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MODULATION OF COLLAGEN METABOLISM BY GLUCOCORTICOIDS

RECEPTOR-MEDIATED EFFECTS OF DEXAMETHASONE ON COLLAGEN BIOSYNTHESIS IN CHICK EMBRYO FIBROBLASTS AND CHONDROCYTES*

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Abstract—The steroid modulation of collagen metabolism was studied by injecting chick embryos with dexamethasone *in vivo*, and collagen synthesis was subsequently assayed by pulse-labeling the tissue with [^{14}C]proline *in vitro*. The synthesis of [^{14}C]hydroxyproline in tendons and sterna from chick embryos treated with dexamethasone was markedly reduced as compared with untreated controls. The inhibition of [^{3}H]hydroxyproline synthesis was accompanied by a similar reduction in type I and II procollagen mRNA levels, as detected by Northern blot and dot blot hybridizations with chick proα1(I), proα2(I) and proα1(II) sequence specific cDNAs. The reduction in type II procollagen mRNA level was shown to be dose dependent. Control experiments indicated that the post-translational hydroxylation of prolyl residues was only slightly decreased in dexamethasone treated animals, and that the specific activity of the intracellular free proline pool and the intracellular degradation of collagen were unchanged.

To address the mechanisms of the inhibition of collagen biosynthesis, specific binding of dexamethasone to glucocorticoid receptors in chick embryo tendon and cartilage cells was studied in a whole cell assay using [^{3}H]dexamethasone as the ligand. Matrix-free tendon and cartilage cells had approximately 19,000 and 15,000 receptor sites per cell, respectively, and the binding affinities (K_d) for dexamethasone in tendon and cartilage cells were 2.9×10^{-9} and 2.3×10^{-9} M. Comparable values were obtained using a cytosol binding assay. The nuclear binding of dexamethasone in tendon and cartilage cells were similar. The results suggest that the dexamethasone-induced inhibition of collagen production is primarily due to decreased levels of funtional procollagen mRNA, possibly resulting from receptor-mediated inhibition of the gene expression on the transcriptional level.

Glucocorticoids have been shown to modulate various aspects of cell metabolism both *in vivo* and *in vitro* (for review, see Refs 2–4). These effects are mediated through binding of the glucocorticoids to specific cytoplasmic receptors, and the steroid–receptor complexes then bind to chromatin influencing the gene expression. Several previous studies have demonstrated that glucocorticoids can affect collagen production by fibroblasts from different sources (for review, see Refs 5 and 6). Most studies suggest that glucocorticoids decrease the production of procollagen and that this decrease is mediated by reduction in the level of corresponding procollagen mRNAs [5, 7, 8]. In other studies, however, conflicting results have been obtained in that increased, unchanged or decreased collagen production as a result of steroid treatment has been noted in fibroblast cultures even under apparently identical conditions (see Ref. 6). A plausible explanation for the conflicting results would be that these studies have not addressed the question of receptor density and steroid-binding affinity in the same cell cultures.

In the present study, we have examined the effects of dexamethasone, a synthetic, high potency glucocorticoid, on collagen gene expression by two different cell types, fibroblasts and chondrocytes, isolated from chick embryo tendons and sterna, respectively. We have also determined the comparative binding affinities of dexamethasone and other steroids for the receptors in these cells.

MATERIALS AND METHODS

Material. [^{14}C]Proline (291 Ci/mol), [6,7-

* A preliminary report of this study has been presented at the Annual Meeting of Western Society for Clinical Investigation, Carmel, California, February, 1984 [1]. This work was supported by U.S. Public Health Service, National Institutes of Health grants GM-28833, AM-28450, AM-35297, and by grants from the Medical Research Council of the Academy of Finland and the Sigrid Juselius Foundation.

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^{3}H]dexamethasone (38–50 Ci/mmol) and [6-^{3}H]thymidine (2.0 Ci/mmol) were purchased from New England Nuclear Corp. (Boston, MA). Purified bacterial collagenase was obtained from Sigma Chemical Co. (Kingston-upon-Thames, U.K.), and trypsin, 2.5% (w/v) solution in 0.9% NaCl, was purchased from Gibco Corp. (Grand Island, NY). Newborn and fetal calf serum and tissue culture media were purchased from Flow Laboratories (Ayrshire, U.K.). Non-labeled steroids were purchased from Steraloids, Inc. (Wilton, NH).

Isolation of matrix-free cells from tendon and cartilage. The cells were isolated from leg tendons and sterna of 17-day-old chick embryos by enzymic digestion using trypsin and collagenase, as described previously [9, 10]. For incubations and receptor assays, the cells were suspended in modified Krebs medium, 0.6–10.0 × 10^6 cells per ml.

Collagen assays. Freshly-isolated tendon and cartilage cells in Krebs medium containing (50 μg/ml) ascorbic acid were preincubated for 1 hr with dexamethasone dissolved in ethanol, and all tubes, including the controls, contained 0.1% (v/v) of ethanol. Two μCi of [^{14}C]proline was then added, and the incubation was continued for 2 hr at 37° with shaking. After incubation, 100 μl of Krebs medium containing cycloheximide and α,α′-dipyridyl was added in final concentrations of 100 μg/ml and 1 mM, respectively. The samples were dialyzed against running tap water, hydrolyzed in 6 M HCl, and the total ^{14}C incorporation and the synthesis of [^{14}C]hydroxyproline were assayed [11].

In similar experiments, tendon and cartilage cells were preincubated in the presence of dexamethasone or ethanol for 0, 2, 4 or 7 hr in Krebs medium containing ascorbic acid (50 μg/ml) and then labeled with 10 μCi of [^{3}H]leucine for 1 hr. The labeling was stopped by adding 0.1 volume of solution containing 1 mg/ml cycloheximide, 10 mM α,α′-dipyridyl and 1 mg/ml unlabeled leucine. The medium was separated from the cells by centrifugation, and cells were sonicated in buffer containing protease inhibitors. Aliquots of the cell homogenates were used for cellular protein [12] and DNA assays [13]. Aliquots of medium and cell fractions were also dialyzed against running tap water, and then digested with bacterial collagenase (Sigma, chromatographically purified, sp. act. 456 U/mg) (100 μg/ml) in buffer containing 0.154 M NaCl, 0.25 M Tris–HCl, pH 7.5 and 10 mM CaCl for 2 hr at 37°. The digestion was stopped by 25 mM EDTA. Albumin was added as a carrier, and undigested proteins were precipitated by adding an equal volume of 40% TCA. The samples were centrifuged and aliquots of the supernatants were counted.

The effect of dexamethasone *in vivo* was studied by injecting the steroid onto the chorioallantoic membrane of 16-day-old chick embryos. Dexamethasone was dissolved in 200 μl of physiological saline containing 6% ethanol. Control embryos received 200 μl saline–ethanol solution alone. Following the incubation at 37°, the tendons and sterna were dissected and collagen synthesis was measured by incubation in Krebs medium with [^{14}C]proline or [^{3}H]leucine, as described above.

