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# GLUCOCORTICOIDS ALTER THE RATIO OF TYPE III/ TYPE I COLLAGEN SYNTHESIS BY MOUSE DERMAL FIBROBLASTS

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**Abstract**—The type of collagen synthesized by primary cultures of dermal fibroblasts from two different strains of mice (A/J and C57BL6/J) was investigated. Confluent cultures were grown for four days with or without $10^{-6}$ and $10^{-7}$ M corticosterone or $10^{-6}$ M dexamethasone. Glucocorticoid treatment results in a lower type III/type I procollagen ratio in both strains. While the amount of type I procollagen remains unchanged, type III is reduced to about 25 per cent of control values in the cells from both mouse strains. Thus, glucocorticoids can modulate the types of collagen synthesized by cultured cells.

Glucocorticoids are used in the treatment of a variety of connective tissue disorders [1–3]. Following their chronic administration *in vivo*, a number of complications occur such as thinning of skin [4, 5], changes in blood vessels [6] and bone [2, 7]. These changes suggest direct effects on matrix production by the cells. Furthermore, the fibroblast, a major connective tissue cell, is one of the primary sites of glucocorticoid actions [1, 2]. It has previously been found that cultures of fibroblasts from human and guinea pig skin, when maintained at high cell density (i.e. no growth), produced proportionally more type III than type I collagen [8]. When cells are cultured for more than four days in the presence of glucocorticoids, cell growth is severely reduced [9]. It thus could be expected that glucocorticoids, by inhibiting cell growth, might alter the type III/type I collagen ratio in fibroblast cultures.

In this study, primary cultures of mouse dermal fibroblasts were tested for the effects of anti-inflammatory steroids on collagen synthesis. Neonatal mice from two inbred strains, A/Jax (A/J) and C57BL6/Jax (C57) were compared. A/J mice have a greater susceptibility to glucocorticoid-induced congenital malformations which are associated with different levels of cytoplasmic glucocorticoid receptors [10]. Moreover, dexamethasone treatment of adult A/J mice produces a greater decrease in skin collagen content after *in vivo* administration than similarly treated adult mice from other strains [11].

## MATERIALS AND METHODS

*Isolation and culture of fibroblasts.* Timed pregnant A/J and C57 mice were obtained from Jackson Laboratories (Bar Harbor, ME). Skins were removed from one to three day old neonates as previously described [12]. Dermis and epidermis were separated [9] and the dermal layers were digested for 40 minutes at 37° in 25 ml of Eagle's Minimum Essential Medium (MEM, NIH Media Unit) containing 0.35% *Clostridium* collagenase (CLS Type III, Worthington Biochemical, Freehold, NJ), 4 mM glutamine and 20 mM HEPES buffer (pH 7.4). The resulting suspension was filtered and centrifuged [12]. The cell pellet was washed with MEM containing 10% (v/v) fetal bovine serum (Microbiological Associates, Walkersville, MD) to inactivate the collagenase [13]. Cells were counted [9] and seeded at a density of $2.7 \times 10^4/cm^2$ in 75 $cm^2$ tissue culture flasks (Corning Glass Works, Corning, NY). Cells were cultured in MEM which contained 10% (v/v) fetal bovine serum, antibiotics, glutamine and 20 mM HEPES buffer (Grand Island Biological Co., Grand Island, NY) [9]

One day after seeding, similar fresh MEM was added (day 1). On day 4, the medium was changed and corticosterone or dexamethasone (9$\alpha$-fluoro-16$\alpha$-methyl-11$\beta$,17$\alpha$,21-trihydroxy-1,4-pregnadiene-3,20-dione) (Sigma Chemical Co., St. Louis, MO) were added in absolute ethanol at appropriate concentrations. The final ethanol concentration was less than 0.1%. Control cultures received the same concentration of alcohol. On day 7, overnight labeling was initiated with fresh medium as above, except 0.4 mM glutamine and ascorbic acid (50 $\mu$g/ml) were added. Control cultures were labeled with 6 $\mu$Ci/ml $^{14}$C proline (280 mCi/mM, Amersham, Arlington Heights, IL) and steroid-treated cultures with 6 $\mu$Ci/ml 2,3-$^3$H proline (20–35 Ci/mM, New England Nuclear, Boston, MA). Unlabeled proline was added to the cultures to correct for the different molarity of the isotopes. In order to avoid differences due to experimental variation, further procedures were performed on pooled samples from glucocorticoid-treated and untreated culture medium.

