A Retinoic Acid-binding Protein from Chick Embryo Skin

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SUMMARY

Previous studies by others have indicated that retinoic acid reverses metaplastic changes caused by chemical carcinogens in vivo and in organ culture. The present study deals with the detection of a specific retinoic acid-binding protein from chick embryo metatarsal skin that may be mediating these biological effects. The protein has an S20w value of 2 and an isoelectric pH of 4.6. Competition experiments with labeled retinoic acid and 200-fold molar excesses of unlabeled retinoic acid, retinol, retinal, methyl retinoate, diethylretinamide, synthetic analogs of retinoic acid, and γ-linolenic acid reveal that only retinoic acid and its analogs with a free carboxyl group bind to this protein. Among the analogs of retinoic acid, a cyclopentenyl analog, a trimethylmethoxyphenyl analog, 13-cis-retinoic acid, and α-retinoic acid compete for the binding site on the protein, with the cyclopentenyl analog having greater affinity than retinoic acid does. Phenyl and pyridyl analogs of retinoic acid are poor binders. In general, the ability of the various analogs to bind to this protein correlates with their biological activity in the reversal of keratinization and in the production of mucous metaplasia by chick embryo metatarsal skin as reported by others.

INTRODUCTION

The role of retinol, retinoic acid, and some of their synthetic analogs in preventing and retarding the growth of epithelial tumors has been documented (3, 30). Administration of retinoic acid p.o. produces regression of chemically induced papillomas on the skin of mice (4); and, in organ culture, retinoic acid inhibits and reverses the hyperplasia induced by 3-methylcholanthrene (6, 15). Spontaneously regressing keratoacanthomas produced on the ears of rabbits by 7,12-dimethylbenz[a]anthracene can be treated by retinoic acid to convert the keratinized lesion into a mucous-producing epithelium (24). The accompanying paper illustrates that in organ cultures of metatarsal skin from 12- to 13-day-old chick embryos the explants undergo a squamous, keratinizing metaplasia in 4 to 6 days. However, explants cultured in the presence of retinoic acid do not keratinize but instead exhibit a mucous metaplasia with increased formation of mucopolysaccharides.

The action of retinoic acid in reversing preneoplastic and neoplastic lesions may be due to a hormone-like effect involving induction and/or depression of gene activity. If so, one would expect binding of retinoic acid to a specific receptor molecule after entry into the target cell, as illustrated for steroid hormones (9, 11, 13). Such a complex might facilitate the entry of retinoic acid into the cell nucleus and allow the necessary interaction with genetic material (20, 31).

We presently report the detection and partial characterization of a specific retinoic acid-binding protein from chick embryo metatarsal skin. The ability of various synthetic analogs of retinoic acid to compete for binding of retinoic acid to this protein has been evaluated with a view that such experiments might allow selection of retinoic acid analogs that could be used for inhibition of epithelial keratinization and, perhaps, for reversal of squamous, preneoplastic lesions. Preliminary reports of part of these studies have appeared (27, 28).

MATERIALS AND METHODS

Retinoic acid, [11,12-3H]retinoic acid, methyl retinoate, ethyl retinoate, diethylretinamide, α-retinoic acid, 13-cis-retinoic acid, and the cyclopentenyl, pyridyl, phenyl, trimethylmethoxyphenyl, and furyl analogs of retinoic acid were prepared by Hoffman-La Roche Inc., Nutley, N. J., and Basel, Switzerland, and supplied to us by the Lung Cancer Segment of the National Cancer Institute, Bethesda, Md. [3H]Retinoic acid used in these studies was homogeneously purified in our laboratory by thin-layer chromatography using the solvent system benzene:chloroform:methanol (4:1:1, v/v). These samples were kept in closed ampuls over liquid nitrogen and freshly prepared solutions were made in dimethyl sulfoxide. Retinol, retinal, γ-linolenic acid, bovine serum albumin, ovalbumin, and Pronase were purchased from the Sigma Chemical Company, St. Louis, Mo.

Specific antiserum to chicken serum albumin (produced in rabbit) and antiserum to whole chicken serum (produced in goat) were purchased from Chappel Laboratories, Inc., Downingtown, Pa. The specificity of antiserum to chicken serum albumin was verified by the vendor and in our laboratory by double immunodiffusion techniques. γ-Globulins from the antisera and the corresponding normal sera were prepared by precipitation with 33% ammonium sulfate. The precipitates were dissolved in 150 mM NaCl containing 10 mM sodium phosphate (pH 7.2) and dialyzed against the same solution to remove the ammonium sulfate. The protein concentration in these γ-globulin preparations was adjusted to 20 mg/ml.

