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DEMONSTRATION OF CELLULAR RETINOIC ACID BINDING PROTEIN (CRABP) IN CHICK EMBRYO TENDON CELLS AND EFFECTS OF RETINOIDS ON COLLAGEN SYNTHESIS IN TENDON AND STERNA*

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Abstract—The binding of all-*trans*-retinoic acid (all-*trans* RA) to specific cytosol proteins and the effects of retinoids on procollagen synthesis were studied in chick-embryo tendon cells. For the receptor assay, tendon cytosols were incubated with [^{3}H]all-*trans*-RA in the presence or absence of 100-fold excess of nonlabeled all-*trans*-RA up to 20 hr at +4° and unbound retinoid was removed by charcoal-dextran treatment or by gel filtration chromatography. The results indicated that chick-embryo tendon cells contained cellular retinoic acid binding protein (CRABP). The binding of [^{3}H]all-*trans*-RA could be displaced by 13-*cis*-retinoic acid, but not by retinol or etretinate. In contrast no CRABP could be found in cartilage cells isolated from sterna or in whole sterna. The treatment of tendon cytosol with proteases (pronase, trypsin, chymotrypsin) abolished the specific binding of [^{3}H]all-*trans*-RA. Gel filtration studies on Sephadex G-100 indicated an apparent molecular weight of 14,500–15,000 daltons for the all-*trans*-retinoic acid binding protein.

All-*trans*-RA markedly decreased procollagen synthesis in isolated chick-embryo tendon cells, the inhibition being concentration dependent; the decrease was about 58% of the control in the presence of 10^{-5} M all-*trans*-RA. Similar decrease was noted with 13-*cis*-RA and etretinate, while retinol was less effective. In isolated cartilage cells the dose of 10^{-5} M of all-*trans*-retinoic acid decreased drastically total protein and collagen synthesis. The mannosylation of procollagen, the conversion of procollagen to collagen and the size of procollagen chains were not significantly affected.

The results of the present study indicate that CRABP is not expressed in sterna of chick-embryos, and in contrast high levels of CRABP could be found in tendons. However, retinoids modulated collagen synthesis in both tissues. Thus it is possible that retinoids can affect the metabolism of different collagen types also in clinical use.

Synthetic retinoids, such as all-*trans*-retinoic acid (RA)†, 13-*cis*-RA, and etretinate (RO-10-9359) have gained considerable use in dermatology for treatment of various hyperproliferative epidermal disorders [2, 3]. These compounds modulate several aspects of the metabolism of epidermal cells, and it has been suggested that these effects are mediated largely through binding of the retinoids to specific cellular binding proteins [4, 5]. In support of this suggestion are demonstrations that epidermal cells, as well as cells from other tissues, contain a cellular retinoic acid binding protein (CRABP), which binds with all-*trans*-RA and 13-*cis*-RA [6, 7]. Similar, yet distinct, cellular binding protein for retinol (vitamin A) has been demonstrated in several tissues [8, 9].

Recently, retinoids have also been shown to affect the connective tissue metabolism in fibroblasts from human skin [10] or synovial tissue [11]. For example, all-*trans*-RA and 13-*cis*-RA have been shown to suppress the production of collagenase by these cells [10–12]. In a recent study, we have also demonstrated that retinoids markedly suppress the production of procollagen by human skin fibroblasts in culture [10] and that human skin fibroblasts contain specific cellular retinoic acid binding protein [13].

Chick-embryo tendon and sterna cells have been extensively used to study biosynthesis of collagen, due to the fact that the major protein produced by tendon cells is type I collagen [14] and by sterna cells type II collagen [15]. In this study we have examined the question as to whether these cells contain specific cellular retinoic acid binding protein and what are the effects of retinoids on procollagen production in these cells.

MATERIALS AND METHODS

Materials. [^{14}C]Proline (specific activity 291 Ci/mol) and [^{3}H]retinol (all-*trans*-vitamin A, 1-^{3}H(N): specific activity 13.6 Ci/mmol) were purchased from New England Nuclear Corp. (Boston, MA). [11-^{3}H]

* A preliminary report of this study was presented at the Annual Meeting of the Western Society for Investigative Dermatology, Carmel, California, 1985 [1].

† Abbreviations used: RA, retinoic acid; CRABP, cellular retinoic acid binding protein; HBSS, Hank's balanced salt solution; NEM, *N*-ethylmaleimide; Na_2EDTA, disodium ethylenediaminetetracetic acid; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum.

all-*trans*-RA (specific activity 2.24 Ci/mmol) and [11-^{3}H] 13-*cis*-RA (specific activity 1.56 Ci/mmol) were obtained from the Chemoprevention Program, National Cancer Institute, National Institutes of Health. The unlabeled retinoids, all-*trans*-retinoic acid and retinol, were purchased from Sigma Chemical Co. (St. Louis, MO); 13-*cis*-retinoic acid and RO-10-9359 (etretinate) were obtained from Hoffmann-LaRoche.

Isolation of matrix-free cells from tendon and cartilage. The cells were isolated from the leg tendons and sterna of 17-day-old chick-embryos by enzymic digestion using trypsin and collagenase, as described previously [14, 15]. For incubations, the cells were suspended in modified Krebs medium or in HBSS, 0.6–10.0 × 10^6 cells per ml.