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*DEAE-cellulose chromatography.* Collagenous proteins were precipitated from the medium with $(NH_4)_2SO_4$ (1.14 g/10 ml) in the presence of protease inhibitors: 20 mM EDTA, 1 mM *p*-hydroxymercuribenzoate and 10 $\mu$M phenylmethylsulfonylfluoride [8]. Collagenous proteins were redissolved and dialyzed against the starting buffer, 50 mM Tris–HCl (pH 7.4), containing 2 M urea [8]. Diethylaminoethylcellulose (DEAE) (DE 52, Whatman, W. & R. Balston, Maidstone, U.K.) column (1.7 × 7 cm) chromatography was performed at 4° with a 0–0.2 M NaCl linear gradient in starting buffer, over a total volume of 450 ml. Fractions of 5 ml were collected and the radioactivity of aliquots was determined with a Beckman liquid scintillation system LS-335 (Beckman Instruments, Fullerton, CA) in 10 ml Hydromix (Yorktown Research, NY).

*Polyacrylamide gel electrophoresis.* Collagenous protein was precipitated from the culture medium with 30% $(NH_4)_2SO_4$ [14]. Guinea pig skin collagen (1 mg) was added as carrier. The precipitate was dialyzed against 0.5 M acetic acid and was digested with pepsin (1 mg/10 ml) (Sigma, St. Louis, MO) at pH 2.0, 4°, for 6 hr. Pepsin was inactivated by raising the pH to 8.0, collagen precipitated with 20% NaCl, material was redissolved in 0.5 M acetic acid, dialyzed and lyophilized. Aliquots were electrophoresed on 5% polyacrylamide disc gels by the method of Laemmli [15] in the presence of 0.5 M urea, and in reducing conditions. Disc gels were cut to 1 mm slices with a Gilson Automatic Gel Slicer (Gilson Medical Electronics, Middleton, WI). Slices were dried, 75 $\mu$l $H_2O$ and 0.5 ml NCS solution were added (Tissue Solubilizer, Amersham, Arlington Heights, IL), and heated at 60° for 4 hr. After cooling, 10 ml of Liquifluor was added (42 ml Spectrafluor and 1000 ml toluene, Amersham, Arlington Heights, IL). Counts were adjusted to bring $\alpha_1$(I) from treated and control samples to the same height (Fig. 2). Values were compared by paired Student's *t*-test.

## RESULTS

We first measured the amounts of type I and type III collagen present in the cultures. In control cells, the mean total dpm in the type III procollagen peaks from DEAE–cellulose column chromatography is significantly lower in A/J than in C57 ($P < 0.01$) (Table 1). No difference is seen between the strains for type I procollagen. The ratio of type III/type I collagen in medium from C57 cultures is about double that in A/J ($P < 0.001$).

In cultures treated for four days at confluency with corticosterone $10^{-6}$ and $10^{-7}$ M and dexamethasone $10^{-6}$ M, DEAE–cellulose column chromatography shows a reduction of type III procollagen (Fig. 1). Type I procollagen is not inhibited. Although different steroids and different concentrations were used, we have calculated the mean and standard deviation of the glucocorticoid-treated group (Table 1). The mean type III procollagen in the medium is $87 \pm 42 \times 10^3$ dpm in C57, and $37 \pm 15 \times 10^3$ dpm in A/J, or an inhibition by glucocorticoids to about 25% of control values in both strains. The ratio type III/type I procollagen in C57 is $0.39 \pm 0.20$, in A/J $0.16 \pm 0.06$: both are significantly lower than controls ($P < 0.01$). After steroid treatment, no significant difference is seen between the strains. Disc gel electrophoresis was used to confirm the nature of the collagenous chains. A relative decrease of type III collagen after glucocorticoid treatment (Fig. 2) was observed as expected and confirmed the ion exchange data.

## DISCUSSION

We have found that glucocorticoids alter the ratio of type III to type I collagen synthesized by cultured fibroblasts. A significantly lower content of procollagen type III in medium from untreated A/J cultures was observed, compared with C57 (Table 1). The culture medium was analyzed because it contains 80 to 85 per cent of total labeled collagen [16]. The ratios of type III to type I procollagen are 1.45 in untreated C57 and 0.69 in A/J fibroblasts. These ratios are higher than in mature rat dermis [17] and in human fibroblast cultures [8, 18]. The difference between both strains is significant. Since no skins were removed from mice older than three days, and the neonates never differed by more than one day in age, it seems unlikely that age could

