are means for two experiments of duplicate dishes and expressed as cpm/mg protein. Open and closed circles represent the values in the case of pretreatment with EGF plus dexamethasone and EGF, respectively.

stimulated DNA synthesis shown in Fig. 1. Figure 3 shows the effects of dexamethasone (10^{-7} M) on the DNA synthesis stimulated by other growth factors. PDGF (1 half-maximum unit/ml) and Sm-C (100 ng/ml) stimulated DNA synthesis by about 2- and 3-fold, respectively. Dexamethasone (10^{-7} M) inhibited the DNA synthesis stimulated by PDGF or Sm-C almost to the control levels.

Effect of Dexamethasone on the Binding of [^{125}I]-EGF We further studied whether or not the inhibition of the DNA synthesis stimulated by these growth factors was due to the modulation of growth factor receptors. Figure 4 shows the effect of dexamethasone on the binding of [^{125}I]-EGF to its receptors. In these studies it is necessary to design the experimental conditions in which EGF stimulates DNA synthesis. Stimulation of the DNA synthesis requires at least 12 h exposure of the EGF to the cells (data not shown). Similar results were shown in the experiment using cultured human skin fibroblasts.[23] Therefore, EGF was preincubated with or without dexamethasone (10^{-7} M) for 5, 10 and 20 h. [^{125}I]-EGF was then bound to the cell surface receptors for 1 h at 37 °C according to the method of Carpenter.[20] Within 5 h of exposure of EGF alone, the cells lost about 80% of their binding capacity to [^{125}I]-EGF. Thereafter, the cells restored their binding capacity to about 50% of the original level within 10 h. Dexamethasone caused no significant effect in up to 20 h of treatment. It also caused no significant effect on the binding of [^{125}I]-EGF to the cell surface receptors upon preincubation with EGF in the absence of EGF (data not shown).

Effect of Various Glucocorticoids on DNA Synthesis Next, we studied the structural requirements of glucocorticoids for the inhibition of growth factor-stimulated DNA synthesis in cultured rat skin fibroblasts. Figure 5 shows the effects of a variety of natural occurring glucocorticoids and their precursors at several concentrations in the DNA synthesis stimulated by 10% FCS. The values are expressed as % of control of DNA synthesis. There are apparent relationships between the structural features of glucocorticoids and their inhibitory effects. Progesterone (pregn-4-ene-3,20-dione), which is a typical gestagen, had no inhibitory effects up to 10^{-5} M. Re-

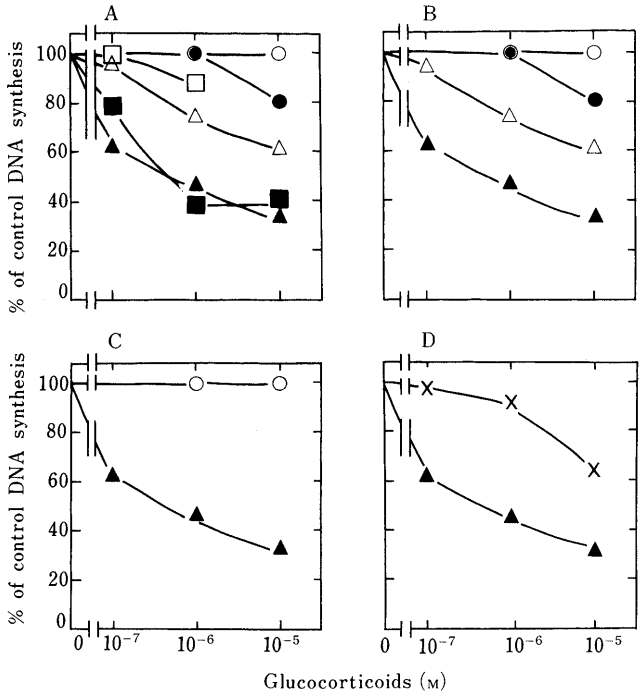

Fig. 5. Effects of a Variety of Natural Occurring Glucocorticoids and Their Precursor on DNA Synthesis Stimulated by 10% FCS in Cultured Rat Skin Fibroblasts

The values show averages from two experiments of duplicate dishes and are expressed as % of control DNA synthesis stimulated by 10% FCS. Experimental conditions are described in Materials and Methods. The steroids are progesterone (○); 11-deoxycorticosterone (●); 21-deoxycortisone (□); 11β-hydroxy progesterone (△); hydrocortisone (×); cortisone (■); corticosterone (▲); A: Effects of substitution of a hydroxymethyl group for a methyl group at C-21 (open *vs.* closed symbol). B: Effects of introduction of 11β-hydroxyl group (circle *vs.* triangle). C: Effects of substitution of hydroxymethyl group for methyl group at C-21 plus introduction of 11β-hydroxyl group. D: Effects of introduction of 17α-hydroxyl group.

