Modulation of Collagen Metabolism in Cultured Human Skin Fibroblasts by Dexamethasone: Correlation with Glucocorticoid Receptor Density*

AARNE OIKARINEN,[1,2] HELJÄ OIKARINEN,[2] ELAINE M. L. TAN[2] and JOUNI UITTO[2]

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

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Connective tissue metabolism was studied in detail in three human skin fibroblast lines, demonstrating low, medium, or high levels of glucocorticoid receptor densities. In the cell lines with low and medium receptor density, dexamethasone, in the range of 10^{-5}–10^{-9} M, had no effect on collagen production, using short incubation time periods and high (20%) fetal calf serum concentration, while in the cells with highest receptor density, a slight stimulation of collagen synthesis was noted in the concentration range 10^{-6}–10^{-9} M. In the presence of low concentration (0.5%) of serum, dexamethasone markedly inhibited collagen production. The production of collagenase, assayed by degradation of ^{3}H-labelled type I collagen substrate with a brief trypsin activation of the enzyme, was reduced in a dose dependent manner in all 3 cell lines, the inhibition with 10^{-5} dexamethasone being up to 56% of the control. Similarly, the activity of an elastase-like neutral protease was decreased in the presence of dexamethasone. Thus the results indicate that glucocorticoids may have profound effects on the degradation of connective tissue components, while the effects on collagen synthesis may be more variable depending on the environmental milieu. (Received July 4, 1986.)

A. Oikarinen, Department of Dermatology, University of Oulu, Kajaanintie 50, SF-90220 Oulu 22, Finland.

Glucocorticosteroids are widely used for treatment of a spectrum of skin disorders either by topical application, intralesional injections or oral administration (2, 3 for reviews). One of the recognized side effects of the prolonged use of glucocorticoids is the development of dermal atrophy (4). Since collagen is the main component of the dermal connective tissue, comprising approximately 80% of the dry weight of the dermis (5), one would predict that a prolonged glucocorticoid treatment leads to loss of collagen either through suppression of the production or enhancement of the degradation of this protein.

Fibroblasts in culture have provided a useful model to study the effects of a variety of pharmacologic agents on the metabolism of collagen (6). In several studies, the effects of glucocorticoids on collagen metabolism have been examined by employing cultured human skin fibroblasts (7). Careful analysis of these studies reveals, however, that the results are conflicting; indeed increased, unaltered and decreased collagen productions have all

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Abbreviations used: SDS, sodium dodecyl fulfate; Na_2 EDTA, disodium ethylenediaminetetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; NEM, N-ethylmaleimide; SAPNA, succinyl-(L-Alanyl)$_3$-paranitroanilide; FCS, fetal calf serum; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate buffered saline; HBSS, Hank's Balanced Salt Solution; Kd, dissociation constant.

been reported, even though apparently similar culture conditions and steroid concentrations have been employed (8, 9 for summary of these studies).

Glucocorticoids mediate their effects on the cellular metabolism by binding to specific cytoplasmic receptors (10–12 for reviews). 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 molecule. The precursor molecule is processed to form the functional mRNA molecule, which serves as a template for the synthesis of a specific protein. A plausible explanation for the conflicting results observed in previous in vitro studies would be that the number and the binding capacity of the glucocorticoid receptors are variable in cultured cells and such variation would then correlate with the changes in collagen metabolism. Thus, in the present study, we have examined dexamethasone modulation of collagen metabolism in fibroblast cultures employing three different cell lines characterized by low, intermediate or high density of glucocorticoid receptors.

MATERIALS AND METHODS

Human skin fibroblast cultures

Cell cultures were initiated from punch biopsies or from skin obtained from surgical procedures performed for cosmetic reasons, after obtaining informed consent. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing glutamine (KC Biologicals) and supplemented with 30 mM Hepes buffer, pH 7.6, 200 units/ml of penicillin, 200 μg/ml of streptomycin, and 20% fetal calf serum (FCS). Confluent primary cultures were trypsinized and subcultured in tissue culture flasks (Falcon Plastics) at 37°C. The cells were studied in passages 3–6.

Glucocorticoid receptor assay.