Receptor assays. For whole cell binding assay [16, 17] the cells were incubated in 1.0 ml HBSS containing various concentrations of [^{3}H]all-*trans*-RA, at 37° for 45 min. The samples were protected from the light during incubation. Parallel tubes containing the same amount of cells were incubated with the same concentration of radioactive all-*trans*-RA and in the presence of a 100-fold excess of an unlabeled retinoid. The unlabeled retinoids were dissolved in dimethylsulfoxide, and the final concentration of the solvent in all samples was 0.1%. At the end of the incubation, 20 μl aliquots were taken for determation of total radioactivity in each sample. Two ml cold (4°) HBSS was added, and the samples were centrifuged at 1000 *g* for 5 min at room temperature. The supernatant was discarded, and the cells were resuspended in HBSS, and the cells were collected by centrifugation, as above. The washing procedure was repeated, and the cell pellets were resuspended in 0.5 ml of HBSS. Aliquots of the suspensions were taken for assay of DNA [18] and protein [19] as well as for cell count by hemocytometer. Aliquots of 0.1 ml were taken for determination of bound radioactivity.

For cytosol binding assay, frozen chick-embryo tissues (0.2 g/ml) were homogenized on ice with a Polytron tissue homogenizer, 2 times for 10 sec in 20 mM Tris–HCl, pH 7.5, containing 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10% glycerol. The homogenates were centrifuged at 100,000 *g* for 60 min at 4°, and the supernatants were used for receptor assay and for protein determination. Chick-embryo liver and skin samples were filtered through cotton wool before the assay in order to remove fat.

For receptor assays, the cytosol preparations were incubated in the presence of [^{3}H]all-*trans*-RA, [^{3}H]retinol or [^{3}H]13-*cis*-RA for 4–20 hr at 4° in the dark. At the end of the incubation, 20 μl aliquots were taken for determination of total radioactivity, and the samples were then mixed with an equal volume of solution containing 1% charcoal, 0.05% dextran and 30% glycerol in 10 mM Tris–HCl, pH 7.5. The samples were incubated 5 min at 4° and centrifuged for 5 min at 10,000 *g*. Aliquots of the supernatants were taken for determination of radioactivity. In parallel samples, the cytosol preparations were incubated with the same concentration of radioactive retinoid in the presence of a 100-fold excess of unlabeled retinoids.

For gel filtration chromatography, cytosol preparations, which were incubated with [^{3}H]all-*trans*-RA or [^{3}H]retinol in the presence or absence of a 100-fold excess of unlabeled all-*trans*-RA or retinol, were chromatographed on Sephadex G-100 (Pharmacia), equilibrated and eluted with 20 mM Tris–HCl, pH 7.5, containing 2 mM $CaCl_2$ and 2 mM $MgCl_2$. Fractions of 1.0 ml were collected, and aliquots were used for liquid scintillation counting of radioactivity.

The protein concentration in the fractions was measured by absorbance at 280 nm. The gel filtration column was calibrated with [^{3}H]H_2O, blue dextran (Amersham), bovine serum albumin, chymotrypsinogen and ribonuclease A (all from Pharmacia).

Competitive retinoid binding studies were conducted according to the methods described above (charcoal-dextran method) using 8 × 10^{-8} M [^{3}H]all-*trans*-RA and 100-fold excess of unlabeled all-*trans*-RA, 13-*cis*-RA, retinol or RO-10-9359.

Proteolytic treatment of tendon cytosol samples was performed using trypsin, chymotrypsin and pronase, all in final concentration of 100 μg/ml. The cytosol fractions were incubated at 37° for various time periods up to 5 hr with proteolytic enzymes, and control samples without proteolytic treatment were incubated in a parallel manner. After incubation, samples were used for receptor assay as described above.

To study the dissociation of retinoic acid–receptor complexes tendon cytosols were first incubated with [^{3}H]all-*trans*-RA for 20 hr at 4°, followed by incubations for various time periods up to 40 min at 37°. The samples were then treated with charcoal-dextran and the bound radioactivity was determined from supernatant fractions as described above.

Collagen studies. Freshly isolated tendon or cartilage cells were preincubated in Krebs medium supplemented with ascorbic acid, 50 μg/ml, and β-aminopropionitrile, 50 μg/ml, and containing retinoids dissolved in DMSO. All tubes including the controls, contained 0.1% (v/v) of DMSO. After 60 min incubation, one μCi of [^{14}C]proline per tube was added, and the incubation was continued for 120 min at 37° with shaking. After incubation, 200 μl of Krebs medium containing cycloheximide and α,α′-dipyridyl was added to yield concentrations of 100 μg/ml and 1 μM, respectively. The samples were dialyzed against running tap water, hydrolyzed in 6 M HCl, and total ^{14}C incorporation and the synthesis of [^{14}C]hydroxyproline were assayed [20]. To study the effect of retinoids on collagen synthesis in whole tissues, tendon and sterna were isolated and preincubated with various concentrations of retinoids for 24 hr in the presence of 1% FCS, 50 μg/ml β-aminopropionitrile and 50 μg/ml ascorbic acid in DMEM. After preincubation samples were labeled with 3 μCi of [^{14}C]proline for 120 min.

The radioactive proteins were then extracted with 2% sodium dodecyl sulphate (SDS) in the presence of protease inhibitors, and total radioactivity and [^{14}C]hydroxyproline were then analyzed after dialysis [20].

The conversion of procollagen to collagen was examined employing pulse-chase techniques [21]. The sterna were pulse-labeled with 3 μCi of [^{14}C]proline for 30 min, as indicated 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 in the presence or absence of 10^{-5} M all-*trans*-RA and the incubation was stopped by the addition of protease inhibitors and 3% SDS. The samples were immediately boiled for 5 min, and homogenized with 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 [22]. The ^{14}C-polypeptides were visualized by fluorography [23], and the bands representing proα, pNα and α-chains of type II procollagen and collagen, were quantitated by scanning densitometry.