placement of a 21-methyl group of progesterone by a 21-hydroxymethyl group (11-deoxycorticosterone; 21-hydroxypregn-4-ene-3,20-dione) caused an increase in the inhibitory effect (Fig. 5A). Similar results were also observed from 21-deoxycortisone (17-hydroxypregn-4-ene-3,11,20-trione) to cortisone (17,21-dihydroxypregn-4-ene-3,11,20-trione) and from 11β-hydroxyprogesterone (11β-hydroxypregn-4-ene-3,20-dione) to corticosterone (11β,21-dihydroxypregn-4-ene-3,20-dione) (Fig. 5A). This indicates that the 21-hydroxymethyl group may enhance the inhibitory effect. Also, the introduction of the 11β-hydroxyl group results in a marked enhancement of the inhibitory effect as seen in the cases from progesterone to 11β-hydroxyprogesterone and from 11-deoxycorticosterone to corticosterone (Fig. 5B). 21-Hydroxymethyl group substitution and the introduction of the 11β-hydroxyl group had an additional effect on the inhibition of the growth factor-stimulated DNA synthesis as seen in the case from progesterone to corticosterone (Fig. 5C). On the other hand, the introduction of the 17α-hydroxyl group rather suppressed the inhibitory effect (corticosterone to hydrocortisone; 11β,17,21-trihydroxypregn-4-ene-3,20-dione) (Fig. 5D). Also, the introduction of the 17α-hydroxyl group may restrict the conformation of the 21-hydroxymethyl group, thereby affecting its contribution to the inhibitory action on the DNA synthesis.

Figure 6 shows the effects of a variety of synthetic glucocorticoids on the DNA synthesis stimulated by 10%

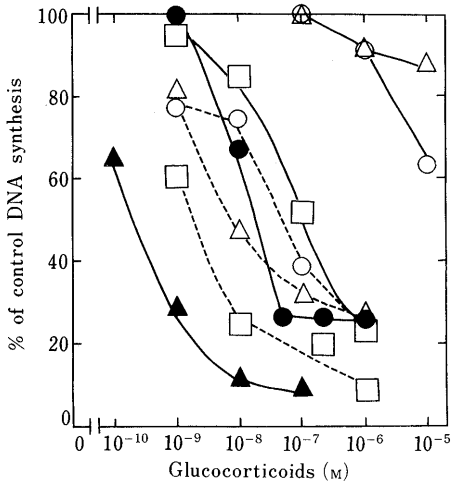

Fig. 6. Effects of a Variety of Synthetic Glucocorticoids on DNA Synthesis Stimulated by 10% FCS in Cultured Rat Skin Fibroblasts

The values show averages from two experiments of duplicate dishes and are expressed as % of control DNA synthesis stimulated by 10% FCS. Experimental conditions are described in Materials and Methods. The steroids are betamethasone 17-valerate (△—△); dexamethasone 17-valerate (□—□); prednisolone (○---○); dexamethasone (△---△); triamcinolone acetonide (□---□); betamethasone (●—●); halcinonide (▲—▲); hydrocortisone (○—○).

FCS. There are also apparent relationships between their structural features and inhibitory effects. Prednisolone, (11β,17,21-trihydroxy-pregna-1,4-diene-3,20-dione), dexamethasone (16α-methyl-9α-fluoroprednisolone) and triamcinolone acetonide (9α-fluoro-16α,17-isopropylidenedioxy-prednisolone) were more potent than the glucocorticoids described above. The concentrations necessary for the half maximum inhibition by prednisolone, dexamethasone and triamcinolone acetonide are estimated as about 7×10^{-8}, 1×10^{-8} and 3×10^{-9} M, respectively. As shown in Fig. 6, the introduction of the double bond between C-1 and C-2 at ring A caused an increase in the inhibitory effect (hydrocortisone to prednisolone). And the introduction of the 9α-fluoro and 16α-methyl groups to prednisolone caused an increase in the inhibitory effect (dexamethasone). Also, the introduction of the 9α-fluoro group and 16,17-acetonide to prednisolone caused more enhancement of the inhibitory effect than the introduction described above (triamcinolone acetonide). These results show that the introduction of the 16,17-acetonide group, the 9α-fluoro and the 16α-methyl group effectively enhances the steroidal inhibition of the growth factor-stimulated DNA synthesis.