Following the incubation with [^{14}C]proline, tendons and sterna were homogenized, dialyzed against running tap water, and total ^{14}C-radioactivity and [^{14}C]hydroxyproline were assayed, as described above. After labeling with [^{3}H]leucine, samples were dialyzed against 0.5 M acetic acid, and an aliquot was incubated with 100 μg/ml of pepsin (sp. act. 2500 U/mg) for 24 hr at 4°. Following the incubation, albumin was added as carrier and non-digested proteins were precipitated with TCA. After centrifugation, aliquots of the supernatants were used for counting. Aliquots of the dialysates were neutralized and used for bacterial collagenase digestion, as described above.

The conversion of procollagen to collagen was examined employing pulse-chase techniques [14]. The sterna were pulse-labeled with [^{14}C]proline for 30 min, as decribed above. Further incorporation of radioactivity was then inhibited by the addition of [^{12}C]proline and cycloheximide in final concentrations of 0.5 mg/ml and 100 μg/ml, respectively. The incubation was continued for 120 min, the incubation was stopped by the addition of protease inhibitors and 3% SDS.* The samples were boiled for 5 min, and homogenized with a Polytron tissue homogenizer. The samples were extracted for 30 min at 37°, centrifuged at 15,000 *g* at room temperature, and the supernatant was examined by SDS–polyacrylamide gel electrophoresis using 6% gels [15]. The ^{14}C-polypeptides were visualized by fluorography [16], and the bands representing proα, pNα and α-chains of type II procollagen and collagen were quantitated by densitometry.

Assay of Type I and II procollagen mRNA. For isolation of RNA, tendons from chick embryos were homogenized with a Polytron tissue homogenizer in 10 mM Tris–HCl, pH 7.4, containing 1 mM EDTA and 1% SDS. Proteinase K (Boehringer), 100 μg/ml, was added and the samples were incubated for 120 min at 65°. NaCl, to a final concentration of 0.4 M, was then added, and poly(A)$^+$–RNA was isolated by affinity chromatography on an oligo-dT cellulose column [17]. For measurements on sternal mRNA concentrations, total RNA was prepared, as described previously [18].

For Northern blot analyses, aliquots of poly(A) or total RNA were electrophoresed on 1.0% agarose gels under denaturing conditions [19]. The RNA was transferred to nitrocellulose paper, air dried and heated at 78° for 120 min in a vacuum oven. For dot blot analyses, RNA samples were mixed with an equal volume of solution containing 3 volumes of 20 × SSC and 2 volumes of 37% formaldehyde. Aliquots were dot blotted on nitrocellulose paper using a vacuum manifold (model SRC-96, Schleicher and Schuell). The filters were air dried and heated at 78°, as above.

The tendon RNA samples bound to the nitrocellulose filters were hybridized with pCg54 and pC45 cDNAs, kindly provided by Dr. Helga Boedtker, Harvard University. These two cDNAs correspond to chick type I collagen proα1 and proα2 sequences,

* Abbreviations used: K_D, dissociation constant; Na_2EDTA, disodium ethylenediaminetetraacetate; SDS, sodium dodecyl sulfate; SSC, 0.15 M NaCl in 15 mM sodium citrate, pH 6.8; Dex, dexamethasone; NEM, *n*-ethylmaleimide; DTT, dithiothreitol.

respectively [20]. The sternal RNA samples were hybridized with cDNA clone pcARI corresponding to chick proα1(II) collagen mRNA [21]. The cDNAs were labeled with α-^{32}P-nucleotides by nick translation to the specific activity of 4×10^8 cpm/μg [22]. The nitrocellulose filters were first pre-hybridized for 16 hr at 42° in a solution containing 4 × SSC, 50% formamide, 0.1 SDS, 50 μg/ml sheared salmon sperm DNA, 0.02% polyvinylpyrolidone, 0.02% bovine serum albumin and 0.02% ficoll, 25 μl/cm^2. Hybridization was carried out in the same solution, 15 μl/cm^2, containing one of the ^{32}P-labeled cDNAs either for proα1(I), proα2(I) or proα(II) at 42° for 48 hr. The filters were washed first twice in 500 ml of 2 × SSC, at 24°, and then twice each with 1 × SSC, 1 × SSC plus 0.1% SDS, 0.5 × SSC plus 0.1% SDS and 0.2 × SSC all at 60°. The dried filters were then exposed to X-ray films. The level of mRNA was quantitated either by scanning the dot blots at 700 nm using an automatic computing densitometer (Gelman ACD 18), or the dots visualized by exposure to X-ray film were cut out and counted in liquid scintillation counting solution (NEN-947, New England Nuclear).

Glucocorticoid receptor assay. For glucocorticoid receptor measurements, two methods were used. First, a whole-cell binding assay, which has been extensively used for steroid receptor determinations in other cell systems [23–27], was adopted. In this assay, either tendon or cartilage cell suspensions containing 0.8 ml aliquots 0.5–8 × 10^6 cells, were pipetted to siliconized glass tubes, followed by 0.2 ml of modified Krebs medium containing [6,7-^{3}H]dexamethasone in final concentrations of 1.1–100 nM. The nonspecific binding of [6,7-^{3}H]dexamethasone was estimated from parallel sets of tubes containing a 200-fold excess of unlabeled dexamethasone. The tubes were incubated for 45 min at 37° with intermittent shaking. Two ml of Krebs medium (4°) were then added to each tube, and the tubes were centrifuged at 1000 *g* at 22°. The supernatants were discarded and the pellets suspended in 3 ml of Krebs medium. The tubes were incubated for 10 min at 22°, and the cells were again collected by centrifugation. The washing procedure was repeated once, and the cell pellets were suspended in 0.5 ml of Krebs medium and aliquots of the sample were used for cell counting by hemocytometer, for assay of cellular protein [12] and for assay of DNA [13]. Aliquots were also dissolved in 10 ml of counting solution for liquid scintillation counting.

Steroid specificity studies were conducted according to the standard procedure described above, using 1.1×10^{-8} M [6,7-^{3}H]dexamethasone and varying concentrations (1.1×10^{-8}–1.1×10^{-5} M) of different unlabeled steroids.