For the glucocorticoid receptor assay, a whole cell assay was adapted (13, 14). Confluent skin fibroblasts were harvested by brief trypsinization in Hank's Balanced Salt Solution (HBSS). Trypsin was inhibited by the addition of soybean trypsin inhibitor, the cells were washed twice by centrifugation, and finally resuspended in HBSS, $0.15–1.5\times10^6$ cells/ml. For the receptor assay, duplicate 0.8 ml aliquots of the cell suspension were pipetted into siliconized glass tubes, followed by 0.2 ml of HBSS containing [6,7-^{3}H] dexamethasone, the final concentration of the steroid varying from 0.5–100 nM. For the estimation of non-specific binding, parallel sets of tubes containing a 200-fold excess of unlabelled dexamethasone were included in each assay. The tubes were incubated for 45 min at 37°C with intermittent shaking. Two ml of HBSS (4°C) were then added to each tube and the tubes were centrifuged at 600×g at 22°C. Supernatants were discarded and the pellets were resuspended in 3 ml of HBSS. The tubes were incubated for 10 min at 22°C, and the cells were collected by centrifugation. The washing procedure was repeated once, and the cell pellets wre resuspended in a mixture of 0.5 ml PBS and 1 ml ethanol. Samples were then dissolved in 10 ml of counting solution for liquid scintillation counting. The maximum binding and the binding affinity (Kd) were estimated from Scatchard plots (15).

Collagen biosynthesis

To measure the rate of procollagen synthesis, two types of experimental designs were employed. First, the cells were plated in 24-well tissue culture plates (Falcon Multiwell Tissue Culture) of 4.2×10^4 cells per well, in 1.0 ml of DMEM containing 20% FCS. Cells were allowed to attach for 24 h, and the medium was replaced by adding fresh DMEM containing 20% dialysed FCS and dexamethasone in different concentrations. Dexamethasone was dissolved in ethanol, and the final concentration of ethanol was adjusted to the same percentage (0.05% v/v) in each well. In all experiments, the control cells were incubated in the presence of the same final concentration of ethanol. The cells were preincubated for 48 h, and the medium was then replaced by fresh DMEM containing 20% FCS, 25 μg/ml of ascorbic acid and dexamethasone in the same concentrations as during the preincubation period. The cells were labelled with [^{3}H]proline (10 μCi/well) for 24 h. After incubation, the plates were cooled on ice and the medium was separated. Cells were briefly sonicated in 0.5 ml of 0.4 M NaCl, 0.1 M Tris-HCl, pH 7.5, containing 10 mM Na_2EDTA, 10 mM NEM, and 1 mM PMSF. The media and the cell sonicates from the corresponding wells were then combined and dialysed in a microdialysis unit (BRL 1200MD) against a buffer containing 1 mM NEM, 0.1 mM PMSF, 1 mM EDTA, 0.15 M NaCl, all in 0.05 M Tris-HCl, pH 7.6, for 72 h. The samples were hydrolyzed and

assayed for total ^{3}H-radioactivity and [^{3}H]hydroxyproline (16). In all experiments, parallel wells were incubated as above, but after preincubation with dexamethasone, the cultures were not labelled with [^{3}H]proline; instead they were incubated with 20% FCS and dexamethasone as indicated, and used for cell number determination by hemocytometer.

In the second type of experiments, cells wre plated in 25 cm^2 plastic flasks (Falcon Plastics). After reaching early confluency, the cells were incubated for 48 h in DMEM containing 5% or 0.5% dialysed FCS and various concentrations of dexamethasone; control flasks contained ethanol instead of dexamethasone. Medium was then replaced with serum-free DMEM containing 25 μg/ml ascorbic acid and dexamethasone in varying concentrations, and the cultures were labelled for 4–8 h with [^{3}H]proline (50 μCi/flask). After incubation, the medium was separated, and the cells were collected and sonicated, as above. Medium and an aliquot of cell suspension were separately dialysed against running tap water for 48 h, hydrolyzed, and assayed for total ^{3}H-radioactivity and [^{3}H]hydroxyproline (16). The remaining of the cell sonicate was used for DNA (17) and protein (18) determinations.