Similar inhibitory effects were also observed in dexamethasone 17-valerate and betamethasone (9α-fluoro-16β-methylprednisolone). From the results shown in Fig. 6, the doses of dexamethasone 17-valerate and betamethasone required for the half maximum inhibition are estimated to be 2×10^{-8} and 3×10^{-8} M, respectively. However, esterification of betamethasone at the C-17 hydroxyl group with valerate (betamethasone 17-valerate) caused a marked decrease in the inhibitory effect. In other words, replacement of the 16α-methyl group of dexamethasone 17-valerate by the 16β-methyl group (betamethasone 17-valerate) caused a marked decrease in the inhibitory effect.

The other steroids having both a 16β-methyl group and a bulky group at C-17 *e.g.*, betamethasone 17,21-dipropionate and beclomethasone dipropionate (9-chloro, 16β-methyl prednisolone 17,21-dipropionate) also showed

TABLE I. Specific Binding of Glucocorticoids in Cultured Rat Skin Fibroblasts

Competing steroids	Specific binding (% competition)	
	10-fold molar excess	100-fold molar excess
Triamcinolone acetonide	92.8± 6.8	89.6± 2.9
Betamethasone	59.3± 7.0	103.8± 7.7
Hydrocortisone	41.7± 8.2	84.1± 5.0
Corticosterone	46.7±12.1	82.1± 8.6
Progesterone	35.6± 4.8	72.8± 9.0
11-Deoxycorticosterone	34.1± 5.1	76.7±12.9
11β-Hydroxyprogesterone	26.7± 9.7	53.1± 9.2
21-Deoxycortisone	4.8± 4.8	1.4± 1.9
Cortisone	0.7± 0.6	43.1± 6.3
Betamethasone 17-valerate	97.6± 2.8	(91.5±6.6)[a]

The values are means±S.D. from two experiments of duplicate dishes and expressed as percentage binding of [^{3}H]-dexamethasone relative to control incubation (no unlabeled steroid added) after substitution of nonspecific binding (in the presence of 100-fold molar excess of unlabeled dexamethasone). [^{3}H]-Dexamethasone was added to the incubation medium at the concentration of 5×10^{-9} M. Other experimental conditions are described in Materials and Methods. *a*) The parenthesized value shows the result on the equal molar condition.

no inhibitory action, even at the concentration of 10^{-5} M (data not shown). These results indicated that the diminishment of inhibitory action of these steroids was neither due to the presence of the 16β-methyl group only nor of a bulky group at C-17, but due to the presence of both 16β- and 17α-substituents. Therefore, the substituents at positions C-16 and C-17 seem to be complexly associated with each other for the inhibitory action of DNA synthesis.

Among these steroids examined, halcinonide (21-chloro-9α-fluoro-11β-hydroxy-16α,17α-isopropylidenedioxy-pregn-4-ene-3,20-dione) caused the most potent inhibitory effects on the DNA synthesis. Its half maximum dose was estimated to be about 3×10^{-10} M. Halcinonide differs from triamcinolone acetonide with respect to the deletion of a double bond between C-1 and C-2 in ring A, which is effective for the increased inhibition of the DNA synthesis as described above, and the replacement of the 21-hydroxymethyl group by the 21-chloryl methyl group. These results show that the replacement of a 21-hydroxymethyl group by a 21-chloryl methyl group is effective for the enhancement of the inhibitory effects of steroids on the DNA synthesis.

Effects of Various Glucocorticoids on the Binding of [^{3}H]-Dexamethasone Table I shows the specific binding of glucocorticoids in cultured rat skin fibroblasts. Specific bindings of glucocorticoids were assayed by competition with the glucocorticoid for [^{3}H]-dexamethasone binding sites. Triamcinolone acetonide almost completely displaced the binding of [^{3}H]-dexamethasone at only 10-fold molar excess. Betamethasone was also effective but less potent than triamcinolone acetonide at 10-fold molar excess. At 100-fold molar excess, betamethasone completely displaced the binding.

Corticosterone showed a relatively weak displacement effect compared with triamcinolone acetonide and betamethasone at 10-fold and 100-fold molar excess. 11β-Hydroxyprogesterone showed about 20 and 30% less potent displacement effects than corticosterone at 10-fold and 100-fold molar excess, respectively. The degree of displacement by these glucocorticoids almost correlated

with the inhibitory effects on the growth factor-stimulated DNA synthesis.

Unexpectedly, the efficient displacements were also found in steroids such as progesterone, 11-deoxycorticosterone which had no, or only slight, inhibitory effects on the growth factor-stimulated DNA synthesis, as already shown in Fig. 5. The degree of the displacement by both steroids was almost the same as that of corticosterone which showed a significant inhibitory effect on DNA synthesis. In this regard, betamethasone 17-valerate was the most interesting example among the steroids examined. Betamethasone 17-valerate almost completely displaced the binding of [^{3}H]-dexamethasone to its receptors even at equal molar. But this steroid has little inhibitory effect on DNA synthesis as shown in Fig. 6.

