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INHIBITION OF COLLAGEN AND SULFATED GLYCOSAMINOGLYCAN SYNTHESIS IN NEONATAL MOUSE DERMAL FIBROBLASTS BY CORTICOSTERONE

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Abstract—The effects of corticosterone on collagen and sulfated glycosaminoglycan production were investigated in primary cultures of mouse neonatal dermal fibroblasts. Two genetically different strains, A/J and C57BL6/J were compared. Fibroblasts were cultured in the absence or presence of corticosterone for two to seven days. In control cultures, [^{35}S] sulfate incorporation into TCA insoluble glycosaminoglycans increased up to the eighth day. Collagen was highest on the fifth day in culture. In the medium, about 15% of the total protein was collagen. After four to seven days in the presence of 10^{-6}M corticosterone, ^{35}S sulfated glycosaminoglycans and collagen in the medium were reduced by up to 70% in both mouse strains. Corticosterone specifically inhibited in a dose-dependent manner collagen over total protein, reducing the percentage of collagen in the medium to 7% of the total protein in both strains.

Administration of anti-inflammatory steroids for treatment of arthritic conditions has been complicated by clinical side effects such as the thinning of skin [1, 2] and changes in blood vessels [3] and bone [4, 5]. Since fibroblasts are one of the primary sites of glucocorticoid action in skin [4, 6], a variety of cellular responses to these hormones have been monitored in these cells both *in vivo* and *in vitro* [4, 6, 7].

In a previous study using comparable growth conditions [8], it had been demonstrated that glucocorticoids can inhibit the growth of primary cultures of mouse neonatal dermal fibroblasts. Furthermore, these effects were apparently mediated by the presence of specific, saturable glucocorticoid receptors in the cytoplasm and nuclei of mouse dermal fibroblasts [8], as was also demonstrated elsewhere [7, 9, 10]. Two inbred strains of mice, A/Jax (A/J) and C57BL6/Jax (C57), were compared due to the documented differences in the levels of cytoplasmic glucocorticoid receptors [11] and because A/J show a more pronounced inhibition of collagen synthesis after dexamethasone treatment *in vivo* [12].

The synthesis of various extracellular matrix components as collagen and glycosaminoglycans (GAG) is inhibited by pharmacological doses of glucocorticoids [6, 13–16], especially after chronic exposure to these compounds [17]. Collagen production is preferentially inhibited over total protein by glucocorticoid treatment [15, 18].

The present study was undertaken to determine whether glucocorticoids differentially affect the production of collagen and sulfated glycosaminoglycans in neonatal dermal fibroblasts obtained from two mouse strains which exhibit response differences to glucocorticoids *in vivo* [11, 12].

MATERIALS AND METHODS

Isolation and culture of dermal fibroblasts

Timed pregnant A/J and C57 mice were obtained from Jackson Laboratories, Bar Harbor, ME. Skins were removed from one to three days old neonates as described by Yuspa and Harris [20]. Dermis and epidermis were separated [8] and dermal layers were digested using 0.35% *Clostridium* collagenase (CLS Type III, Worthington Biochemicals, Freehold, NY) in Eagle's minimum essential medium (MEM, NIH Media Unit) [8]. After filtration and centrifugation, the collagenase was inactivated by adding MEM containing 10% (v/v) fetal bovine serum (Microbiological Associates, Bethesda, MD). Cells were counted on a ZB1 Coulter Particle Counter (Coulter Electronics, Hialeah, FL) and seeded at a density of 3.2×10^4/cm^2 in 25 cm^2 tissue culture flasks (3013 Falcon flasks, Oxnard, CA). Cells were cultured in complete MEM (CMEM) containing 10% (v/v) fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml fungizone, 20 mM HEPES buffer (pH 7.4) and 4 mM glutamine. Cells were grown at 37° in incubators containing 5% CO_2 and humidified to saturation. One day after inoculation (day 1), fresh culture medium was added.