To study the effects of dexamethasone on the synthesis of genetically distinct collagen types, the cells were preincubated for 48 h in the presence of dexamethasone (10^{-7} M] or with ethanol. The medium was replaced by fresh DMEM containing 20% dialyzed FCS, dexamethasone and ascorbic acid (25 μg/ml), and the cells were labelled with 50 μCi of [^{3}H]proline for 24 h, as above. The media were removed into tubes containing protease inhibitors (see above), dialysed against 0.5 N acetic acid at 4°C for 20 h, and pepsinized for 6 h at 22°C. The collagenous proteins were precipitated by adding 1.8 M NaCl, and the precipitates were recovered by centrifugation at 15000×g for 30 min at 4°C. The precipitates were heated in 10% SDS for 5 min at 100°C, and dialysed against 0.125 M Tris-HCl, pH 6.8, containing 2% SDS, 0.01% bromophenol blue, and 10% glycerol. The proteins were electrophoresed on 6% polyacrylamide gels (19) and ^{3}H-polypeptides were visualized by fluorography (20). The bands representing α-chains of type I and type III collagens were quantitated by densitometer.

Thymidine incorporation studies

For DNA synthesis synthesis studies, cells were plated in 24-well microwell plates as described above, and preincubated in the presence of 20% dialysed FCS, DMEM and dexamethasone in varying concentrations, for 48 h. The medium was changed to fresh DMEM containing 20% FCS and the steroid, and the cultures were labelled with [^{3}H]thymidine (3 μCi/well) for 6 h. The medium was removed, and the cells were incubated in 0.5 ml of 0.25% trypsin solution for 20 min at 37°C. Cells were harvested by miltiple automatic sample harvester (MASH II, M.A. Bioproducts), collected on filters, and the radioactivity on the filters was determined by liquid scintillation counting.

Enzyme assays

For assay of collagenase and elastase activity, cells were plated in 25 cm^2 flasks and preincubated in DMEM containing 20% FCS and dexamethasone (or ethanol) for 48 h. The media were then changed, and the cells were rinsed twice with serum-free DMEM. Thereafter, 3 ml of fresh DMEM containing dexamethasone (or ethanol) was added, and the cultures were incubated for 6 h. After incubation, the media were collected and assayed for collagenase and elastase activity. Cells were used for protein (18) and DNA (17) assays, as described above.

For collagenase assay, the enzyme preparations were activated by trypsin proteolysis (5 μg/ml) for 10 min at 25°C, and trypsin was then inactivated by soybean trypsin inhibitor. Collagenase activity was assayed by incubating samples with ^{3}H-labelled type 1 collagen. In a standard incubation, 40 μl aliquots of medium were incubated in 50 mM Tris-HCl, pH 7.6, containing 10 mM $CaCl_2$, 0.15 M NaCl, and 20 μg/ml bovine serum albumin, in a final volume of 100 μl, for 4 h at 37°C (21). The reaction was stopped by the addition of 40 mM Na_2EDTA, and the samples were treated by a mixture of trypsin and α-chymotrypsin, employing conditions under which the undegraded collagen substrate resists proteolysis, while the collagen degradation products are digested to TCA-soluble peptides. The enzyme activity was expressed as degradation of ^{3}H-labelled collagen, cpm per μg DNA/h.

Elastase activity was assayed by incubating 50–100 μl aliquots of the media with a synthetic substrate, Succinyl-L-(Alanyl^3)-PNA (SAPNA) in 50 mM Tris-HCl, pH 7.8, up to 24 h. The reaction was monitored by the change in the absorbance at 410 nm (22). The enzyme activity was expressed as hydrolysis of SAPNA, nmol per mg DNA/h.

For statistical analyses, Student's *t*-test was used.

RESULTS

Assay of receptor densites in fibroblast cultures

In initial experiments, the glucocorticoid receptor densities and binding affinities were assayed in 16 human skin fibroblast cultures by a whole-cell binding assay using [^{3}H]dexa-

methasone as ligand (23). After initial screening, three cell lines were selected for detailed studies on collagen metabolism; these cell lines contained either low, intermediate or high levels of receptors (Table I).

Correlation between receptor density and glucocorticoid-mediated changes in procollagen production

The effects of dexamethasone on collagen production and total protein synthesis were studied by incubating fibroblast cultures with [^{3}H]proline. Collagen production was assayed by the synthesis of nondialysable [^{3}H]hydroxyproline and the total protein synthesis was measured by incorporation of ^{3}H-radioactivity into nondialysable fraction. Since optimal collagen production by human skin fibroblasts in culture requires the presence of serum (24), and FCS clearly does not influence the binding of dexamethasone to the receptors (23), the first experiments were performed in the presence of 20% dialysed FCS. The results indicated that total protein synthesis was slightly increased in cell lines demonstrating the low and high receptor densities, when incubated with dexamethasone in concentrations varying from 10^{-6} to 10^{-9} (Fig. 1 A and C). No clear effect was detected in cell cultures with intermediate receptor density (Fig. 1 B). When [^{3}H]hydroxyproline was assayed as an index of collagen production, no changes as a result of dexamethasone treatment could be observed in cultures with low and intermediate receptor density, while a slight stimulation in collagen production with dexamethasone in 10^{-6} to 10^{-8} M concentrations was noted in the cell cultures with the highest receptor density (Fig. 1).