On the other hand, the steroids having an 11-keto group, such as 21-deoxycortisone or cortisone, showed rather poor displacement effects compared with their inhibitory effects on DNA synthesis. In contrast to the case of the effects on the inhibitory action on DNA synthesis (Fig. 5D), introduction of the 17α-hydroxyl group did not inhibit the binding ability of a steroid to the receptor (*e.g.* corticosterone→hydrocortisone).

Discussion

In this work, we prepred rat skin fibroblasts at a relatively low passage number by means of enzymatic dispersion of the skin of adult rats and used them as an *in vitro* model system for studying the mode of action of glucocorticoids on DNA synthesis stimulated by various growth factors.

In most cases, skin fibroblasts are prepared by the method using the migration of fibroblasts from the skin explants.[24] At first we tried to prepare rat skin fibroblasts by this method. However, because of a low yield of cells, this method was not so useful as compared with enzymatic digestion. Probably, fibroblasts do not migrate well from rat skin explants.

In our system, it was found that some glucocorticoids, like dexamethasone, had an inhibitory effect on DNA synthesis stimulated by growth factors such as 10% FCS, EGF, PDGF or Sm-C. The results of EGF receptor assay suggest that the inhibition of the EGF-stimulated DNA synthesis by dexamethasone is not due to the modulation of cell surface receptors for EGF. Probably, glucocorticoids suppress certain growth signal transduction which might be common to several growth factors.

Recently, it has been shown that c-*fos* and c-*myc* genes are expressed by the addition of several growth factors in cultured cells and these proto-oncogene expressions are important for cellular growth signal transduction.[25-28] It was also reported that the c-*fos* messenger ribonucleic acid (mRNA) level increased, reached a peak at 30 min, and then declined to the control level after 60 min in normal human fibroblasts exposed to EGF or PDGF.[29] But the c-*myc* mRNA level increased at later times compared with the c-*fos* mRNA expression and remained elevated throughout 24 h in the similar experiment.[29] In our experiments, the inhibition of DNA synthesis by dexamethasone was found more than 8 h later, after the addition of EGF (data not shown). In more recent studies, it is reported that glucocorticoids also reduce c-*myc* mRNA level in human T lymphoblastic leukemic cell line CCRF-CEM and avian oviduct tissue, as well as inhibit the growth of the cells.[30,31] From the close correlation of the inhibition of growth and c-*myc* expression, it is suggested that regulation of c-*myc* expression by glucocorticoid may be a key event mediating steroid control of the proliferation. These findings suggest that it is possible that dexamethasone may inhibit c-*myc* gene expression in rat skin fibroblasts and that the inhibition may be one of the mechanisms of the growth inhibitory action of dexamethasone in this cell.

There are certain structural requirements of glucocorticoids for the inhibition of the DNA synthesis. The presence of 11β-hydroxyl and 21-hydroxyl groups on the steroid nucleus were necessary to the inhibition, while the introduction of the 17α-hydroxyl group rather suppressed the inhibitory effect (Fig. 5). Introduction of the double bond between C-1 and C-2 at ring A, the 16,17-acetonide group, the 9α-fluoro and the 16α-methyl group is effective for the enhancement of steroidal inhibition of the growth factor-stimulated DNA synthesis (Fig. 6). The latter findings are well correlated with the reports using cultured fibroblasts.[1,2] Therefore, the necessity and efficacy of these structures seem to be common to some extent, independent of the kinds of fibroblasts. However, the mechanism is unknown.

On the other hand, the inhibitory action of 16α-methyl substituted glucocorticoids was markedly diminished by the replacement of the 16α-methyl group by the 16β-methyl group in the presence of a bulky group at C-17 *e.g.* 17α-valerate (Fig. 6). Interestingly, the effect on DNA synthesis doesn't always correlate with the binding to glucocorticoid receptors in some steroids as shown in Figs. 5, 6 and Table I. These results suggest that steroid binding to glucocorticoid receptors seems to be necessary but not enough for the inhibition of DNA synthesis. In other words, it is highly possible that there may be at least two kinds of structural requirements of glucocorticoids for their inhibitory action on the growth factor-stimulated DNA synthesis in cultured rat skin fibroblasts: one is for binding to glucocorticoid receptors, another is for post-receptor-binding events.