Table 1. DEAE–cellulose column chromatography of medium from fibroblast cultures

| Mouse strain | Steroid | (M) | Procollagen Type III dpm × $10^{-3}$ | Procollagen Type I dpm × $10^{-3}$ | Type III/I | *n* |
|---|---|---|---|---|---|---|
| C57 | Corticosterone | $10^{-6}$ | 80 | 214 | 0.38 | 1 |
| | | $10^{-7}$ | 132 | 226 | 0.59 | 1 |
| | Dexamethasone | $10^{-6}$ | 49 | 238 | 0.20 | 1 |
| | Controls | | 324 ± 58 | 223 ± 29 | 1.45 ± 0.08 | 3 |
| A/J | Corticosterone | $10^{-6}$ | 42 | 245 | 0.17 | 1 |
| | | $10^{-7}$ | 49 | 238 | 0.21 | 1 |
| | Dexamethasone | $10^{-6}$ | 21 | 202 | 0.10 | 1 |
| | Controls | | 134 ± 49 | 189 ± 40 | 0.69 ± 0.10 | 3 |

Control values are the mean ± S.D. of the appropriate controls of each steroid treated culture. Dpm $^{14}C$ and $^{3}H$ proline were calculated from DEAE–cellulose column chromatography eluates (Fig. 1).

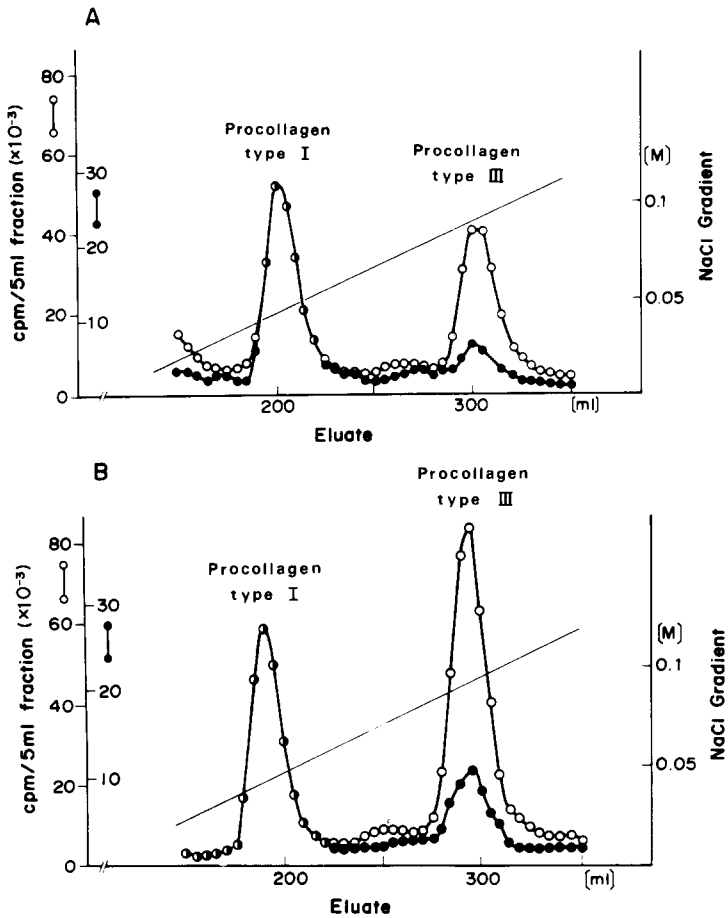


Fig. 1. DEAE–cellulose column chromatography of material from culture medium from A/J (A) and C57 (B) fibroblasts. Corticosterone ($10^{-6}$ M) was added with fresh medium on day 4. Cultures were labeled overnight on day 7. After terminating the growth experiment (day 8), control (○——○) and treated (●——●) media of each strain were processed together. All steroid values were adjusted to equalize procollagen type I peaks in control and treated cultures.

explain the difference in type III procollagen levels between both strains [19]. No significant difference in cell counts could be demonstrated between the strains under the growth conditions used here. However, total protein was about 15 per cent lower in untreated A/J than in C57 cultures ($P < 0.01$) [20].

Anti-inflammatory steroids generally inhibit synthesis of collagen and glycosaminoglycans, and decrease cell growth and DNA synthesis in primary fibroblast cultures [1, 9, 16, 21–23]. In the present experimental system, four days treatment with $10^{-6}$ and $10^{-7}$ M corticosterone or dexamethasone reduced the cell number, and total DNA and protein content in both strains to 60 to 80 per cent of control cultures [9, 20]. Conditions being equal, incorporation of [$^3$H] thymidine into DNA and of radiolabeled proline into collagenous protein were reduced to 20 to 30 per cent of untreated cells by pharmacological doses of glucocorticoids [9, 16, 20]. About 15 per cent of the total protein in untreated medium was collagen, as opposed to about 1.4 per cent in the cell layer. Corticosterone $10^{-6}$ M specifically reduced collagen to 7 and 0.8 per cent of total protein in the medium and cell layer respectively [16]. Growth experiments correlate with steroid binding to specific, saturable receptors in the cytoplasm and nuclei of these fibroblasts [9].