To study the effect of all-*trans*-RA on the mannosylation of type I procollagen, the tendon cells were preincubated for 1 hr in the presence or absence of 10^{-5} M all-*trans*-RA. The cells were then labeled simultaneously with [2-^{3}H]mannose (100 μCi/tube) and [^{14}C]proline (2 μCi/tube) for 2 hr. The incubation was stopped by cooling and adding [^{12}C]proline, cycloheximide and protease inhibitors to final concentrations (EDTA 20 mM, NEM 10 mM, PMSF 2 mM). The cells and medium were separated by centrifugation. Cells were dissolved in 3% SDS containing protease inhibitors, boiled and homogenized, and dialyzed against 0.125 M Tris–HCl, pH 6.8, containing 2% SDS, 0.01% bromphenol blue, and 10% glycerol. The cell and medium fractions were then electrophoresed after reduction with 2-mercaptoethanol on 6% polyacrylamide gel [22] and radioactive peptides were visualized by fluorography [23]. Bands corresponding to proα-chains of procollagen were cut out from the gel and treated with protosol tissue solubilizer (New England Nuclear). The radioactivities of ^{3}H and ^{14}C were then counted by liquid scintillation counting using two-channel double-label counting program.

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

RESULTS

Receptor studies

Two different methods were utilized for assay of retinoid binding receptors. First isolated chick-embryo tendon fibroblasts in suspension culture were incubated with [^{3}H]all-*trans*-RA in the presence or absence of a 100-fold excess of unlabeled retinoid. After a 45 min incubation, the cells were carefully washed and counted for bound ^{3}H-radioactivity, as described in Materials and Methods. Significant amounts of radioactive all-*trans*-RA remained bound after the radioactivity which was not incorporated into the cells was removed by washings. The binding of radioactive [^{3}H]all-*trans*-RA could be markedly reduced by incubation of the cells in the presence of a 100-fold excess of unlabeled all-*trans*-RA (Table 1).

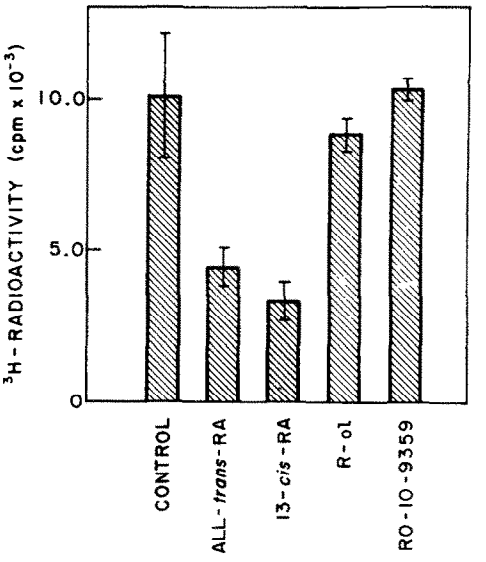

Fig. 1. Competitive inhibition of [^{3}H]all-*trans*-RA binding to retinoid receptors by other unlabeled retinoids. Tendon cytosol samples were incubated with 80 nM [^{3}H]all-*trans*-RA in the presence of 100-fold excess of unlabeled retinoids. Control samples were incubated with [^{3}H]all-*trans*-RA alone. The values are the mean ±SD of triplicate samples.

Secondly, chick-embryo tendons were homogenized, and the 100,000 *g* supernatants were incubated with [^{3}H]all-*trans*-RA at 4°. Unbound radioactivity was then removed by charcoal-dextran treatment, and the supernatants were counted for ^{3}H-radioactivity. Considerable amount of ^{3}H-radioactivity remained in the supernatant after charcoal-dextran precipitation (Fig. 1). This binding could be reduced by about 55% when the incubation with radioactive all-*trans*-RA was performed in the presence of a 100-fold excess of unlabeled all-*trans*-RA,

Table 1. Binding of [^{3}H]all-*trans*-RA to chick-embryo tendon cells

Unlabeled retinoid added	[^{3}]radioactivity bound d.p.m. × 10^{-3}/sample
None	18.9
All-*trans*-RA	10.8

Chick-embryo tendon cells (3.0×10^6 in tube) were incubated with 3.2×10^{-7} M [^{3}H]all-*trans*-RA in the presence or absence of 100-fold excess of unlabeled retinoid for 45 min at 37° and the samples were treated thereafter as described in the Materials and Methods. The values are the mean of duplicate assays.

Table 2. Binding of [^{3}H]13-*cis*-retinoic acid to chick-embryo tendon and skin cytosols

Sample	Unlabeled 13-*cis* retinoic acid added	[^{3}H]radioactivity bound d.p.m./μg protein
Tendon	None	930
	100 × 13-*cis*-RA	190
Skin	None	420
	100 × 13-*cis*-RA	160

Cytosol fractions were incubated with 4.3×10^{-7} M [^{3}H]13-*cis*-RA for 4 hr at +4°, and thereafter samples were treated as described in Materials and Methods.