Glucocorticoid receptors were also assayed using a cytosol assay. Isolated tendon or cartilage cells were homogenized by Teflon and glass homogenizer (20 strokes) in hypotonic buffer (20 mM Tris–HCl, pH 7.5, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10% glycerol, 10 mM sodium molybdate, and 2 mM DTT) at 4°. The samples were centrifuged for 60 min at 100,000 *g* at 4°, and the supernatants were used for receptor assay and for protein measurements [12]. For receptor assay, aliquots of cytosol were incubated with 6, 21, or 50 nM [^{3}H]dexamethasone in the presence or absence of a 200-fold excess of cold dexamethasone

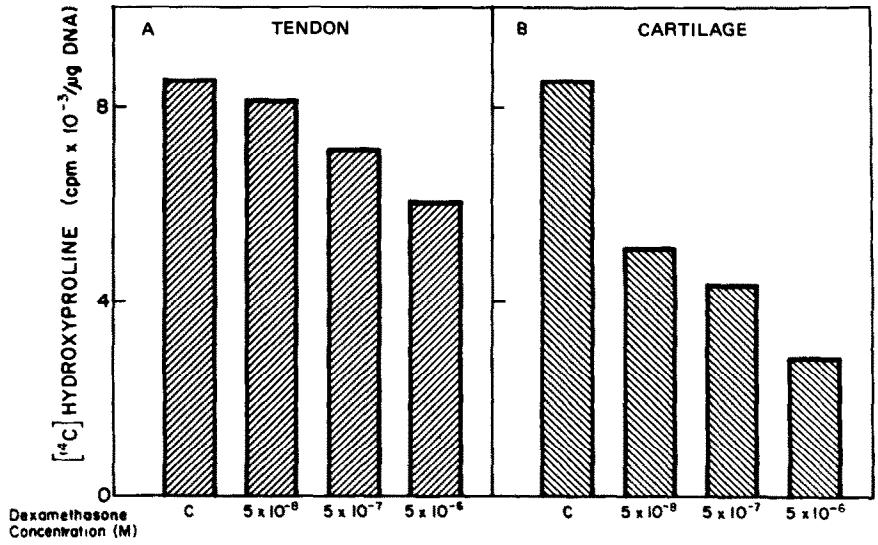

Fig. 1. Inhibition of collagen synthesis in chick embryos injected with dexamethasone. Varying concentrations of dexamethasone were injected onto the chorioallantoic membrane of 16-day-old chick embryos. Control embryos (C) received a corresponding amount of solvent. Tendons and sterna were removed at 24 hr, and collagen synthesis was assayed by incubation with [^{14}C]proline for 90 min at 37° [^{14}C]Hydroxyproline in the non-dialyzable fraction and tissue DNA were determined, as indicated in Materials and Methods. The concentrations of dexamethasone indicated in the figure are calculated as final concentrations *in vivo*. The values are the means of three parallel determinations on two separate samples. The variation within the assay was less than 10%. A. [^{14}C]Hydroxyproline in tendons. B. [^{14}C]Hydroxyproline in sterna.

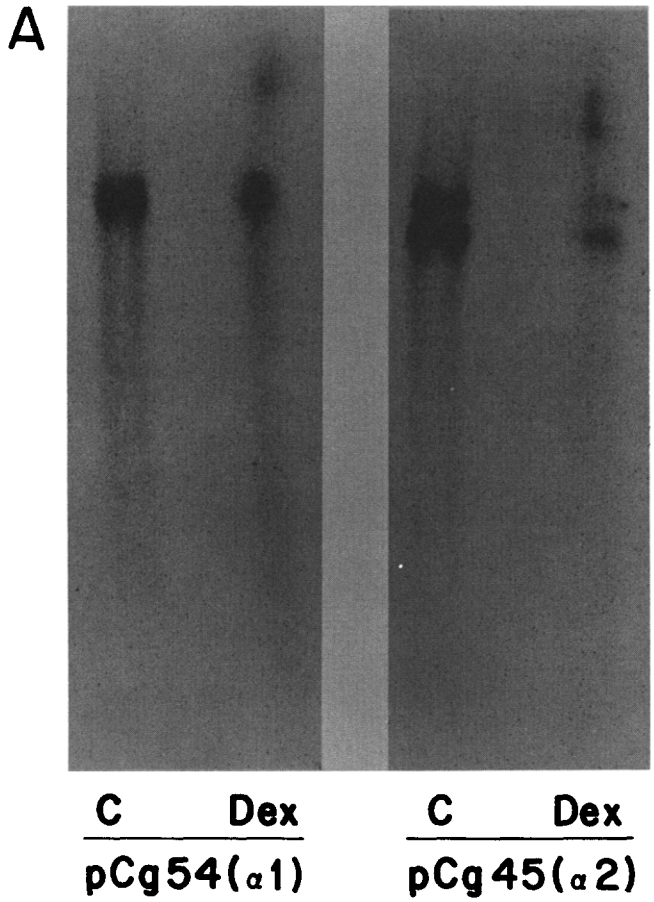

Fig. 2. (*continued on facing page*).

for 4–20 hr at 4° in the final volume of 200 μl. After incubation, small aliquots were taken for total radioactivity determinations, and 200 μl of charcoal–dextran solution (1% charcoal, 0.05% dextran in 10 mM Tris, pH 7.5, and 30% glycerol) was added [28]. The samples were vortexed and incubated at cold for 5 min, and centrifuged for 5 min. Aliquots of the supernatant were used for counting of total binding and nonspecific binding of [^{3}H]dexamethasone. The specific binding of [^{3}H]dexamethasone was expressed per mg cytosol protein. Cytosols were also analyzed by gel filtration on Sephadex G-100 column (5.0 × 0.5 cm) in a buffer containing 20 mM Tris–HCl, pH 7.5, 2 mM $CaCl_2$, and 2 mM $MgCl_2$; fractions of 0.2 ml were collected and counted for radioactivity.

Nuclear binding. Tendon and cartilage cells were isolated as described above. The cells were resuspended in Krebs buffer and incubated with 11 nM [^{3}H]dexamethasone in the presence or absence of a 200-fold excess of unlabeled dexamethasone for 45 min at 37°. The incubation was stopped by adding 2 ml cold Krebs buffer, and cells were washed three times as described above. After the washings, the cell pellets were suspended in hypotonic buffer (20 mM Tris–HCl, pH 7.5, containing 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10% glycerol and 2 mM DTT) [29]. After a 10 min incubation at cold, cells were lysed by freeze-thawing in a dry ice–ethanol bath. The nuclei were collected by centrifugation and washed two times with hypotonic buffer. Small aliquots were then taken for radioactivity counting. The binding of [^{3}H]dexamethasone was expressed per 10^6 nuclei.

Other assays. For thymidine incorporation studies, the matrix-free tendon or cartilage cells were preincubated in Krebs medium for 60 min with or without dexamethasone. [^{3}H]Thymidine, 0.5 μCi/ml, was added and the incubation was continued for 120 min. The samples were filtered on glass-fiber filters (Millipore), and the ^{3}H-radioactivity recovered on the filters was determined by liquid scintillation counting.