1 Supported by Contract NO1-CP-22064 with the Lung Cancer Segment, Division of Cancer Cause and Prevention, National Cancer Institute, NIH, Department of Health, Education and Welfare.
2 L. J. Wilkoff, J. Peckham, E. A. Dulmadge, R. W. Mowry, and D. P. Chopra. Evaluation of Vitamin A Analogs in Modulating Epithelial Differentiation of 13-Day Chick Embryo Metatarsal Skin, submitted for publication to CANCER RESEARCH.

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The LKB 8100 isoelectric focusing column (110 ml) and ampholines were purchased from LKB Instruments, Chapel Hill, N. C.

Preparation of Chick Skin Extracts. Metatarsal skins were dissected from 12- to 13-day-old chick embryos and washed thoroughly with PBS. The skins were then suspended in 2 mM Tris-chloride (pH 7.2), collected by centrifugation at 1000 x g for 10 min, and dried between filter paper folds. Skin (3 g net weight) was homogenized using a VirTis Teflon homogenizer in 15 ml of 2 mM Tris-chloride (pH 7.2) and lyophilized to dryness. The powder was homogenized with 30 ml of chilled acetone at -50° using a Dounce homogenizer and collected by filtration. The exposure to acetone was for 3 min. The dried acetone powder was further homogenized, using a VirTis Teflon homogenizer, in 5.0 ml of PBS and centrifuged at 100,000 x g for 60 min. The supernatant contained 40 mg of protein.

Unless otherwise mentioned, all the procedures were accomplished at 4°. The protein determinations were accomplished by the method of Lowry et al. (18).

Skin Extract-Retinoic Acid Incubations. Twenty-four pmol of [11,12-3H]retinoic acid (30 nCi) in 3 μl of dimethyl sulfoxide were added to 2.5 mg protein of skin extract in a total volume of 0.4 ml. In competition experiments a 200-fold molar excess of the respective nonlabeled compound in 5 μl of dimethylsulfoxide was added 2 min after the labeled retinoic acid, and the preparations were incubated in the dark at 25° for 60 min. Although a 3-hr incubation was sufficient to obtain maximum binding, all samples were kept at 4° overnight (16 hr) and then dialyzed, except where indicated, for 6 hr against PBS in the dark.

Sucrose Density Gradient Sedimentation. Binding of [3H]retinoic acid to macromolecules in the skin extracts was analyzed on linear 5 to 20% (w/v) sucrose density gradients in 50 mM sodium phosphate (pH 7.2). The skin extracts (0.4 ml), after incubation with [3H]retinoic acid, were layered on top of the gradients and centrifuged at 180,000 x g for 18 hr in a Spinco SW 50.1 rotor at 4°. The total volume in each gradient was 4.5 ml, which was fractionated in portions of 5 drops by puncturing the bottom of the tubes. Bovine serum albumin (molecular weight, 68,000) and ovalbumin (molecular weight, 45,000) were used as standards for determination of sedimentation constants of the radioactive peaks (19). All the binding experiments were accomplished in duplicate.

Pronase Digestion. A 1.0-ml portion of the skin extract (4.5 mg protein) was mixed with 5 μl of [11,12-3H]retinoic acid (50 nCi), incubated at room temperature for 60 min, and left overnight for 16 hr at 4°. The preparation was then passed through a Sephadex G-25 column (10.5 x 1.5 cm) previously equilibrated with PBS; protein fractions, free from small molecules, were collected. A fraction of 2.5 ml with a protein concentration of 1.0 mg/ml contained 4500 cpm/ml. A portion (1.0 ml) of this fraction was divided. One half was treated with 100 μg of Pronase (not self-digested) in 50 μl of PBS, and 50 μl of PBS were added to the other. The preparations were kept at room temperature for 16 hr and then at 4° for 48 hr. Both were passed through Sephadex G-25 columns (15 x 1.6 cm) equilibrated with PBS. The 280 nm absorbance and the radioactivity of the eluted fractions were measured. The entire procedure was performed under subdued light.

Isoelectric Focusing Procedure. A portion of the skin extract (12 mg of protein) was mixed with 160 pmol of [3H]retinoic acid (0.2 μCi), incubated as described above, and dialyzed against 5 mM Tris-Cl (pH 7.2) prior to application to the isoelectric focusing column. Ampholines of pH 3 to 10 were used, and the anode was at the bottom of the column. Electrophoresis was accomplished in 65 hr at 5°, and fractions of 2.0 ml were collected.

RESULTS

Detection of the Retinoic Acid-binding Protein. A sucrose density gradient pattern of 2.5 mg of the skin extract plus [3H]retinoic acid after incubation for 16 hr, but without dialysis, is shown in Chart 1. The peak in this radioactivity profile marked with a solid arrow represents the retinoic acid-binding protein peak and has a calculated S20,w value of 2 (3 separate determinations). The retinoic acid-binding 2 S peak is located in fractions where the protein concentration is low. In Fraction 19 the amount of radioactivity bound is 50 nCi/mg of protein which is equivalent to 250 pmol of retinoic acid bound per mg of the 2 S peak protein. The peak at the top of the gradient corresponds to free retinoic acid, and retinoic acid bound to small molecules and is greatly reduced when the sucrose gradient centrifugation is accomplished after dialysis of the preparation (Chart 1).