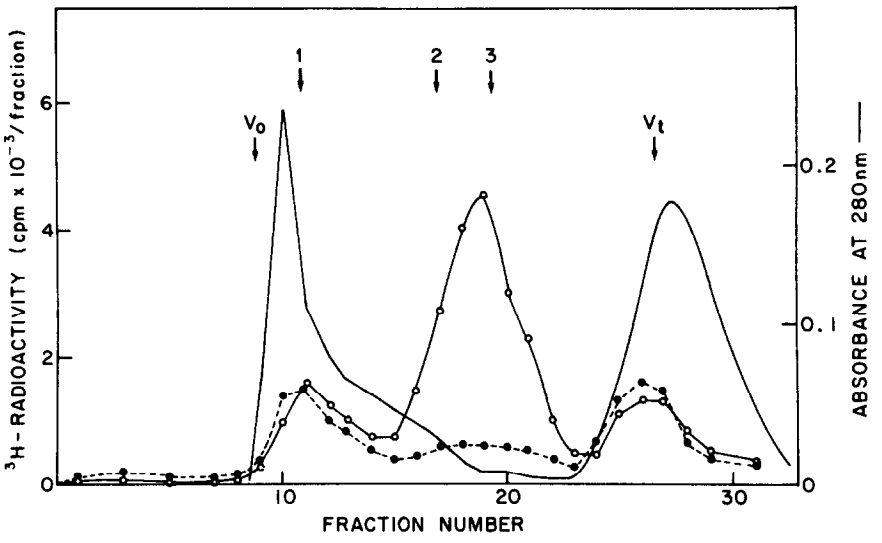

Fig. 2. Demonstration of retinoic acid binding protein in chick-embryo tendon fibroblasts by gel filtration chromatography. Tendon cytosol samples were incubated with 80 nM [^{3}H]all-*trans*-RA with or without a 100-fold excess of unlabeled retinoid for 20 hr at 4°. The samples were then briefly treated with charcoal-dextran, centrifuged and supernatant fractions were then chromatographed on Sephadex G-100 gel filtration column. The fractions were then counted for radioactivity and the protein concentration was measured by absorbance at 280 nm. The column was standardized by using the dextran (V_0), [^{3}H] $-H_2O$ (V_t), bovine serum albumin (standard 1; MW 66,000), chymotrypsinogen (standard 2; MW 25,000), and ribonuclease A (standard 3; MW 13,700). Symbols: O——O, Sample incubated with [^{3}H]all-*trans*-RA alone; ●----●, Sample incubated with [^{3}H]all-*trans*-RA in the presence of a 100-fold excess of unlabeled all-*trans*-RA; ——, absorbance at 280 nm.

while retinol only slightly reduced the binding and RO-10-9359 (etretinate) had no effect (Fig. 1). Similar assays demonstrated binding of [^{3}H]13-*cis*-RA into cytosol preparations, and this binding could be displaced by the addition of a 100-fold excess of unlabeled 13-*cis*-RA (Table 2). Further experiments using [^{3}H]retinol as the ligand failed to demonstrate any specific binding which could be displaced by unlabeled retinol.

To characterize the retinoid binding proteins further, cytosol preparations, after incubations with [^{3}H]all-*trans*-RA or [^{3}H]retinol, were chromatographed of Sephadex G-100, and the fractions counted for ^{3}H-radioactivity. Three major peaks of radio-

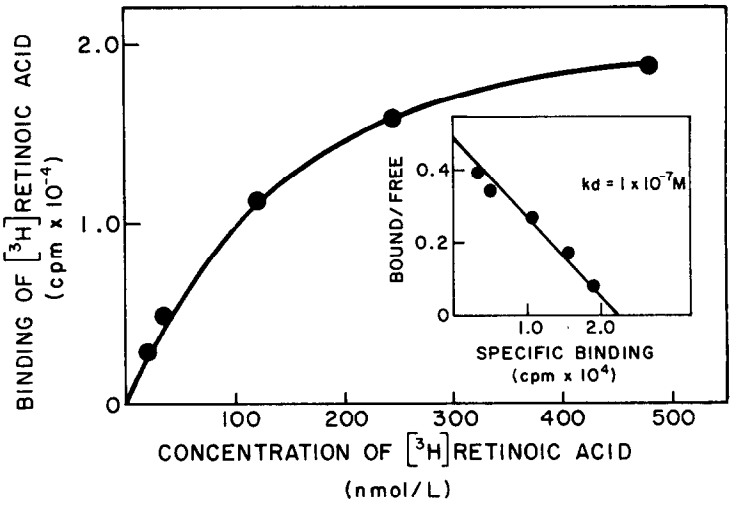

Fig. 3. The typical binding curve of [^{3}H]all-*trans*-RA to receptors in tendon cytosol samples and determination of binding affinity by Scatchard plot. For the assay tendon cytosols were incubated with various concentrations of [^{3}H]all-*trans*-RA in the presence or absence of 100-fold excess of unlabeled all-*trans*-RA for 20 hr at 4°. The unbound [^{3}H]all-*trans*-RA was removed by charcoal-dextran treatment as described and the specific binding was calculated by deducting non-specific binding from the total binding. The binding affinity (K_d) was esimated from the Scatchard plot (insert) [31].