To investigate macromolecular synthesis at different time intervals during logarithmic growth and at confluency, corticosterone dissolved in absolute ethanol was added to cultures on day 1. Control cultures received the same concentration of ethanol which never exceeded 0.1 per cent. The time–course

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experiments were terminated after 2, 4 and 7 days in the presence of the glucocorticoids. No additional changes of culture medium were performed in these studies.

Confluency was reached after around four days in culture, as determined by visual control and cell count of sample cultures (data not shown). In separate assays, confluent cell layers were exposed to appropriate concentrations of corticosterone in fresh CMEM from days 4 to 8.

Collagen biosynthesis

At appropriate times, cultures were labeled for 12 hr with 7.5 μCi/ml [^{14}C]proline (280 mCi/mmole, Amersham Searle, Arlington Heights, IL) in fresh CMEM containing 0.4 mM glutamine and 50 μg/ml of ascorbic acid. Following labeling, the cell layer was washed with 0.15 M phosphate buffered saline (PBS) and solubilized with 0.2 N NaOH. Medium and cell layer were separately dialyzed for 2 to 3 days against distilled water, at 4°, and lyophilized. The dried residue was redissolved in 0.2 N NaOH for 24 hr and neutralized with 0.2 N HCl and 1 M HEPES buffer. Duplicate samples were incubated with or without protease-free bacterial collagenase (Advanced Biofactures Corporation, Lynbrook, NY) following the method of Peterkofsky and Diegelmann [21]. After precipitation of samples with trichloroacetic acid (TCA) and tannic acid, the radioactivity in the supernatant was counted: the difference between collagenase treated and blank tubes gives the collagen content (C). Counts in the precipitate of collagenase treated tubes represent noncollagenous protein (NCP). The percentage collagen of total protein was calculated as follows: $100 \times C/(NCP \times 5.4) + C$ [21]. Using [^{3}H]tryptophan-labeled protein, the proteolytic activity of the collagenase was negligible in each experimental group. Blank values always were significantly lower than after collagenase treatment.

Incorporation of [^{35}S]sulfate into glycosaminoglycans

Cultures were labeled for 12 hr with 5 μCi/ml of ^{35}S-sulfate (sp. act. 500 mCi/mmole, New England Nuclear Corporation, Boston, MA). The cell layer was washed with PBS and sequentially extracted at 4° with 10% TCA and three times with 5% TCA, in the presence of 10 mM Na_2SO_4 [22]. The material from the first three washes was pooled and counted. Radioactivity in the fourth supernatant was insignificant. The pellets were solubilized in 0.5 M NaOH and duplicate aliquots were counted.

After exhaustive dialyzation, the culture medium was extracted three times with 10% TCA, containing 10 mM Na_2SO_4. The tubes were centrifuged at 10,000 rpm at 4°. The final pellet was solubilized with 1 N NaOH and duplicate aliquots were counted in a Beckman LS 300 liquid scintillation spectrometer.

RESULTS

Syntheses of noncollagenous protein and collagen in the cell layer and medium were maximal five days after plating in both strains, when the cultures were in the early stages of confluency (Table 1) and decreased after eight days. [^{35}S]Sulfate incorporation into glycosaminoglycans increased during the culture period.

The amount of NCP in the culture medium was 3 to 4 times lower than in the cell layer. After three days in culture, the collagen content was 1.5 to 3 times higher in the medium than in the cell layer. After eight days in culture, and following the addition of fresh CMEM on day four, 4 to 5 times more collagen was found in the medium than in the cell layer (Table 1). Whereas the collagen content of the cell layer constituted 1.4 per cent of the total protein, it reached about 15 per cent in the medium demonstrating that under the present culture conditions collagen is preferentially secreted into the culture medium (Table 2). [^{35}S]Sulfate incorporation into

Table 1. Noncollagenous proteins (NCP), collagen and sulfated glycosaminoglycans in mouse dermal fibroblast cultures