It is generally agreed that glucocorticoids penetrate the membrane of the target cells and associate with specific cytoplasmic receptor proteins.[32,33] Subsequently, the receptor–steroid complexes are thought to undergo conformational changes which allow them to associate with the nucleus and bind to specific DNA sequences at the nucleus.[32,33] These events result in the alteration of specific gene expression.

On this hypothesis, one explanation of the efficient displacement, but slight or no inhibition, on DNA synthesis by some steroids may be as follows: Progesterone and 11-deoxycorticosterone bind to glucocorticoid receptors but don't, or only slightly, inhibit the DNA synthesis. Therefore, the structures of these steroids are enough for the binding, but not enough for causing the conformational changes. Since the introduction of the 11β-hydroxyl group and the 21-hydroxymethyl group to these structures resulted in no significant change of the displacement but caused the efficient inhibition of the DNA synthesis, the 11β-hydroxyl group and the 21-hydroxymethyl group seem to be necessary for causing the subsequent conformational changes. When the conformational changes are caused by these sub-

stituents, the receptor–steroid complexes may be able to associate with the nucleus to alter specific gene expressions and thereby to inhibit DNA synthesis. But we can't exclude the possibility that these structures affect the stability of the complex and the steroids themselves. For further discussion, it is necessary to study whether or not the binding of the glucocorticoid receptor complex to the glucocorticoid responsible genes and the transcription of such genes depends on the structure of a given stetoid *e.g.* progesterone or 11-deoxycorticosterone and its 11β-hydroxyl group and 21-hydroxymethyl group derivatives.

In relation to this, the action of betamethasone 17-valerate seems to be interesting. Betamethasone 17-valerate had almost no inhibitory effect on DNA synthesis, but highly efficiently displaced the binding of [^{3}H]-dexamethasone to its receptors as shown in Fig. 6 and Table I. This discrepancy is probably because the conformational changes of receptor–steroid complexes may be disturbed by the 16β-methyl group in the presence of 17-valerate or *vice versa*. This seems to be highly probable from the comparison of the structure of betamethasone 17-valerate with those of betamethasone and dexamethasone 17-valerate which show marked inhibitory action on the DNA synthesis (Fig. 6).

On the other hand, 21-deoxycortisone had no significant displacement effect even at 100-fold excess molar. Cortisone had no significant effect at 10-fold excess molar and caused only 40% displacement even at 100-fold excess molar. Cortisone and 21-deoxycortisone have 11-ketone. This substituent was absent in the steroids which show efficient displacement effects as described above. These results suggest that the presence of 11-ketone decreases in the binding of glucocorticoids to their receptors. Although cortisone showed rather weak displacement effects in this experiment, it inhibited DNA synthesis efficiently as shown in Fig. 5. On the other hand, the displacement effect of hydrocortisone was higher than that of cortisone. However, the inhibitory effect of hydrocortisone was weaker than that of cortisone as shown in Fig. 5A and D. In L-929 fibroblasts, cortisone had no effect on the growth of the cells.[1] It is known that cortisone has an anti-inflammatory activity *in vivo* only when it can be transformed to hydrocortisone.[34] It was also shown that the biotransformation of cortisone and hydrocortisone was minimal in L-929 fibroblasts.[35] In rat skin fibroblast, however, the mode of action of cortisone seems to be dfferent from that in L-929 fibroblast as mentioned above. Although the binding of cortisone is low in rat skin fibroblast, cortisone itself, or the binding of cortisone to its receptors, may be relatively stable during the assay of DNA synthesis. A rather long culture time is needed and consequentially cortisone may cause the efficient inhibition of DNA synthesis.

In this paper, we showed that various glucocorticoids inhibited the DNA synthesis stimulated by 10% FCS or various growth factors in cultured skin fibroblasts, and that the inhibitory effect was not caused by the modulation of cell surface receptors for EGF. Furthermore, we clarified the structures which are necessary, or conversely suppressive, for the inhibitory action of DNA synthesis and binding to glucocorticoid receptors. On the basis of these results, steroids can be classified at least into three groups: 1) steroids which possess no, or little, binding ability to the glucocorticoid receptor as well as no, or little, inhibitory action on the DNA synthesis; 2) steroids which possess significant binding ability, but no, or little, inhibitory action on the DNA synthesis; 3) steroids which possess binding ability that is comparable to the inhibitory activity on the DNA synthesis. These results suggest that there are at least two kinds of structural requirements for the inhibitory action of glucocorticoids on DNA synthesis, namely, at the stage of receptor binding and at the stage of post-receptor binding events. Various steroids which possess different biological features will be useful as tools for the study of the action mechanisms of glucocorticoids in DNA synthesis.

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