Further experiments were performed in the presence of 20% FCS to examine the effects of dexamethasone on DNA synthesis by these cells, as detected by [^{3}H]thymidine incorporation. In all three cell lines, only small variations in the rate of DNA synthesis was noted in the presence of dexamethasone in the concentration range of 10^{-5} to 10^{-9} M (Fig. 2). Also, the number of cells in dexamethasone treated cultures was unchanged, as assayed by hemocytometer. Since it was possible that the relatively high serum concentration used in the study above might maximally stimulate collagen synthesis, thus masking any inhibitory effects of dexamethasone, further experiments were performed using reduced serum concentrations. The cells demonstrating high levels of receptor density (Control 3) were preincubated for 48 h with 10^{-5}, 10^{-7} or 10^{-9} M dexamethasone, and 5% FCS. The media were then changed and the cells were labelled with [^{3}H]proline for 4 h in fresh media without serum, but containing the steroid. In this experiment, collagen synthesis was increased by 55 to 61% in the presence of 10^{-5} to 10^{-9} M dexamethasone (Table II).

In further experiments, the same cell line was continuously cultured for 6 days in the

Table I. *Glucocorticoid receptor binding and dissociation constants (K_d) in fibroblast lines established from the skin of three healthy subjects*

Cell line	Age (yrs)/ sex of subjects	Dexamethasone binding Fmol/μg DNA [a]	K_d (10^{-9} M) [a]
1	12/M	3.2	8.1
2	61/F	8.8	8.8
3	22/F	16.8	17.6

[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.