Days in Culture	Donor Strain	Cpm x 10^{-3}/μg DNA					
		Culture Medium			Cell Layer		
		NCP	Collagen	Sulfate	NCP	Collagen	Sulfate
3	A/J (3)	91.7 ± 9.8	15.1 ± 1.5	1.43±0.11	272.6±6.9	5.97±0.64	0.48±0.05
	C57 (3)	69.8 ± 9.4	9.6 ± 1.5	1.73±0.07	281.4±8.0	7.33±0.27	0.71±0.03
5	A/J (6)	111.9 ± 17.6	20.5 ± 3.6	1.52±0.03	373.4±59.7	6.91±1.11	0.53±0.03
	C57 (6)	113.3 ± 15.0	18.2 ± 2.6	1.17±0.05	437.9±67.0	8.79±1.51	0.53±0.01
8	A/J (9)	101.9 ± 3.9	17.4 ± 1.3	2.74±0.18	271.9±23.3	3.68±0.48	1.01±0.15
	C57 (9)	109.8 ± 7.4	19.6 ± 1.4	3.07±0.23	307.4±21.2	4.27±0.37	1.33±0.21

Values represent the mean ±S.E. obtained from n number of samples in parentheses. Cell preparation, culture and overnight labeling were as described in Materials and Methods. Fresh CMEM was added on day 1 to all culture flasks. For assays after 8 days in culture, an additional change of culture medium was performed on day four.

Table 2. Per cent collagen of total protein

Treatment	Culture medium		Cell layer	
	A/J	C57	A/J	C57
Control				
(9)	14.5 ± 0.7	15.1 ± 0.2	1.38 ± 0.25	1.43 ± 0.14
Corticosterone				
10^{-6}M	7.1 ± 1.6	7.7 ± 1.2	0.94 ± 0.10	0.78 ± 0.13
(9)	***	***	NS	**
10^{-7}M	12.5 ± 0.1	11.1 ± 0.2	1.11 ± 0.06	0.83 ± 0.10
(4)	***	***	NS	*
10^{-8}M	12.2 ± 0.7	14.7 ± 0.5	0.94 ± 0.06	1.38 ± 0.09
(7)	NS	NS	NS	NS
10^{-9}M	14.6 ± 0.3	17.3 ± 0.3	1.54 ± 0.04	1.37 ± 0.02
(3)				
10^{-10}M	15.1 ± 0.5	16.7 ± 0.1	1.63 ± 0.06	1.52 ± 0.03
(3)				

Values are mean ±S.E. of *n* number of samples in parentheses after eight days in culture. Corticosterone was added after four days. Cells were labeled overnight with [^{14}C]proline before terminating the experiment. The collagen content was calculated after incubation with protease-free collagenase [21]. *P<0.05, **P<0.01, ***P<0.001, NS: not significant; comparison with controls by Student's *t*-test.

TCA insoluble GAG's was 2 to 3 times higher in the medium than in the cell layer of both strains.

The addition of 1 μM corticosterone 24 hr after cell seeding affected all parameters of macromolecular synthesis as measured in the culture medium (Fig. 1). After a two day exposure to corticosterone, NCP, collagen and GAG production expressed per μg DNA [8] were inhibited by only 20 per cent in A/J cells. In C57, sulfate incorporation into GAG's was inhibited by about 30 per cent of controls, while collagen and NCP were unaffected.

Inhibition was enhanced by longer exposure to steroids. After eight days distinct inhibition (40–60% of controls) of NCP, collagen and sulfate incorporation was observed in both strains. In the cell layer (data not shown) a comparable inhibition was observed for all parameters three days after plating, which further decreased to about 60 per cent of control values on day eight.

The reduction of NCP, collagen and sulfate incorporation into the medium in A/J and C57 fibroblasts exhibited a dose–response relationship at different concentrations of corticosterone (Fig. 2). Confluent fibroblast cultures were exposed to different concentrations of corticosterone for four days. In the medium of both strains, all parameters were significantly decreased by 10^{-6} and 10^{-7} M corticosterone. Some stimulation, especially of collagen and sulfate incorporation, was observed between 10^{-9} and 10^{-10} M corticosterone. In the cell layer (data not shown), 10^{-6} M corticosterone significantly decreased NCP, collagen and sulfate incorporation to 40–60 per cent of controls.