activity could be detected (Fig. 2): a small peak of radioactivity appeared in the total volume of the column, corresponding to free [^{3}H]all-*trans*-RA; a second peak in the void volume of the column with a MW in excess of that of bovine serum albumin, i.e. >66,000 daltons; a third peak of radioactivity chromatographed in a position corresponding to an approximate MW of 15,000 daltons. The incubation of the cytosol preparations with [^{3}H]all-*trans*-RA in the presence of a 100-fold excess of unlabeled all-*trans*-RA had no effect on the radioactivity in the void volume or total volume peaks (Fig. 2). However, the radioactivity in the 15,000 MW peaks was abolished in the presence of unlabeled retinoid (Fig. 2). Thus, the radioactivity in the latter peak represents specific binding of [^{3}H]all-*trans*-RA. In cytosol preparations from chick-embryo skin or liver, gel filtration studies revealed a similar retinoic acid binding protein of 15,000 daltons (data not shown). In contrast cytosol preparations from chick-embryo sterna, or cartilage cells isolated from sterna, failed to show a cellular retinoic acid binding protein (not shown). The incubation of tendon cytosols with [^{3}H]retinol demonstrated two major peaks in Sephadex-100 column. The first peak corresponded to a void volume of the column and another large peak eluted in the total volume of the column. Only very small peak was found in the fractions 18–22, which was partially abolished when cytosols were incubated in the presence of 100-fold excess of unlabeled retinol (not shown). The amount of CRABP was 94 pmol/mg cytosol protein in chick-embryo tendons. The corresponding values in chick-embryo skin and liver being 46 and 13 pmol/mg cytosol protein. The apparent binding affinity (K_d) determined by Scatchard plot analysis was 1×10^{-7} M in tendon cytosols (Fig. 3).

In order to study the details of the binding of [^{3}H]all-*trans*-RA to cellular retinoic acid binding protein in chick-embryo tendons, the effects of various reagents as well as proteases were examined. The omission of glycerol from reaction mixture decreased the specific binding by over 70% (not shown). The treatment of cytosol with 10 mM *N*-ethylmaleimide had no effect on the binding (not shown). The effect of various proteases on the cellular retinoic acid binding protein were further studied by incubating tendon cytosol fractions in the presence of pronase, trypsin or chymotrypsin (all in concentration of 100 μg/ml) up to 5 hr at 37° before the assay. After 90 min incubation with pronase, there was no specific binding of all-*trans*-RA in the cytosol (Fig. 4). Treatment with trypsin (Fig. 4) or chymotrypsin (not shown) were less effective in decreasing the binding of retinoic acid to cellular retinoic acid binding protein in cytosol fractions.

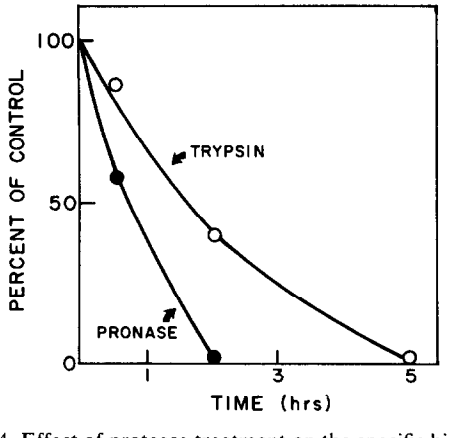

Fig. 4. Effect of protease treatment on the specific binding of [^{3}H]all-*trans*-RA. Tendon cytosols were treated with pronase or trypsin as described, and thereafter the samples were incubated with [^{3}H]all-*trans*-RA as described and the specific binding of [^{3}H]all-*trans*-RA was determined. The values are the mean of duplicate samples, and the values present the relative specific binding in compared to control samples which were not incubated with enzymes.

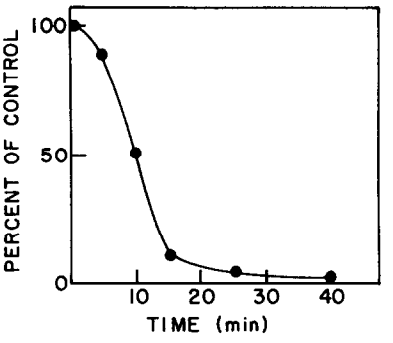

Fig. 5. Dissociation of retinoic acid-receptor complex at 37°. Tendon cytosols were incubated for 20 hr at 4° with [^{3}H]all-*trans*-RA in the presence or absence of 100-fold excess of unlabeled all-*trans*-RA. After that samples were incubated up to 40 min at 37° treated with charcoal-dextran, centrifuged and specifically bound [^{3}H]all-*trans*-RA was determined from supernatant. The values are the mean of duplicate samples and the values present the relative specific binding in compared to control samples which were treated at cold with charcoal-dextran.

The stability of the complex of retinoid and CRABP was studied by increasing the temperature to 37° after incubation at +4°. At 37° the specific binding decreased by over 90% in 15 min (Fig. 5).

Table 3. Effect of all-*trans*-retinoic acid on collagen and total protein synthesis in isolated chick-embryo cartilage cells*

All-*trans*-RA (M)	Total ^{14}C d.p.m. × 10^{-3}	[^{14}C]hydroxyproline d.p.m. × 10^{-3}
None	50.3 ± 8.9	12.4 ± 2.5
10^{-9}	46.6 ± 1.1	8.8 ± 1.6
10^{-7}	60.1 ± 8.1	12.8 ± 2.1
10^{-5}	18.8 ± 3.8†	2.6 ± 0.1†

* Freshly isolated chick-embryo cartilage cells were pre-incubated with all-*trans*-RA or solvent for 60 min and labeled for 120 min with [^{14}]proline and the incorporation of total ^{14}C-radioactivity and the synthesis of [^{14}C]hydroxyproline were assayed as described in Materials and Methods. The values are the mean ± SD of three parallel samples.

† $P < 0.005$ compared to control.