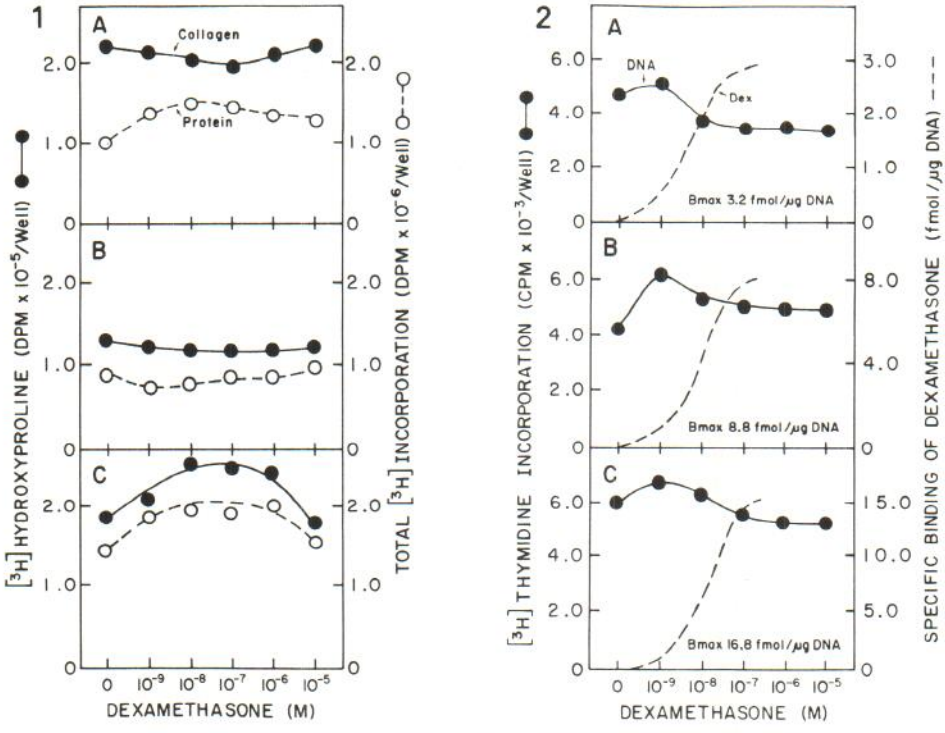

Fig. 1. Effect of dexamethasone on total protein and collagen synthesis. Three normal cell lines were preincubated for 48 h with varying concentrations of dexamethasone. The media were changed and cells were labelled with [^{3}H]proline for 24 h in the presence of dexamethasone and 20% FCS. Total nondialyzable radioactivity as a measure of total protein synthesis, and [^{3}H]hydroxyproline, as an index of collagen production, were assayed. The values are mean of triplicate parallel cultures. ○—○, Total radioactivity; ●—●, [^{3}H]hycroxyproline; (A) control line 1 with receptor density of 3.2 fmol/µg DNA; (*b*) control line 2 with receptor density of 8.8 fmol/µg DNA; (*c*) control line 3 with receptor density of 16.8 fmol/µg DNA.

Fig. 2. Effect of dexamethasone on DNA synthesis. Three different normal cell lines were preincubated for 48 h with various concentrations of dexamethasone. The media were changed and the cultures were labelled with [^{3}H]thymidine. The values are the mean of triplicate wells. In the same figure the specific binding of dexamethasone is shown. The lines utilized are the same as in Fig. 1. ●—●, Thymidine incorporation; ---, Dexamethasone binding.

presence of 10^{-5} M dexamethasone and 0.5% FCS. The media were then changed and the cells were labelled for 8 h with [^{3}H]proline in fresh media without serum but containing 10^{-5} M dexamethasone. Under these experimental conditions, collagen synthesis was decreased by 50% in the presence of 10^{-5} M dexamethasone ($p<0.001$, Fig. 3).

To study the effect of dexamethasone on the level of post-translational hydroxylation of prolyl residues in collagen α-chains, the newly synthesized collagen polypeptides were isolated by limited pepsin proteolysis and differential salt precipitation. Amino acid analyses indicated that the ratio of [^{3}H]hydroxyproline/[^{3}H]proline in collagen α-chains synthesized in the presence of 10^{-7} M dexamethasone was 0.69 while the corresponding value in control cultures was 0.62. To study the possibility that dexamethasone might change the relative synthesis of genetically distinct procollagens, two cell lines, Control 1 and Control 3, were incubated with [^{3}H]proline, and the synthesis of type I and type III

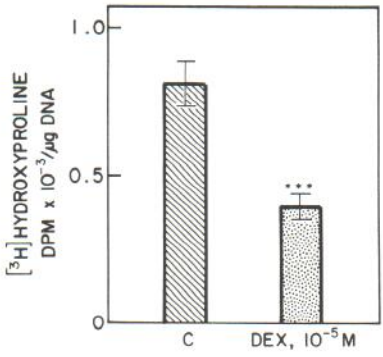

Fig. 3. Effect of dexamethasone on collagen synthesis in the presence of low serum concentration. Human skin fibroblasts (Control 3) were preincubated with dexamethasone for 6 days in medium containing 0.5% FCS; the controls and the experimental cultures contained the same concentration (0.05%) ethanol. The media were changed after 3 days preincubation. After 6 days preincubation, the media were changed to fresh DMEM without serum, and the cultures were labelled with [^{3}H]proline; [^{3}H]hydroxyproline was assayed as an index of collagen synthesis. The values are the mean of triplicate samples. *** $p<0.001$.

procollagens was quantitated by SDS-polyacrylamide slab gel electrophoresis (Fig. 4). The ratio of type I/III collagens in Control 1 cell culture, incubated with ethanol alone, was 92:8, and the corresponding ratio in cultures incubated with 10^{-7} M dexamethasone was 90:10 (Fig. 4). Similar results were observed by incubating Control 3 cells with 10^{-7} M dexamethasone. Also, the ratio of α1(I) to α2(I) chains of type I collagen in all cultures studied was approximately 2:1 (see Fig. 4).