This is the first demonstration of the effect of a steroid on a specific collagen type. Corticosterone $10^{-6}$ and $10^{-7}$ M and dexamethasone $10^{-6}$ M clearly decrease type III procollagen in the medium of fibroblast cultures. In A/J and C57 mice, the exposure of confluent cultures to anti-inflammatory steroids decreases the mean level of type III procollagen which bound to the DEAE columns to about 25 per cent of controls, while no decrease in type I is observed (Table 1, Fig. 1). The ratio of type III to type I procollagen, which is lower in untreated A/J cultures than in C57, equally decreases in glucocorticoid-treated culture medium from both strains. The front peak of the DEAE–cellulose column contained a substantial amount of radioactivity which in SDS gels was type I collagen (data not shown). This may contribute to the lower ratio of type III to type I collagen in disc gel electrophoresis (Fig. 2). Moreover, some degradation of type III collagen may be due to pepsinisation [24]. In cultures of fibroblasts from human and guinea pig skin, the ratio of collagen type III to type I is lower at low cell density [8]. The question arises to what

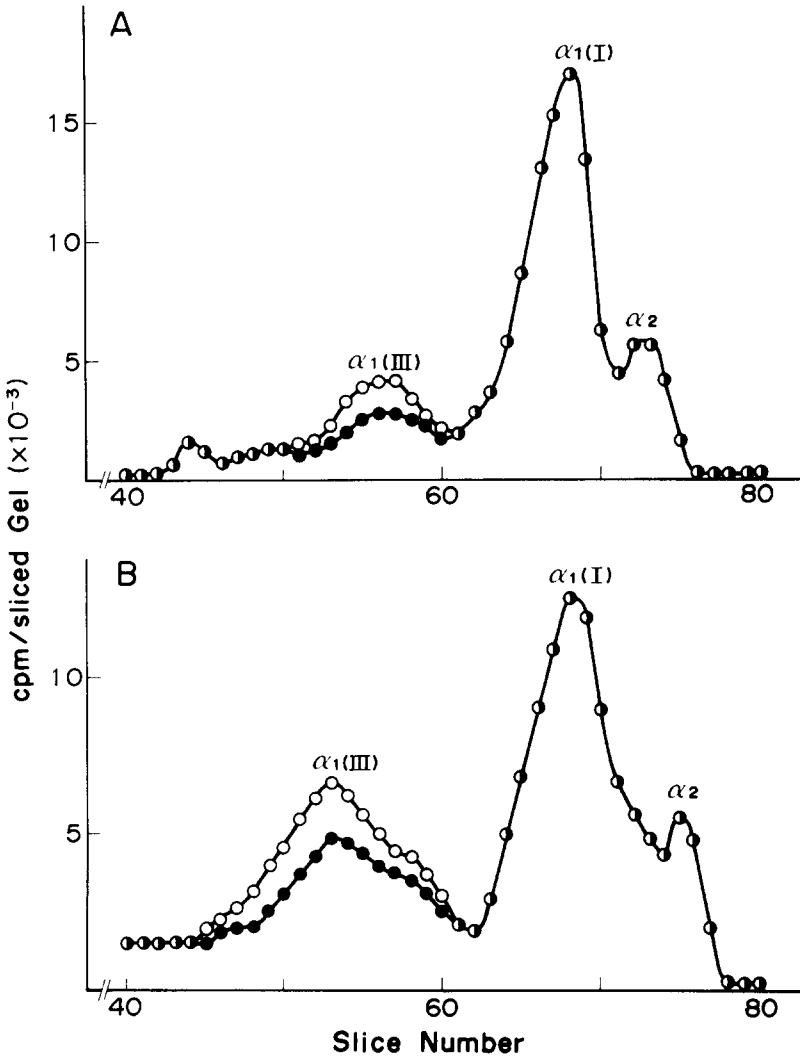


Fig. 2. A/J fibroblast culture medium from control cells (○——○) and fibroblasts treated with corticosterone $10^{-7}$ M (A) and dexamethasone $10^{-6}$ M (B) (●——●). Disc gel electrophoresis was performed as in Materials and Methods. Gels were cut to 1 mm thickness and radioactivity was counted. All steroid treated values were adjusted to equalize $\alpha_1$ (I) peaks in control and treated cultures: values for corticosterone $10^{-7}$ M were multiplied by about 1.4, and values for dexamethasone $10^{-6}$ M by about 1.7.

extent the glucocorticoid-induced inhibition of synthesis of type III collagen is due to a specific effect of the lower cell density.

Since type III collagen is reduced with glucocorticoid treatment, and type III collagen appears elevated in early wound healing [17, 25], it is possible that the poor wound healing seen in long-term anti-inflammatory steroid treatment is due to the lack of sufficient type III in the wound site and poor cell divison.

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