Protein Nature of the Binding Molecule. Digestion with Pronase suggested that the macromolecule to which [3H]retinoic acid was bound in the 2 S peak was a protein. After Pronase treatment of the chick skin extract-[3H]retinoic acid complex, radioactivity was eluted from a Sephadex G-25 column in fractions containing mate-
rial of lower molecular weight as compared to the control in which macromolecules as well as radioactivity were eluted in the same, but earlier, fractions.

**Isoelectric pH of the Retinoic Acid-binding Protein.** Chart 2 shows the isoelectric profile of the skin extract-[3H]retinoic acid complex using ampholines of pH 3 to 10. For radioactivity, there is a sharp peak at pH 4.6. Fractions containing this radioactivity were collected and dialyzed against PBS, and 0.5 ml of these fractions was placed on sucrose density gradients as described. The principal radioactive peak that was observed on these profiles corresponded to an S_{20,w} value of 2 which allows the conclusion that the retinoic acid-binding protein of the 2 S peak has an isoelectric pH of 4.6.

**Characterization of the 1st Radioactive Peak.** Retinoic acid is known to bind to bovine serum albumin (16, 29). In order to establish that the 1st radioactive peak in the radioactivity profile (Chart 1), which corresponds to the sedimentation rate of bovine serum albumin, is due to chick serum albumin present in the skin extracts, immunological studies were performed. Chart 3 shows the results of immunoprecipitation of the skin extract-[3H]retinoic acid complex by immune γ-globulins isolated from anti-chicken serum albumin. The immunoprecipitation almost eliminates the 1st peak, whereas normal γ-globulin has no effect. Also, immunoprecipitations with immune γ-globulins isolated from antisera to the whole chicken serum eliminates only the 1st radioactive peak. Again the 2 S peak remains virtually unchanged, indicating that this retinoic acid-binding protein is of chick skin origin and is not a protein from chick serum.

**Specificity of the Binding Protein.** The ligand specificity of the protein was assessed by challenging the [3H]retinoic acid binding with 200-fold molar excesses of various unlabeled test compounds, dialysis, and subsequent sucrose density gradient analysis. A 200-fold excess of unlabeled retinoic acid almost eliminates the radioactive peak at 2 S, whereas a 200-fold excess of retinol did not alter the binding of [3H]retinoic acid (Chart 4A). A similar excess of retinal showed little or no competition for the retinoic acid binding site. This implies that a carboxyl group is essential for binding.

In order to test whether any carboxylic acid group is a sufficient requirement for competition at this site, the [3H]retinoate binding was challenged with γ-linolenic acid which has 3 double bonds and a free carboxylic acid group. As illustrated in Chart 4B, a 200-fold molar excess of γ-linolenic acid showed minimal competition for binding to this protein. A similar excess of diethylretinamide also failed to compete for the retinoic acid-binding site (Chart 4B). However, 13-cis-retinoic acid competed as efficiently as retinoic acid for the binding site. The methyl ester of retinoic acid, like the diethylretinamide, also was found to be noncompetitive (Chart 4C). The ethyl ester (not shown) behaves like the methyl ester also. Among the other analogs of retinoic acid tested in the competition experiments, the cyclopentenyl analog showed the best affinity for the 2 S protein (Chart 4C). We have noticed, in a separate experiment (not shown), that the cyclopentenyl analog competes even better than retinoic acid for the binding site in the protein. The pyridyl analog was not a good competitor of retinoic acid (Chart 4D). A 200-fold excess of the phenyl analog, like the pyridyl analog, also poorly competes for the retinoic acid binding site (Chart 4E). However, if the phenyl ring is substituted, as in the trimethylmethoxyphenyl analog, competition for binding is greatly enhanced (Chart 4E). As shown by Chart 4F, a 200-fold excess of furyl analog of retinoic acid, unlike the phenyl and pyridyl analogs, competes well for the retinoic acid binding site at the 2 S peak.