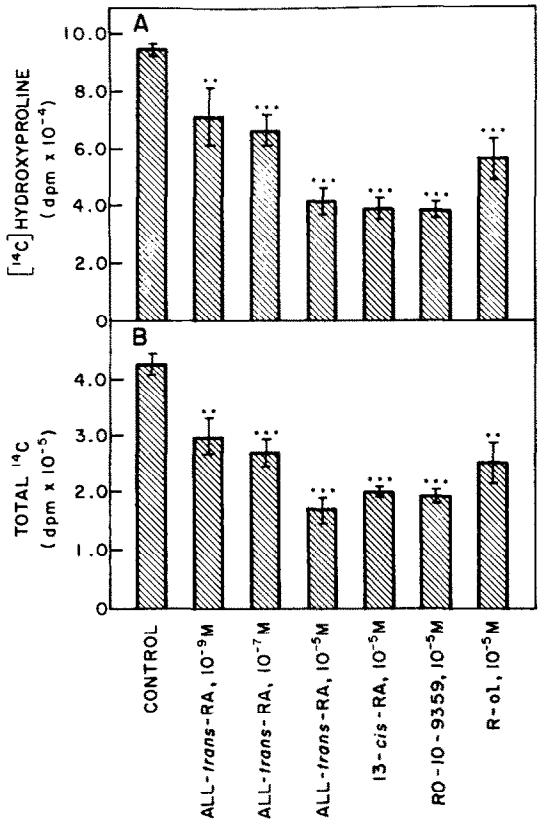

Fig. 6. The effect of various retinoids on the synthesis of procollagen and the incorporation of total ^{14}C-radioactivity in isolated chick-embryo tendon cells. Tendon cells were preincubated for 60 min in the presence of various retinoids. The cells were then labeled with [^{14}C]proline for 120 min, and the synthesis of [^{14}C]hydroxyproline and the incorporation of total ^{14}C-radioactivity into nondialyzable fraction were assayed, as described in Materials and Methods. The values are the mean ± SD of three parallel samples. **$P < 0.005$ compared to control samples; ***$P < 0.001$ compared to control samples.

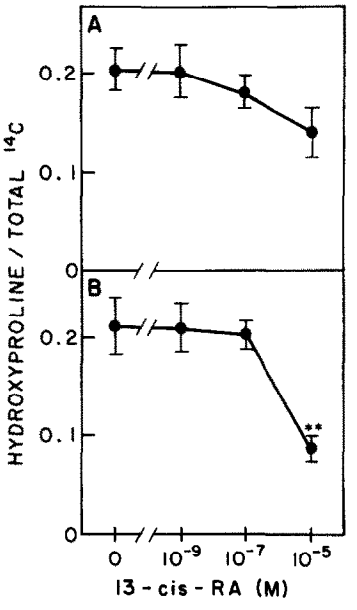

Fig. 7. Effect of 13-*cis*-RA on relative collagen synthesis in chick-embryo tendon and sterna. Chick-embryo tendon and sterna were prepared as described and preincubated for 24 hr with various concentrations of 13-*cis*-RA. After preincubation the samples were labeled with [^{14}C]proline and after incubation total radioactivity and [^{14}C]hydroxyproline were determined as described. The values are the mean ± SD of triplicate samples: (A) Tendon; (B) Sterna; **$P < 0.005$ compared to control samples.

Collagen synthesis studies

In further studies, the effects of retinoids on procollagen synthesis were studied in isolated chick-embryo tendon fibroblasts. All-*trans*-RA markedly decreased the procollagen synthesis, the decrease being 58% with 10^{-5} M all-*trans*-RA. 13-*cis*-RA and etretinate were equally effective, while retinol decreased less efficiently the procollagen production (Fig. 6). The effects of retinoids on collagen synthesis in tendon cells in suspension conditions were not specific, however, since total protein synthesis was similarly affected. In isolated chondrocytes, 10^{-5} M all-*trans*-RA markedly decreased total protein and collagen synthesis (Table 3).

The effect of 13-*cis*-RA on relative collagen synthesis was studied by preincubating whole tendons and sterna in the presence of various concentrations of retinoid, and the labeling samples for 120 min with [^{14}C]proline. Relative collagen synthesis was then assayed from SDS extractable protein by determining the ratio of [^{14}C]hydroxyproline/total ^{14}C-radioactivity. In tendon this ratio was in control specimens 0.20, and was decreased to 0.14 in samples

Table 4. The effect of all-*trans*-RA on the synthesis of [^{14}C]hydroxyproline and [^{3}H]mannose incorporation into medium proteins in tendon cells

	[^{3}H]mannose, d.p.m. × 10^{-5} in media	[^{14}C]hydroxyproline, d.p.m. × 10^{-3} in media
None	11.2 ± 2.1	2.5 ± 0.8
All-*trans*-RA 10^{-5} M	9.9 ± 1.9	1.0 ± 0.1

Freshly isolated tendon cells were preincubated for 1 hr in the presence or absence of 10^{-5} M all-*trans*-RA and thereafter labeled with 1.5 μCi of [^{14}C]proline or 30 μCi of [^{3}H]mannose for 2 hr. The cells and medium fractions were then separated, and dialyzed against running tap water, and assayed for [^{14}C]hydroxyproline or for nondialyzable [^{3}H]mannose. The values are mean ± SD of triplicate samples.