In the medium of both strains, corticosterone 10^{-6} and 10^{-7} M significantly decreased the collagen expressed as a percentage of total protein from normal values of about 15 per cent to 7.5 and 12 per cent respectively (Table 2). In the cell layer, corticosterone 10^{-6} and 10^{-7} M reduced the collagen content to about 0.8 per cent. Corticosterone (10^{-8} M) decreased the collagen content of medium and cell layer in A/J, but not in C57. In contrast, collagen increased to about 17 per cent in the medium of C57 cells after 10^{-9} and 10^{-10} M corticosterone, while no difference was seen from control values in A/J. However, in the cell layers treated with 10^{-9} and 10^{-10} M corticosterone, collagen respectively increased to 1.54 and 1.63 per cent of total protein in A/J, while no clear increase was seen in C57.

DISCUSSION

Synthesis of collagen and NCP by neonatal dermal fibroblasts obtained from A/J and C57 mouse strains (Table 1) was highest just prior to confluency and decreased thereafter whereas sulfate incorporation into glycosaminoglycans increased throughout the culture period, suggesting different control mechanisms. Preferential secretion of collagen and sulfated GAG's in the culture medium corresponds with the role of fibroblasts in the synthesis of connective tissue [6]. After expression of collagen as a percentage of total protein (Table 2), its relative amount in the culture medium is about 10 times higher than in the cell layer: about 14.5 and 1.4 per cent respectively.

Corticosterone decreased collagen content and the incorporation of sulfated GAG's in TCA insoluble material after four to seven days in the culture medium from both mouse strains. In contrast, the

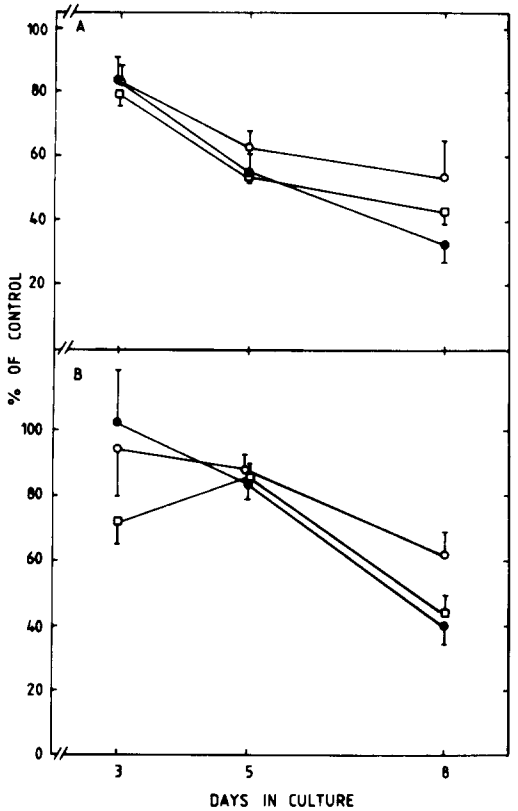

Fig. 1. Effect of corticosterone on noncollagenous protein (NCP), collagen and sulfated glycosaminoglycans in the medium of neonatal mouse dermal fibroblast cultures. A/J (A) or C57 (B) fibroblasts were grown in the presence of 1 μM corticosterone (added 1 day after seeding) for 2, 4 or 7 days, after which incorporation of [^{14}C]proline was determined into non-collagenous (○—○) and collagenous protein (●—●) and of [^{35}S]sulfate into TCA-insoluble material (□—□). After correction for the DNA content of cultures, values are expressed as a percentage of controls (100%, in the absence of steroid see Table 1) and represent the mean ± S.E. of 3 cultures.