RESULTS

Dexamethasone inhibits collagen synthesis in chick embryos injected in vivo

The effects of glucocorticoids on collagen metabolism were studied both *in vivo* and *in vitro*. In the first set of experiments, dexamethasone was injected onto the chorioallantoic membrane of the chick embryos. After 24 hours, the tendons and sterna were dissected and incubated *in vitro* for 120 min in a medium containing [^{14}C]proline. The total incorporation of ^{14}C into proteins and the synthesis of [^{14}C]hydroxyproline were markedly reduced by

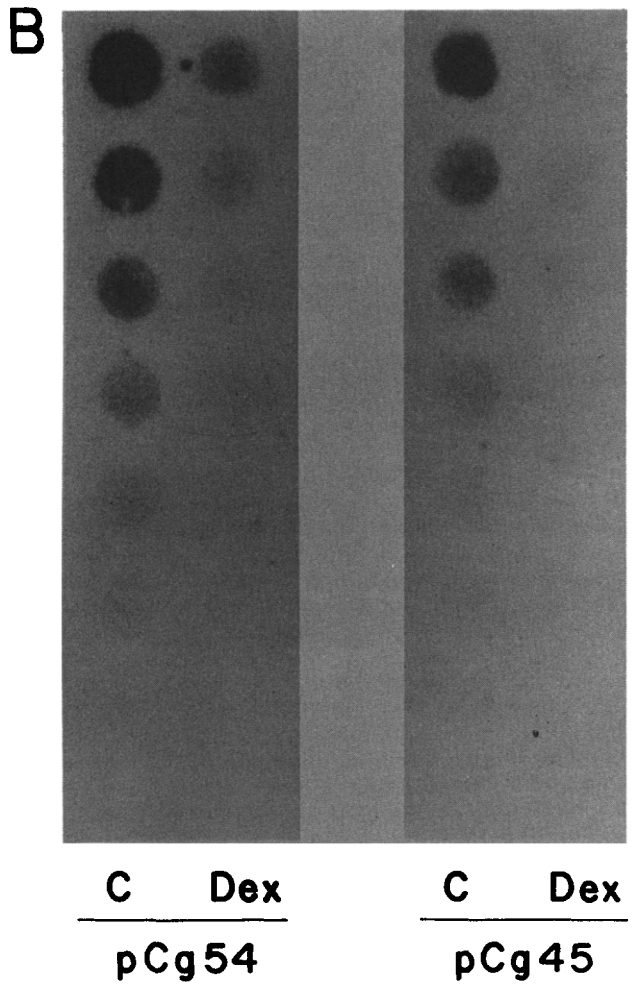

Fig. 2. Northern blot and dot blot hydridizations of poly(A)$^+$–RNA isolated from tendons of chick embryos injected with dexamethasone. Sixteen-day-old chick embryos were treated with dexamethasone in a final concentration of 5×10^{-6} M, as indicated in Fig. 1. After a 24 hr incubation, the tendons were removed, and poly(A)$^+$–RNA was isolated, as described in Methods. Poly(A)$^+$–RNA, 0.3 μg, from dexamethasone-treated embryos (Dex) or from the controls (C) were electrophoresed on 1% agarose gel under denaturing conditions and transferred to nitrocellulose filters (Frame A). Also, poly(A)$^+$–RNA varying from 100 ng to 0.78 ng, in 1:2 dilutions, were dot blotted on the nitrocellulose filters (Frame B). The filters were hybridized with ^{32}P-labeled cDNAs corresponding to chick proα(I) (pCg54) and proα2(I) (pCg45) sequences.

dexamethasone in tendons and cartilage, when the values were expressed per μg DNA in the corresponding sample (Fig. 1). The synthesis of [^{14}C]hydroxyproline was reduced more than the incorporation of total ^{14}C, and consequently, the ratio of [^{14}C]hydroxyproline/total ^{14}C was markedly decreased in dexamethasone-treated animals; this effect was particularly striking in cartilage tissues (see also Table 3).

Since the synthesis of [^{14}H]hydroxyproline may be affected on the post-translational level, similar experiments were performed using [^{3}H]-leucine as a precursor amino acid and by assaying collagen synthesis as bacterial collagenase digestable ^{3}H-peptides or as pepsin-resistant triple-helical ^{3}H-collagen (see Materials and Methods). Employing bacterial collagenase, the synthesis of collagenous polypeptides was shown to be $5.4 \pm 0.3 \times 10^2$ cpm/μg protein, when tendons from control chick embryos were incubated with [^{3}H]leucine *in vitro*. When the embryos were injected with 5×10^{-6} M dexamethasone for 24 hr *in vivo*, the *in vitro* synthesis of collagen was reduced to $4.3 \pm 0.3 \times 10^2$ cpm/μg protein. In this experiment, $47.4 \pm 4.1\%$ of the [^{3}H]leucine incorporation was in collagenous protein in the control tendon. The corresponding value in dexamethasone-treated embryos was $39.6 \pm 1.6\%$. Essentially similar results were obtained when limited pepsin proteolysis was used as probe for collagen production.

To examine the post-translational hydroxylation of prolyl residues in collagen, [^{14}C]proline-labelled collagen synthesized by sterna from control and dexamethasone-(5×10^{-6} M) treated animals was subsequently isolated by limited pepsin proteolysis and differential salt precipitation. Assay of