Table 5. Effect of all-*trans*-RA on the mannosylation of procollagen

Group	Ratio of [^{3}H]mannose/[^{14}C]proline			
	Cells		Medium	
	Proα 1(I)	Proα 2(I)	Proα 1(I)	Proα 2(I)
Control	2.7 ± 0.1	3.0 ± 0.3	14.8 ± 3.1	30.9 ± 4.7
RA 10^{-5} M	1.9 ± 0.2	2.4 ± 0.2	13.7 ± 1.6	25.2 ± 0.8

Isolated tendon cells were preincubated for 1 hr at 37° with or without 10^{-5} M all-*trans*-RA. The cells were then double-labeled with 100 μCi [^{3}H]mannose and 2 μCi [^{14}C]proline for 2 hr. After incubation cells and media fractions were separated, and analysed by 6% SDS gel electrophoresis. Bands corresponding to proα 1 and proα 2 regions were isolated and assayed for [^{3}H] and [^{14}C]radioactivity. The values are the mean of triplicate assays.

incubated with 10^{-5} M 13-*cis*-RA (Fig. 7A). In sterna, the ratio was in controls 0.22, and in samples incubated with 10^{-5} M 13-*cis*-RA the ratio was 0.08 (Fig. 7B).

Since retinoids have been suggested to affect the glycosylation of proteins [24], the incorporation of [^{3}H]mannose to nondialyzable medium proteins was studied. The results indicated that total mannose incorporation was not markedly altered under conditions in which procollagen synthesis was significantly decreased (Table 4). In order to study directly the mannosylation of pro1α(I) and proα2(I) chains, the tendon cells were simultaneously labeled with [^{3}H]mannose and [^{14}C]proline. The proα chains were isolated by SDS-PAGE and the degree of mannosylation was estimated from the ratio of ^{3}H/^{14}C, assayed by liquid scintillation counting. The results indicated that the degree of mannosylation of proα1(I) or proα2(I) chains either from cell or medium fraction was not significantly affected (Table 5). The results also demonstrated that overall mannosylation of proα2(I) chains recovered in the medium was higher than the corresponding values in the proα1(I) chains.

In the same studies, the size of proα chains was analysed by SDS-gel electrophoresis. The results indicated that proα1(I) and proα2(I) migrated in the same positions in retinoic acid treated samples as those in control samples incubated with solvent alone.

To study the effect of all-*trans*-RA on the conversion of procollagen, chick-embryo sterna were pulse-labeled as described in Materials and Methods and then chased in the presence of 10^{-5} M all-*trans*-RA. The analysis of SDS gel electrophoresis revealed that 62% of collagenous material was recovered as α-chains in control samples and 59% in all-*trans*-RA treated samples. Thus, all-*trans*-RA did not significantly affect the conversion of procollagen to collagen.

DISCUSSION

In the present study, we have demonstrated that chick-embryo tendon fibroblasts contain a specific cellular all-*trans*-RA binding protein (CRABP). The binding of radioactive all-*trans*-RA was demonstrated by a whole cell assay and a cytosol binding assay. The specific binding of [^{3}H]all-*trans*-RA could be displaced by unlabeled all-*trans*-RA or by 13-*cis*-RA. Similar binding could be demonstrated using [^{3}H]13-*cis*-RA as the ligand, and this binding could be displaced by unlabeled 13-*cis*-RA. Therefore, it seems plausible, that these two retinoids may bind to the same or closely related cellular binding proteins with comparable affinities. In contrast to these retinoids, unlabeled retinol or RO-10-9359 were unable to displace any specific binding of the [^{3}H]all-*trans*-RA. Furthermore, only little, if any, specific binding of [^{3}H]retinol could be demonstrated using cytosol assay. The amount of CRABP in tendons was even higher than in other tissues (in skin or liver). Recently a very high amount of CRABP (100 pmol/mg cytosol protein) was reported from the skin of 8-day-old chick-embryos [25]. Since CRABP was measured in our study from 17-day-old chick-embryos, the values are not entirely comparable. However, it seems that chick-embryo tendons are a rich source of CRABP.

The binding of radioactive all-*trans*-RA was further characterized by a gel filtration chromatography of the cytosol proteins. Specific binding which could be abolished by a 100-fold excess of unlabeled all-*trans*-RA was found in a peak with an apparent molecular weight of approximately 15,000 daltons. The value agrees well with previously published molecular weight values for CRABP; which are in the range of 14,000–16,000 daltons [5]. Thus, the cellular retinoic acid binding protein demonstrated here in chick-embryo tendon fibroblasts appears to be similar to those reported to be present in various tissues in different animal species.

Further characterization of CRABP revealed that it was highly sensitive to treatment with pronase, which totally inhibited the specific binding. NEM, a reagent blocking sulphydryl groups had no effect on retinoid binding, indicating that free sulphydryl groups are not needed to retinoid binding. This contrasts with observations on glucocorticoid receptors: the binding of glucocorticoid to the receptor molecule can be blocked by NEM [26].

The effects of various retinoids on procollagen synthesis were studied first by using isolated tendon fibroblasts in short suspension labeling experiments. All-*trans*-RA, and 13-*cis*-RA markedly decreased procollagen production in isolated tendon cells up to 58% in 10^{-5} M concentration. However, the inhibition in the suspension culture was not specific,

since total protein synthesis was similarly inhibited. This contrasts with studies using human skin fibroblasts in culture, in which collagen production was selectively affected [10]. Possible explanations could be different cell type, and different experimental system. Recently it was reported that retinoids decreased collagen production slightly more than other protein synthesis in cultured chick tendon fibroblasts [27]. This could indicate that retinoids need a certain time period in affecting specifically the collagen gene expression. This was also confirmed in our experiments utilizing longer incubation. The preincubation of tendons for 24 hr with 13-*cis*-RA decreased the relative synthesis of collagen. Etretinate, which was not bound to the same binding protein as all-*trans*-RA, also inhibited collagen and protein synthesis, in tendon cells. The mechanisms for this inhibitory effect of etretinate are unknown. It is possible that etretinate modulated cellular functions by some other unknown binding proteins or that etretinate has a direct effect on the metabolism of cells.