**DISCUSSION**

Retinoic acid, which cannot be biologically reduced to either retinal or retinol, can maintain the general well being of animals with the exception of vision and reproductive capacity (1, 7) and can counteract the effects of chemical carcinogens both in vitro and in vivo (8, 15, 25). Recent
Chart 4. Sucrose density gradient profiles showing the effect of competition of 200-fold molar excesses of various unlabeled analogs of retinoic acid on the binding of [3H]retinoic acid with proteins of the skin extract. X, controls containing 2.5 mg of protein of the skin extract + 24 pmoles of [3H]retinoic acid. All the other radioactivity profiles are control + 200-fold molar excesses of the following unlabeled compounds. A, Δ, retinol; ◆, retinal; ○, retinoic acid. B, Δ, γ-linolenic acid; ◆, diethylretinamide; ○, 13-cis-retinoic acid. C, Δ, methyl retinoate; ○, cyclopentenyl analog of retinoic acid. D, Δ, α-retinoic acid; ○, pyridyl analog of retinoic acid. E, Δ, phenyl analog of retinoic acid; ○, trimethylmethoxyphenyl analog of retinoic acid. F, Δ, furyl analog of retinoic acid; ○, retinoic acid. SA, serum albumin peak.

studies indicate that retinoic acid and some of its synthetic analogs are more effective than retinol in reversing the effects caused by carcinogens (6, 15). This is also true in the reversal of keratinization in chick embryo skin in organ culture, where retinoic acid is at least 4 times more active than retinol.3 Presently, there are no available data concerning the concentration of retinoic acid in chick skin. Hence, no comparison of the concentration of retinoic acid used in our studies can be made with the in vivo concentration.

Retinol is transported in the plasma by a specific binding protein with a molecular weight of 21,000 (14, 23). Retinoic acid binds almost as effectively as retinol to this protein (10). In the plasma, 1 molecule of retinol-binding protein is tightly bound to 1 molecule of prealbumin (26). Bashor et al. (2) have presented evidence for a retinol-binding protein present in the cytosol of rat tissues. Recently, Ong and Chytil (21, 22) detected specific retinol-binding proteins in rabbit lung and rat testis, and a small retinol-binding protein with a molecular weight of 4,800 was purified from testis seminiferous tubules (8).

The retinoic acid-binding protein presently described is distinct from the retinol-binding protein of serum, although both have similar molecular weights. The immunoprecipitation studies with antiserum to whole chicken serum confirms that this protein is genuinely of chick skin origin. The exact nature of binding of retinoic acid to this protein is unknown, but a high affinity for the ligand is inferred from the fact that the complex is stable to dialysis, sucrose gradient sedimentation, and Sephadex column chromatography. This stability of the complex is consistent with the protein being a tissue "receptor," as suggested for various steroid hormones (9, 11, 13). However, the retinoic acid-binding protein is considerably smaller than steroid receptors, which have sedimentation constants of 4 to 8 S (12, 17, 20, 31).

The retinol-binding protein of tissue cytosol also sediments in the 2 S region of sucrose gradients, but competition studies using unlabeled retinoic acid reveal that netinol had no effect on the binding of [3H]retinol (2). Our results indicate that retinol does not compete for the binding of [3H]retinoic acid on the retinoic acid-binding protein. Hence it is likely that the tissue retinol-binding protein and the retinoic acid-binding protein are distinct.

The binding constant of the 2 S protein and netinoic acid is presently not known. However, the binding to the protein occurs at sites specific for the retinoic acid structure; even 200-fold molar excesses of retinal, retinol, esters of retinoic acid, diethylretinamide, and γ-linolenic acid did not show...
any displacement of [3H]retinoic acid. To be active, generation of free retinoic acid from esters and amides, either by enzymatic hydrolysis or oxidation, may be required.

If retinoic acid is functioning as a differentiation hormone and if the retinoic acid-binding protein is essential for mediating its biological activity within the cell, then it should be possible to design, synthesize, and test new analogs of retinoic acid that have even greater potency. Affinity for binding to this specific protein could be used as an assay procedure. Indeed, a correlation has been observed between the biological activity of various retinoic acid analogs in reversal of chick skin keratinization and their ability to bind to retinoic acid-binding protein. Thus, the cyclopentenyl analog, which is most active biologically, also has the greatest affinity for the binding protein. The phenyl and pyridyl analogs, which are not very active in the production of mucous metaplasia, are found to be poor binders. However, if the phenyl ring is substituted, as in the trimethylmethoxyphenyl analog, which is most active biologically, also has the greatest affinity for the binding protein. The phenyl and pyridyl analogs, which are not very active in the production of mucous metaplasia, are found to be poor binders. However, if the phenyl ring is substituted, as in the trimethylmethoxyphenyl analog, it more effectively displaces [3H]retinoic acid from the binding protein. This observation parallels the findings that the trimethylmethoxyphenyl analog is quite active in relation to the unsubstituted analog in reversal of keratinization in the organ culture system for chick skin. The only exception that has been observed thus far is with the furyl analog of retinoic acid, which competes for the binding protein but shows little activity to reverse keratinization. This analog, even after complexing with the retinoic acid-binding protein, may lack the ability to activate genes by a specific interaction.

REFERENCES

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