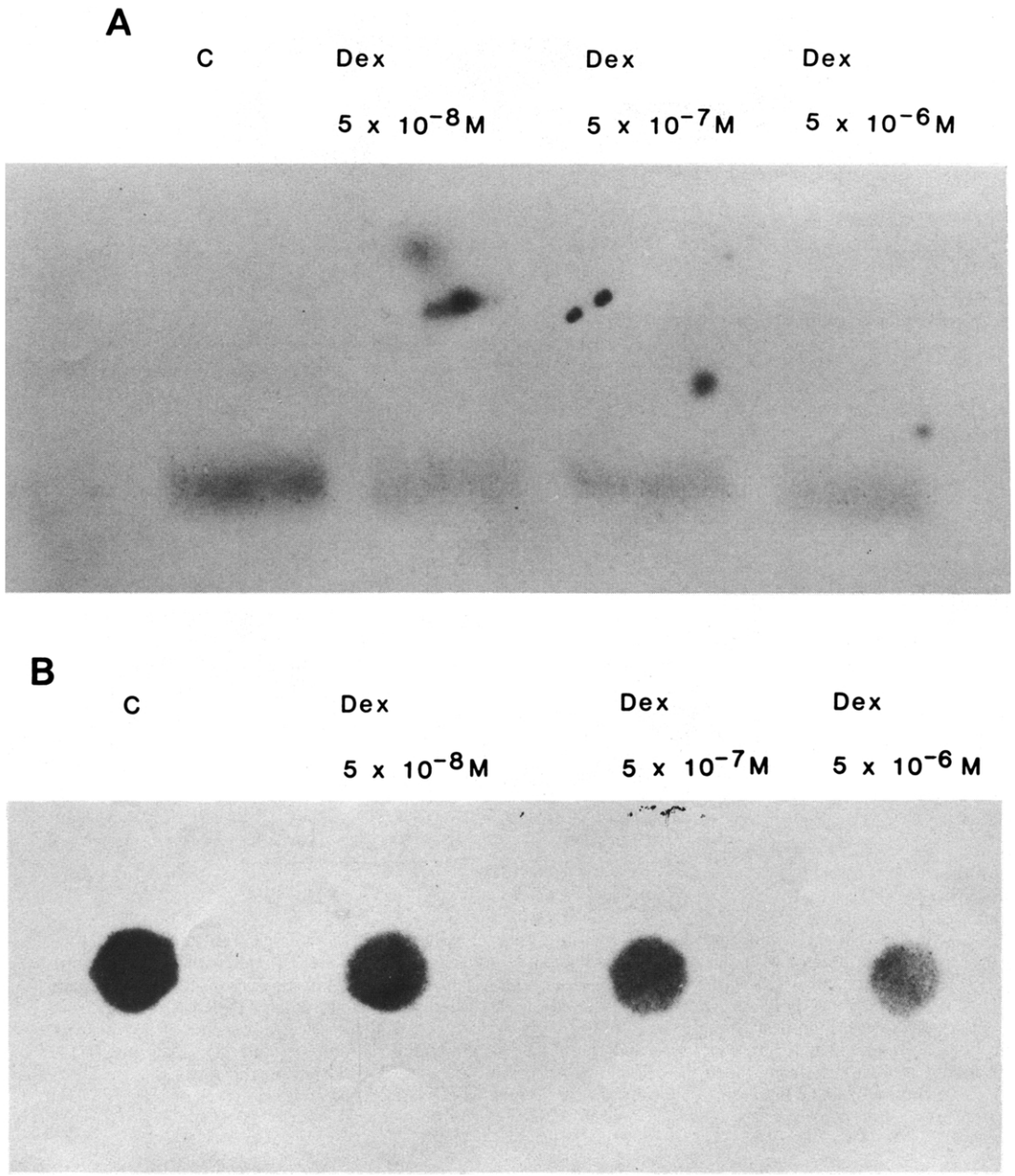

Fig. 3. Northern blot and dot blot hybridizations of RNA isolated from sterna of chick embryos treated with dexamethasone. The 16-day-old chick embryos were treated with varying concentrations of dexamethasone as described in Fig. 1. Total sternal RNA was isolated after a 24 hr incubation from dexamethasone-treated (Dex) or from controls (C) and used for northern blot (A) or dot blot (B) hybridizations using nick-translated pCARI DNA as probe. Same amounts of total RNA were analyzed in northern blot and dot blot assays in each lane.

[^{14}C]hydroxyproline and [^{14}C]proline by an amino acid analyzer indicated that in controls, the ratio of [^{14}C]hydroxyproline/[^{14}C]proline was 1.08 ± 0.03 (mean ± SD; N = 3); while the corresponding value in ^{14}C-collagen isolated from dexamethasone treated sterna was 0.93 ± 0.03.

Examination of the newly-synthesized procollagens by SDS–polyacrylamide gel electrophoresis revealed that there were no changes in the relative mobilities of proα1(I), proα2(I) or proα1(II) chains of type I and type II procollagen, respectively, synthesized by tendon or sterna in the presence of 5×10^{-6} M dexamethasone, as compared to the controls. The ratio of proα1(I):proα2(I) synthesized by the control and dexamethasone-treated tendons was 1.99 and 1.95, respectively. Also, there was no evidence of the synthesis of proα2(I) chains either by control or dexamethasone treated sterna. Thus, no evidence of a structurally-abnormal procollagen or a change in the expression of genetically-distinct

Table 1. Effect of dexamethasone on proα1(II) collagen mRNA levels in chick embryo sterna

Treatment	proα1(II) Collagen mRNA level relative to control
Control	1.0
Dexamethasone	
5×10^{-8} M	0·59
5×10^{-7} M	0·47
5×10^{-6} M	0·42

Dexamethasone was injected onto the chorioallantoic membrane of 16-day chick embryos as described in Fig. 1. Total sternalRNA was isolated at 24 hr and the levels of proα1(II) collagen mRNA were estimated in a dot blot hybridization assay using nick-translated pCARI DNA as the probe. The concentrations of dexamethasone indicated are calculated as final concentrations *in vivo*. The values are the means of three separate hybridization assays.

collagen genes was seen in dexamethasone treated animals.

Procollagen mRNA levels are reduced in tendons and sterna of embryos injected with dexamethasone

Since the synthesis of ^{14}C-procollagen was markedly reduced in tendons and sterna isolated from embryos following a 24 hr injection with dexamethasone, we further assayed type I and II procollagen-specific mRNA levels in tendon and sternal samples employing chick proα1(I), proα2(I) and proα1(II) sequence specific DNA probes.

The relative levels of proα1(I)- and proα2(I)-specific mRNAs in tendons were quantitated by dot blot analyses. The results indicated that the ratio of proα1(I)/proα2(I)-specific mRNA in control tendons was approximately 2:1. However, both proα1(I) and proα2(I) mRNAs in dexamethasone-treated samples were reduced to about 22% of the level noted in controls (Fig. 2B). As the procollagen synthesis was inhibited to a greater degree in sterna than in tendons, the dose-dependency of the inhibition was tested also at the level of type II procollagen mRNA. Total RNA isolated from sterna of chick embryos exposed to different concentrations of dexamethasone were analyzed for the abundance of proα1(II) mRNA in a dot blot assay (Fig. 3B). The results, shown in Table 1, indicated that the reduction in proα1(II) collagen mRNA levels was dose-dependent and closely parallels the reduction observed in procollagen synthesis (see Fig. 1).

The specificity of hybridization was confirmed by Northern blot hybridizations. In accordance with earlier demonstrations [20, 21], pCg54 hybridized with one major and one minor proα1(I) specific poly $(A)^+$-RNA species migrating close to each other in the 4.9 kb region and pCg45 revealed two distinct proα2(I) specific mRNAs in the 5.1 kb region (Fig. 2A), while pCARI, hybridized with one major proα1(II) mRNA transcript of the size of 5.3 kb (Fig. 3A) and a minor one of ~7 kb (not shown). It should be noted that in tendons after dexamethasone treatment extra high molecular weight hybridization bands were observed (Fig. 2A).

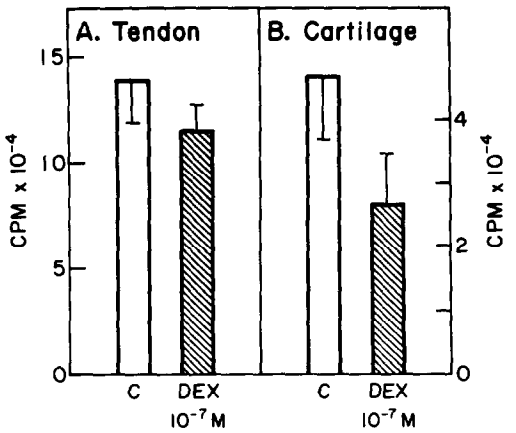

Fig. 4. Inhibition of collagen synthesis in chick embryo tendon and cartilage cells *in vitro*. Isolated chick embryo tendon and cartilage cells were preincubated in the presence of 10^{-7} M dexamethasone for 7 hr and then labeled for 1 hr with [^{3}H]leucine. Collagenous proteins were assayed by using bacterial collagenase, as described in Materials and Methods, and the values are the means of three parallel samples (mean ± SEM).