Effects of dexamethasone on collagenase and elastase-like enzyme activities

To examine the degradative aspects of collagen metabolism in fibroblast cultures, the activity of collagenase in the serum-free culture medium was assayed by using radioactive type I collagen as substrate. Significant collagenase activity, after brief proteolytic activation of the latent collagenase, could be detected in all 3 cell lines tested. In two cell lines, dexamethasone in 10^{-5} or 10^{-7} M concentrations markedly decreased the collagenase activity (Fig. 5 A). In one cell line tested, little, if any, effect could be noticed with the same concentrations of dexamethasone. In order to test the specificity of the dexamethasone--induced suppression of collagenase activity, elastase activity was assayed in the same cell culture media using a synthetic substrate, SAPNA. The degradation of SAPNA

Table II. *Effect of dexamethasone on collagen biosynthesis in human skin fibroblast cultures labeled in the absence of serum*[a]

Dexamethasone concentration (M)	Total ^{3}H-radioactivity		[^{3}H]Hydroxyproline	
	(DPM×10^{-3}/µg DNA)[b]	(%)[c]	(DPM×10^{-3}/µg DNA)[b]	(%)[c]
Control	24.4±5.8	100	2.8±0.8	100
10^{-5}	41.1±9.7	119	4.4±0.9	155
10^{-7}	49.1±9.5	143	4.5±1.2	159
10^{-9}	57.1	154	4.5	161

[a] Normal human skin fibroblasts (Control 3) were preincubated with dexamethasone in a medium containing 5% FCS for 48 h; the controls and the experimental media contained the same concentration (0.05%) ethanol. After preincubation the media were changed to fresh DMEM without serum, and cultures were labelled with [3H]proline, as described in Material and Methods.
[b] The values are total nondialyzable ^{3}H-radioactivity or [^{3}H]hydroxyproline in medium plus cell fractions; mean ± SD of three parallel flasks, except in 10^{-9} M dexamethasone concentration, $n=2$.
[c] The values are percent of the means from the control incubated with ethanol alone.

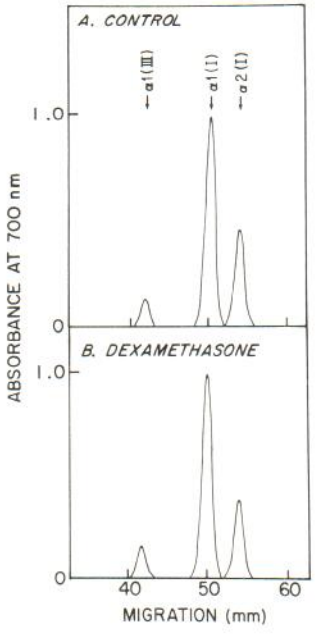

Fig. 4. Effect of dexamethasone on the synthesis of Type I and III collagens. The cultured fibroblasts (Control 1) were preincubated for 48 h with 10^{-7} M dexamethasone or solvent, and labelled for 24 h with [^{3}H]proline. Medium proteins were submitted to limited pepsin proteolysis, collagen molecules were isolated by salt precipitation and examined by 6% SDS-slab gel electrophoresis. The collagen α-chains were visualized by fluorography, and the bands were quantitated by scanning densitometry as 700 nm. The migration position of α1(III), α1(I) and α2(I) chains are shown.

was significantly decreased in all three control cell lines when incubated in the presence of 10^{-7} or 10^{-5} M dexamethasone (Fig. 5 B).

DISCUSSION

The human skin fibroblasts contain specific, high affinity glucocorticoid receptors. As demonstrated here and elsewhere (23) the density of receptors between different fibroblast cell lines can vary markedly. Since fibroblasts synthesize collagen, it was of interest to examine the effects of dexamethasone on collagen productions by human skin fibroblasts. The results indicated that in the cell lines with low and intermediate receptor densities dexamethasone in 10^{-5} to 10^{-9} M concentration using short incubation time periods had no effect on collagen production, as assayed by the synthesis of [^{3}H]hydroxyproline. In the cell line with highest receptor density, a slight stimulation of collagen synthesis was noted with concentrations of 10^{-6} to 10^{-9} M. The latter same cell line was further studied by incubating cells in low 0.5% FCS for 6 days and labelling the cells thereafter in serum-free conditions with [^{3}H]proline. A marked inhibition in collagen production was noted in this experiment. Further studies indicated that the ratio of type I/III collagen was unchanged suggesting co-ordinate regulation of type I and III procollagen synthesis (25). Also, the hydroxylation of prolyl residues in collagen α-chains was unchanged in the presence of dexamethasone as demonstrated previously (26).