Since chondrocytes were shown to have neglible amounts of CRABP, it was of interest to study the effects of retinoids on collagen production in these cells. In suspension conditions or in longer incubations with whole sterna, the total protein and collagen synthesis were clearly decreased in the presence of 10^{-5} M all-*trans*-RA or 13-*cis*-RA. However, lower concentrations were ineffective. Currently, the mechanism by which retinoids decrease collagen synthesis in chondrocytes is not known. It is possible, however, that retinoids have a direct toxic effect on chondrocytes which then leads to decreased protein and collagen synthesis. Recently it has been shown in chondrocyte cultures that retinoids decrease collagen synthesis [28] indicating that retinoids modulate the metabolism of cartilage cells. However, the lack of connection between receptor binding and response in chondrocytes further confirms earlier studies, which have demonstated that the levels of CRABP or CRBP in cells do not always correlate with the biologic responses of the cells by retinoids [29].

Since it has been suggested that one mechanism of retinoid action could be that retinoids might serve as a coenzyme in the membrane-mediated synthesis of glycoproteins [24], and specifically serve as a carrier of mannose into glycoproteins, it was of interest to study the degree of mannosylation of procollagen synthesized in the presence of a retinoid. The overall mannosylation was higher in proα1(I) collagen than in proα2(I) collagen. This finding thus supports a previous study, in which it was shown that mannosylation is higher in proα2(I) propeptides [30]. The mannosylation of procollagen type I was not significantly affected by all-*trans*-RA. The possible explanation could be that in tendon cells, the mannosylation of procollagen is already maximal and thus no free sites are available for further mannosylation.

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REFERENCES

1. A. Oikarinen, H. Oikarinen and J. Uitto, *Clin. Res.* **33**, 157A (1985).
2. J. S. Strauss, D. B. Windhorst and G. A. Weinstein (Ed.), *J. Am. Acad. Dermatol.* **6**, 573 (1982).
3. G. L. Peck, *Prog. Dis. Skin* **1**, 227 (1981).
4. D. F. Chytil and D. W. Ong, *Fedn Proc.* **38**, 2510 (1979).
5. D. S. Goodman, *J. Am. Acad. Dermatol.* **6**, 583 (1982).
6. S. Puhvel and M. Sakamoto, *J. invest. Dermatol.* **82**, 79 (1984).
7. D. E. Ong and F. Chytil, *J. biol. Chem.* **250**, 6113 (1975).
8. D. E. Ong and F. Chytil, *Nature, Lond.* **255**, 74 (1975).
9. M. M. Bashor and F. Chytil, *Biochim. biophys. Acta* **411**, 87 (1975).
10. H. Oikarinen, A. I. Oikarinen, E. M. L. Tan, R. P. Abergel, C. A. Meeker, M. L. Chu, D. J. Prockop and J. Uitto, *J. clin. Invest.* **75**, 1545 (1985).
11. C. E. Brinkerhoff, H. Nagase, J. E. Nagel and E. D. Harris, Jr., *J. Am. Acad. Dermatol.* **6**, 591 (1982).
12. E. A. Bauer, J. L. Seltzer and A. Z. Eisen, *J. invest. Dermatol.* **81**, 162 (1983).
13. A. I. Oikarinen, H. Oikarinen and J. Uitto, *Br. J. Dermatol.* **113**, 529 (1985).
14. P. Dehm and D. J. Prockop, *Biochim. biophys. Acta* **240**, 358 (1971).
15. P. Dehm and D. J. Prockop, *Eur. J. Biochem.* **35**, 959 (1973).
16. M. Lippman and R. Barr, *J. Immunol.* **118**, 1977 (1977).
17. K. Kontula, L. C. Andersson, T. Paavonen, G. Myllylä, L. Teerenhovi and P. Vuopio, *Int. J. Cancer* **26**, 177 (1980).
18. K. Burton, *Methods Enzymol.* **12B**. 163 (1968).
19. M. M. Bradford, *Analyt. Biochem.* **72** 248 (1976).
20. K. Juva and D. J. Prockop, *Analyt. Biochem.* **15**, 77 (1966).
21. L. Ryhänen, E. M. L. Tan, S. Rantala-Ryhänen and J. Uitto, *Archs Biochem. Biophys.* **215**, 230 (1982).
22. J. King and U. K. Laemmli, *J. molec. Biol.* **62**, 465 (1971).
23. M. W. Bonner and R. A. Laskey, *Eur. J. Biochem.* **46**, 83 (1974).
24. L. M. De Luca, *J. Am. Acad. Dermatol.* **6**, 611 (1982).
25. R. E. Gates and L. E. King, *J. invest. Dermatol.* **85**, 279 (1985).
26. A. M. Rees and P. A. Bell, *Biochim. biophys. Acta* **411**, 121 (1975).
27. R. Hein, H. Mensing, P. K. Mueller, O. Braun-Falco and T. Krieg, *Br. J. Dermatol.* **111**, 37 (1984).
28. R. Hein, T. Krieg, P. K. Mueller and O. Braun-Falco, *Biochem. Pharmac.* **33**, 3263 (1984).
29. R. Lotan, D. E. Ong and F. Chytil, *J. natn. Cancer Inst.* **64**, 1259 (1980).
30. C. C. Clark, *J. biol. Chem.* **254**, 10798 (1979).
31. G. Scatchard, *Ann. NY Acad. Sci.* **51**, 660 (1949).