Effect of dexamethasone on collagen synthesis by tendon and cartilage cells in vitro

To study the effects of dexamethasone on collagen production *in vitro*, matrix-free cells isolated from tendon and cartilage, respectively, were incubated with [^{14}C]proline and the total incorporation of ^{14}C-radioactivity into nondialyzable fraction, as well as the formation of [^{14}C]hydroxyproline were assayed. The results indicated that the addition of dexamethasone in 10^{-8} or 10^{-6} M concentration, when present during a 120 min preincubation and subsequent 120 min labeling period, had no effect on total incorporation of ^{14}C or on the synthesis of [^{14}C]hydroxyproline. A small inhibition with 10^{-5} and 10^{-4} M concentrations of dexamethasone was noted in both tendon and cartilage cells. The latter effect was not specific to collagen, however, since the total incorporation of ^{14}C and the synthesis of [^{14}C]hydroxyproline were reduced to the same extent. Furthermore, the inhibition noted with 10^{-4} M dexamethasone could be explained, in part, by a generalized inhibition of cell metabolism, since the same concentration of dexamethasone decreased the [^{3}H]thymidine uptake both in tendon and cartilage cells by about 20%. Lower concentrations of dexamethasone did not affect the [^{3}H]thymidine uptake *in vitro*.

Since no effect on collagen synthesis by dexamethasone was noted after a 120 min preincubation, in further experiments the preincubation time was extended up to 7 hours. Collagen synthesis, assayed by bacterial collagenase digestion after a 60 min pulse-labeling with [^{3}H]leucine at the end of the 7 hr preincubation period, was reduced in tendon cells by 17.2% and in cartilage cells by 43.6% (Fig. 4).

Dexamethasone does not inhibit the conversion of type II procollagen to collagen

Previous studies have suggested that glucocorticoids in relatively high concentrations may affect

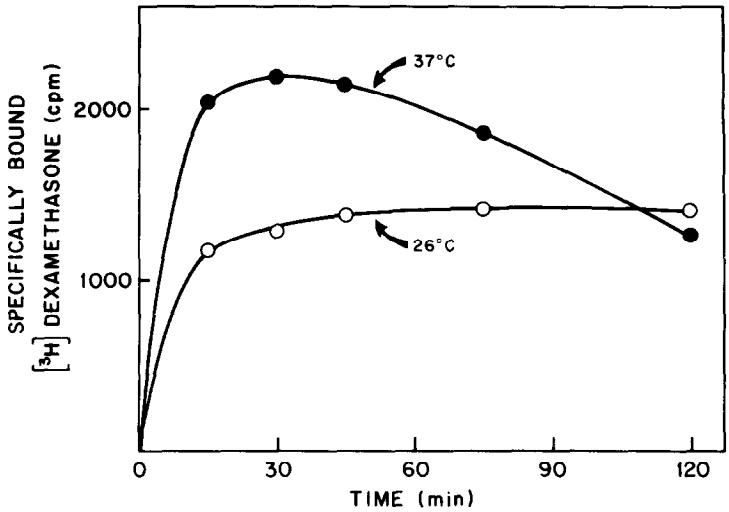

Fig. 5. Time-course for the specific binding of [^{3}H]dexamethasone to freshly isolated chick embryo tendon cells. Freshly isolated tendon cells (2.2×10^6 per tube) were incubated at 37° or 26° in the presence of 11 nm [^{3}H]dexamethasone for the time periods indicated. A parallel set of tubes with [^{3}H]dexamethasone and a 200-fold excess of unlabeled dexamethasone was incubated for the estimation of non-specific binding. The specific binding was calculated by deducting the non-specific binding from total binding.

the activities of enzymes participating in the post-translational modifications of collagen [5]. Also, the results above indicated that the hydroxylation of prolyl residues was slightly suppressed by dexamethasone. Here, the effects of dexamethasone on the conversion of procollagen to collagen, catalyzed by two separate proteinase *in vivo* [30] were studied in two sets of experiments. First, the chick embryos were injected with dexamethasone *in vivo*, the sterna were removed 24 hr later, and the conversion of type II procollagen to collagen was studied using pulse-chase techniques. Dexamethasone treatment *in vivo*, in 5×10^{-6} and 5×10^{-7} concentrations, had no effect on the conversion of procollagen to collagen *in vitro*, and at the end of the 120 min chase period, over 80% of the collagenous polypeptides were recovered as α-chains both in control and dexamethasone-treated tissues. Secondly, dexamethasone in concentrations as high as 10^{-4} M, had no effect on the conversion of type II procollagen to collagen when added directly to the

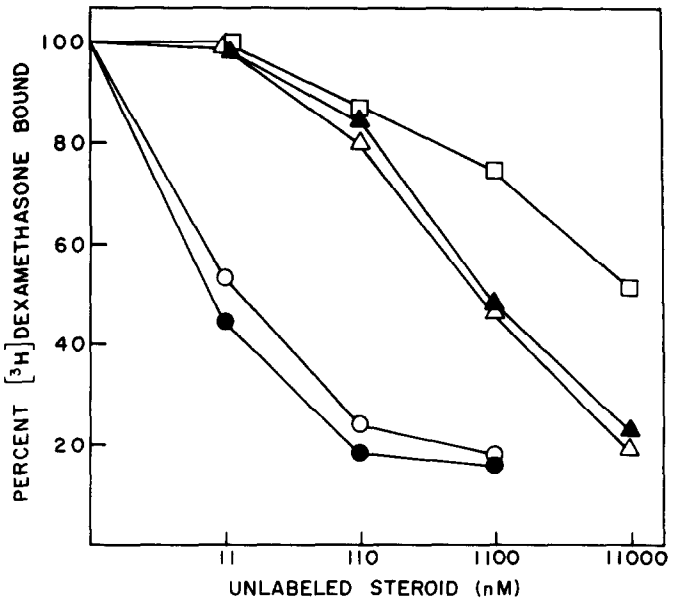

Fig. 6. Competitive inhibition of the [^{3}H]dexamethasone binding to isolated chick embryo tendon cells by unlabeled steroids. Cells, 2.5×10^6 per tube, were incubated at 37° for 45 min in the presence of 11 nM [^{3}H]dexamethasone and varying concentrations of unlabeled steroids. The total binding was determined as in Fig. 5. The unlabeled steroids used were: dexamethasone (○), triamcinolone acetonide (●), progesterone (△) deoxycortisone (▲) and estradiol (□).

incubation medium after pulse-labeling of sterna with [^{14}C]proline.

Assay for glucocorticoid receptors

Since the same concentration of dexamethasone consistently suppressed the collagen production more in cartilage cells than in tendons, in further studies we tested the hypothesis that differences in glucocorticoid receptor characteristics might explain this difference. The glucocorticoid receptors were assayed by two independent methods, whole-cell and cytosol binding assays, using [^{3}H]-dexamethasone as the ligand.