The reasons for these apparently contradictory observations could be manifold. First, glucocorticoids could affect the transport of amino acids, including proline uptake to the cells. It has been shown that in keloid fibroblast cultures, hydrocortisone increases the transport of specific amino acids (27). Thus it could be possible that in different fibroblast lines glucocorticoids affect differently the amino acid transport and thus could influence the results. Secondly it appears that culture conditions can profoundly affect glucocorticoid mediated changes in collagen synthesis (8). As shown in this study, longer incubation in the presence of low serum concentrations leads to inhibition of collagen synthesis by

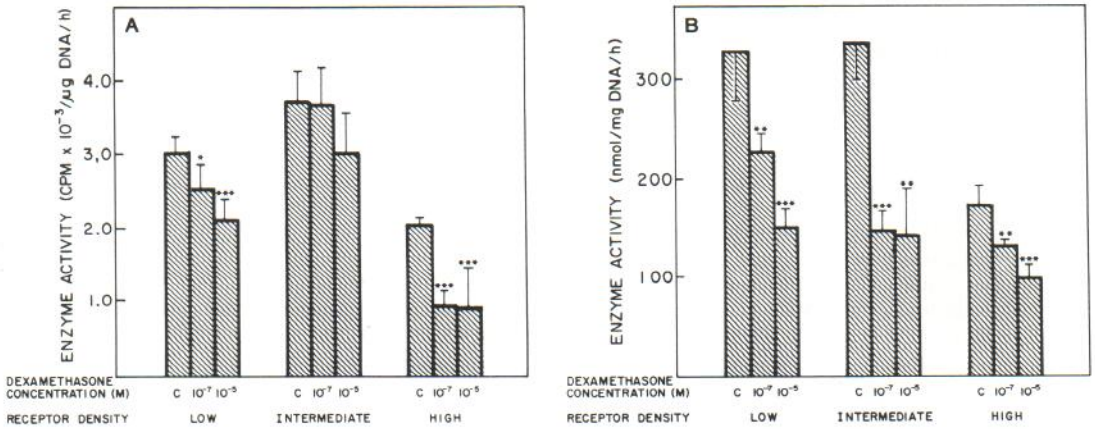

Fig. 5. Effect of dexamethasone on collagenase and elastase-like enzyme activities in cultured fibroblasts. Human skin fibroblasts were incubated with dexamethasone, as indicated in Material and Methods. Aliquots of serum-free medium were assayed for collagenase activity (A) following a brief proteolytic activation of latent collagenase, and for elastase-like enzyme activity (B) by incubating with a synthetic substrate, SAPNA. The values are mean ± SD of three parallel flasks, each assayed as triplicate. Collagenase activity is expressed as degradation of [^{3}H]proline-labelled type I collagen, cpm $\times 10^{-3}$/µg cell DNA/h. Elastase activity is expressed as hydrolysis of SAPNA, nmol/mg DNA/h, as monitored at 410 nm. *** Statistically different from the control, $p<0.001$. ** Statistically different from the control, $p<0.005$. * Statistically different from the control, $p<0.01$.

dexamethasone. Similarly it has been shown that under nutritionally enriched culture conditions dexamethasone increased thymidine incorporation in various cell lines, while in depleted media (28), a marked inhibition was observed. This could indicate that the energy metabolism of the cells or perhaps some proteins synthesized in the presence of glucocorticoid could alter the glucocorticoid response. It should be noted that the receptor density of fibroblasts or the age of the donors did not influence the dexamethasone effects on fibroblasts.

The effects of dexamethasone on connective tissue degrading enzymes collagenase and elastase were inhibitory. Previously, some studies have suggested that glucocorticoids increase collagenolytic activity in fibroblast cultures (29). However, in other studies glucocorticosteroids have been shown to inhibit collagenase activity (30). In the present study, dexamethasone in 10^{-5} or 10^{-7} M concentrations markedly decreased the collagenase activity.

The effect of dexamethasone on collagenase activity was not specific since the activity of an other enzyme, elastase-like neutral protease, which is synthesized and secreted by the fibroblasts, was also decreased. This enzyme has recently been characterized in human skin fibroblast cultures (31). Glucocorticoids have also inhibitory effect on macrophage elastase (32). Thus, the results further suggest that glucocorticoids may profoundly affect the degradation of connective tissue components, the effect being a consistent inhibition of the protease activity. At the same time, the effects of glucocorticosteroids on collagen synthesis may be more variable and dependent of the environmental milieu of the fibroblasts.

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