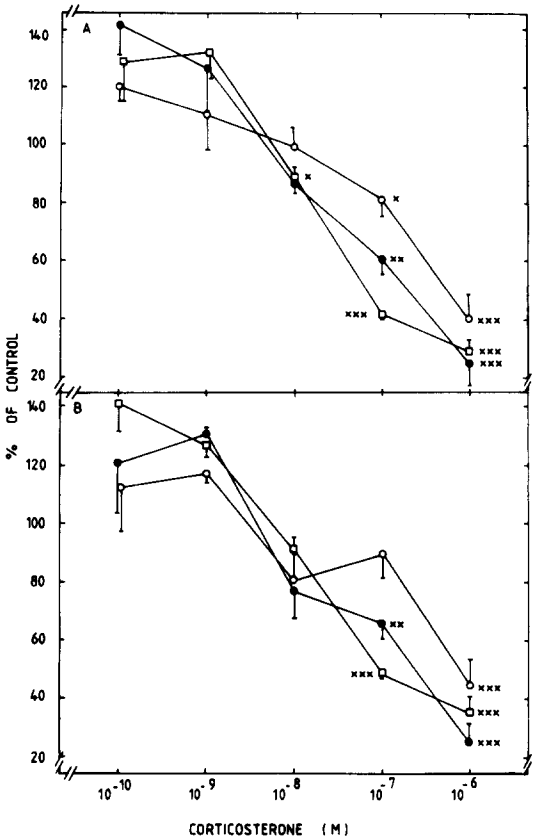

Fig. 2. Effect of corticosterone concentration on noncollagenous protein (NCP), collagen and sulfated glycosaminoglycans in the medium of neonatal mouse dermal fibroblasts. Confluent A/J (A) and C57 (B) fibroblasts were cultured for four days in the presence of different concentrations of corticosterone, after which incorporation was determined of [^{14}C]proline into noncollagenous (○—○) and collagenous protein (●—●) and of [^{35}S]sulfate into TCA insoluble material (□—□). As in Fig. 1 values are calculated per μg DNA and expressed as a percentage of controls (Table 1), and represent the mean ±S.E. of 3 to 9 cultures. × $P<0.05$; ×× $P<0.01$; ××× $P<0.001$: comparison with controls by Student's t-test.

NCP content was only clearly decreased after seven days of steroid exposure (Fig. 1). The dose-response of the cells to corticosterone (Fig. 2) revealed that an equivalent degree of inhibition of GAG's and collagen could be observed in the medium following the addition of 10^{-6} and 10^{-7} M corticosterone. Glucocorticoids have been shown to inhibit GAG synthesis in other systems [16, 25, 26]. NCP was significantly reduced by the higher steroid concentrations although to a lesser extent than collagen. In the medium of both strains, 10^{-6} and 10^{-7} M corticosterone significantly and specifically reduced the collagen content. A specific decrease of collagen over total protein synthesis by glucocorticoids has also been reported in several systems including human skin [15, 18, 27] and chick embryo fibroblasts [28]. Although not statistically significant, collagen in the medium and cell layer of A/J appears to be lower than in C57 (Table 2), after treatment with 10^{-8} M corticosterone. Using adult mice, comparable findings were published previously [12]. Some stimulation of collagen production was observed with lower concentrations of corticosterone (10^{-9} and 10^{-10} M). At these concentrations collagen increased more than NCP, especially in C57. These findings agree with earlier reports on stimulation of DNA and collagen synthesis by physiologic doses of glucocorticoids in human fibroblasts [13]. To account for the presence of hormones and growth factors in fetal calf serum [23, 24], growth conditions were standardized, and serum from the same batch was used in the cultures that were compared.

A selective reduction in collagen content in dermal fibroblasts following chronic administration of glucocorticoids may be related to the thinning of skin observed *in vivo* after therapeutic steroid administration [1–3]. The use of primary cultures of dermal fibroblasts may provide a suitable model system for

studying the effects of glucocorticoids and other anti-inflammatory agents on collagen and other extracellular matrix component synthesis and degradation. Moreover, in the conditions described here, the importance of genetic factors can be studied in control conditions and after exposure to glucocorticoids.

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