For whole-cell assay, a method, which has been extensively used for steroid receptor assay in other cell systems, was optimized for tendon and cartilage cells. First, the time-course of the binding was determined at two different temperatures, 26° and 37°, by incubating tendon fibroblasts with [^{3}H]-dexamethasone. The specific binding of the steroid to the cells rapidly increased with time, and it was maximal at both temperatures after 30–45 min incubation (Fig. 5). At 37°, the binding somewhat decreased after 60 min incubation. The binding after 30 min incubation at 26° was only 59% of the binding at 37°. Therefore, 45 min incubation at 37° was chosen for the standard assay condition.

In further control experiments, the effect of washings on the specific binding was examined. The results indicated that two careful washings were sufficient to remove unbound [^{3}H]dexamethasone, the nonspecific binding being 14% of the specific binding (data not shown).

Table 2. [^{3}H]Dexamethasone binding sites in chick embryo cells freshly isolated from tendon and cartilage

Cells	Sites/cell*
Tendon cells	19520 ± 2440
Cartilage cells	14800 ± 5400

* The values are mean ± SD of three separate assays.

Finally, the effect of fetal calf serum, necessary for cell isolation and efficient collagen synthesis [31], on the steroid binding was tested. The results indicated that nondialyzed fetal calf serum in 10% concentration had no effect on the receptor binding, as compared to the binding in a medium devoid of fetal calf serum. Dialysis or charcoal treatment of the serum did not change the binding. In contrast, nondialyzed human serum in 10% concentration reduced the specific binding by 73%. Incubation of the cells with 10 mM NEM completely abolished the specific binding of [^{3}H]-dexamethasone, suggesting the participation of free sulfhydryl groups in the binding [32].

Specificity of the glucocorticoid receptors in tendon fibroblasts

To examine the specificity of dexamethasone binding, competitive binding studies using several other steroids were performed. These studies were done with [^{3}H]dexamethasone in a final concentration of 11 nM so as to guarantee the saturation of the receptor sites. The results indicated that triamcinolone acetonide displaced dexamethasone in equimolar concentration (Fig. 6). Deoxycortisone and progesterone also reduced the binding of dexamethasone, but a 100-fold excess of each steroid was required to displace more than 50% of the [^{3}H]dexamethasone molecules (Fig. 6). Estradiol and testosterone had significant effects on dexamethasone binding only in a 1000-fold excess.

Specific binding of dexamethasone to receptors in chick embryo fibroblasts and chondrocytes

Specific glucocorticoid receptors could be demonstrated in matrix-free cells isolated both from tendons and sterna of the 17-day-old chick embryos using the whole-cell binding assay (Fig. 7). The receptor density and the binding constant (K_d) were estimated from Scatchard plots [33]. Freshly isolated tendon fibroblasts had approximately 19,500 receptor sites per cell (Table 2). The receptor number,

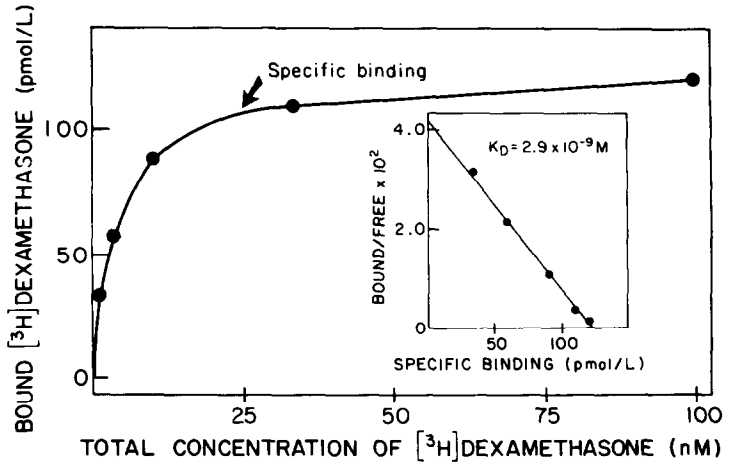

Fig. 7. Specific binding of [^{3}H]dexamethasone to isolated chick embryo tendon cells. Freshly isolated tendon cells were incubated with varying concentrations of [^{3}H]dexamethasone, and the specific binding of the steroid was determined as indicated in Fig. 5 and in Materials and Methods. The maximum binding and the binding affinity (K_D) were estimated from Scatchard plots (inset).

which corresponds to 0.34 fmol/μg cell protein, remained approximately the same when the cells were placed in primary culture. The number of receptor sites in freshly isolated chondrocytes was about 15,000 per cell. The K_D for freshly isolated tendon cells was approximately 3 nM and similar values were detected in freshly isolated chondrocytes.

The receptor determinations were also performed using cytosol binding assay. Cytosol preparations were incubated in the presence of [^{3}H]dexamethasone, with and without a 200-fold excess of unlabeled dexamethasone; the bound and free ^{3}H-radioactivity was then separated either by charcoal–dextran treatment or by gel filtration chromatography. Gel filtration chromatography of tendon cytosols revealed a distinct peak of ^{3}H-radioactivity corresponding to specific binding near the V_0 of the column (not shown). Assay of [^{3}H]dexamethasone binding by charcoal–dextran treatment indicated a value of 0.41 fmol/μg cytosol protein in tendon cells. In cartilage cell preparations, the corresponding value was 0.37 fmol/μg.

Translocation of steroid–receptor complexes into nucleus

Since no marked differences in the receptor densities or the binding affinities between tendon and cartilage cells were noted, we further examined the nuclear binding of [^{3}H]dexamethasone in these two types of cells; as indicated elsewhere [2–4], this step is necessary for receptor-mediated action by the steroid. After a 45 min incubation of the cells at 37°, a significant amount of ^{3}H-radioactivity was detected in the nuclei isolated both from tendon and cartilage cells (Fig. 7). In both cells, most of the ^{3}H-radioactivity could be displaced by a 200-fold excess of unlabeled dexamethasone. The calculated specific binding was 9.0 ± 0.6 fmol/10^6 nuclei in tendon cells, and 8.0 ± 3.3 fmol/10^6 nuclei in cartilage cells. Thus, no significant difference in nuclear translocation of the steroid–receptor complexes was noted.

Effect of deoxycortisone on collagen biosynthesis

The initial studies described above indicated that deoxycortisone in approximately 100-fold excess could compete with dexamethasone for the specific binding. It was, therefore, of interest to determine whether deoxycortisone might elicit an inhibition of collagen synthesis in a similar manner as dexamethasone *in vivo*. Chick embryos were injected with deoxycortisone and dexamethasone in concentrations of 2.5×10^{-5} and 5×10^{-7} M, respectively; the concentrations were comparable in their affinity for glucocorticoid receptors, as estimated from competitive binding studies (see Fig. 6). Both steroids reduced the total incorporation of [^{14}C]proline into nondialyzable protein to the same extent (Table 3). The inhibition of [^{14}C]hydroxyproline synthesis was even more pronounced, and consequently, the ratio of [^{14}C]hydroxyproline/total ^{14}C decreased in both dexamethasone- and deoxycortisone-treated sterna. Treatment of the embryos with a combination of the two steroids did not reduce the ^{14}C incorporation or [^{14}C]hydroxyproline synthesis more than each one of the steroids alone, suggesting that the effects of both steroids were mediated through a shared mechanism.

DISCUSSION

The results indicated that administration of dexamethasone to chick embryos *in vivo* resulted in a markedly decreased protein synthesis, as measured by incorporation of radioactive proline into protein. The synthesis of radioactive hydroxyproline, a marker for collagen production, was reduced even more relative to the decreased total protein synthesis. The reduction in collagen production in tendons was accompanied by a marked decrease in the levels of type I procollagen mRNAs coding for proα1(I) and proα2(I) polypeptides. Similar decrease was recently noted in the skin of neonatal chicks treated with dexamethasone [34]. Even more interestingly, a concomitant dose-dependent reduction in type II procollagen production on one hand and the type II procollagen mRNA abundance on the other was noted in sterna of chick embryos treated with dexamethasone. Thus, a parallel reduction in the synthesis of the procollagen and the corresponding mRNA levels was observed in two tissues tendons and cartilage, expressing genetically different procollagen genes, type I and II procollagen, respectively. Since the post-translational hydroxylation of prolyl residues in newly-synthesized collagen was only slightly decreased in chick embryos injected with dexamethasone, these observations suggest that the reduced collagen gene expressed in this experimental system results primarily from reduced transcription of the genomic DNA coding for the corresponding procollagen. These results do not exclude, however, the possibility that the degradation and turnover of procollagen mRNAs are

Table 3. Effects of dexamethasone and deoxycortisone on collagen synthesis by chick embryo sterna

Treatment	Total ^{14}C (dpm × 10^3/μg DNA)	[^{14}C]Hydroxyproline (dpm × 10^3/μg DNA)	[^{14}C]hydroxyproline/ Total ^{14}C
Control	28.9	8.4	0.29
Dexamethasone 5×10^{-7} M	22.7	5.0	0.22
Deoxycortisone 2.5×10^{-5} M	18.2	4.0	0.22

Deoxycortisone and dexamethasone were injected onto the chorioallantoic membrane of 16-day-old chick embryos as described in Fig. 1. Sterna were isolated at 24 hr, and collagen synthesis was assayed by incubation with [^{14}C]proline for 90 min at 37°. The values are means of three parallel determinations of two separate samples. The variation within the assay was less than 10%.

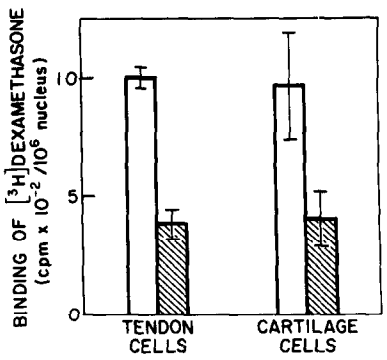

Fig. 8. Nuclear uptake of [^{3}H]dexamethasone in isolated tendon and cartilage cells. Isolated chick embryo tendon and cartilage cells were labeled for 45 min at 37° with 11 nM [^{3}H]dexamethasone in the presence (hatched column) or absence of a 200-fold excess of unlabeled dexamethasone (open column), and nuclearly bound [^{3}H]dexamethasone was assayed as described in Materials and Methods. The values are mean ± SD of three parallel samples.

selectively increased in the presence of dexamethasone [35]. Control experiments with matrix-free cells indicated that the reduction in total protein synthesis was not a result of changes in the specific radioactivity of intracellular free proline pool, nor was the proliferation of cells affected, as determined by the uptake of radioactive thymidine. Of particular interest was the observation that the synthesis of hydroxyproline, in response to dexamethasone injection, was decreased significantly more in cartilage than in tendon cells. The reasons for this observation are unclear, but it cannot be explained on the basis of differences in the receptor densities, binding affinities or nuclear binding of steroid–receptor complexes, since they were essentially the same in these two cell types. The results of this study indicate that matrix-free cells isolated from chick embryo tendons or cartilage contain high affinity receptors for glucocorticoids. The estimated number of receptor sites in freshly isolated tendon cells was about 19,500 sites per cell, and the corresponding number in cartilage cells was approximately 15,000. These values are in the same range as has been reported for other fibroblastic cell lines previously [36–38]. It is thus possible that some post-receptor mechanisms contribute to the differences in the response to steroids in different target tissues.

The specificity of the glucocorticoid receptors was examined by competitive binding studies employing a variety of steroids. The results indicated that dexamethasone, which had a K_D of 2.9×10^{-9} M in tendon cells, could be competitively displaced from the receptors by triamcinolone acetonide in an apparent equimolar concentration. A displacement of dexamethasone from the receptors was also noted with progesterone and deoxycortisone, but the concentrations required for 50% displacement were more than 100-fold in excess of dexamethasone. No significant displacement was noted with estradiol or testosterone in concentrations up to 1000-fold molar excess of dexamethasone. *N*-ethylmaleimide completely abolished the specific dexamethasone binding, suggesting that the receptor function required free sulfhydryl groups [32].

The reduction in total incorporation and hydroxyproline synthesis noted following *in vitro* injection of dexamethasone was achieved in the concentration range 5×10^{-8}–5×10^{-6} M. Strikingly, no effect was observed when matrix-free cells were incubated *in vitro* in a medium containing the same concentrations of dexamethasone for up to 4 hr. This indicates that a certain period is needed until steroids elicit their effects on collagen synthesis *in vitro* [34, 35].

Of interest was the observation that dexamethasone, either after administration *in vivo* or when present *in vitro* had no effect on the conversion of type II procollagen to collagen. This observation indicates that dexamethasone in the concentrations used did not directly inhibit the enzymes catalyzing the conversion process. In addition, even if the enzyme production might have been reduced in proportion to the reduction of total protein synthesis, sufficient levels of active enzymes were present to efficiently catalyze the conversion reactions.

Deoxycortisone, a synthetic steroid, was also shown to bind to glucocorticoid receptors, but its binding affinity was considerably less than that of dexamethasone. When these two steroids were administered to chick embryos *in vivo* in concentrations corresponding to their comparable receptor binding affinities, an equal inhibition of collagen production was noted. This observation suggests that the inhibition of collagen production, as a result of steroid treatment, is receptor-mediated. Thus, assay of receptor binding affinities by different steroids, using methodology outlined in this study, might be helpful in assessing the relative potential of these steroids to suppress collagen production *in vivo*, resulting in connective tissue atrophy and delayed wound healing, known complications of prolonged steroid therapy [6, 39–42].

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