Characterization of the Retinol and Retinoic Acid Binding Proteins in the Human Prostate

Douglas Boyd, Leo Beynon, Geoffrey D. Chisholm, and Fouad K. Habib

Department of Surgery, University Medical School, Teviot Place, Edinburgh EH8 9AG, Scotland

ABSTRACT

A retinol-binding protein has been detected in the cytosol of human prostates with benign hyperplasia. The binding was of high affinity and specific for retinol (Kd = 35 nM), with other retinoids such as trans-retinoic acid, retinal, and the synthetic analogues, all-trans-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8-nonatetraenoic acid and p-[(E)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthyl)-1-propenyl]benzoic acid, showing little or no competition. The retinol binding, which sedimented as a 2S component on sucrose density gradients, was also unaffected by the addition of excess unlabeled steroid hormones. Furthermore, pretreatment of the cytosol proteins with heat and/or trypsin totally abolished the retinol binding.

Parallel experiments with trans-retinoic acid suggest that the hyperplastic prostate possesses a second retinoid-binding site which is specific for retinoic acid and distinct from the retinol-binding component. Experiments with serum from patients with benign prostate hyperplasia revealed no binding at the 2S sedimentation position; this suggests that the retinoid-binding proteins were exclusively associated with prostatic tissue and were not therefore derived from serum.

INTRODUCTION

Vitamin A is an important regulator of epithelial differentiation. In 1925, Wolbach and Howe (23) demonstrated widespread epithelial lesions in rats fed vitamin A-deficient diets. A year later in 1926, Fujimaki (9) confirmed these findings interpreting this pathological state to be a prerequisite for neoplastic transformation.

In contrast to a vitamin A deficiency, hypervitaminosis induced by synthetic analogues and natural metabolites of vitamin A appeared protective against the development of papillomas in mice (3). Furthermore, carcinogen-induced benign and malignant states in a number of in vitro and in vivo systems were prevented by vitamin A and analogues (8, 13, 21); this was particularly evident in the male secondary sex organs. In this regard, Laznitski (12) reported that chemically induced squamous metaplasia in cultured mouse prostate was reversed by the addition of vitamin A to the culture medium. Recently, Reese et al. (16) have found that the sensitivity of rat prostate adenocarcinoma cell lines to retinoids manifests as a 40% decrease in cell saturation density.

The molecular mechanisms by which vitamin A directs differentiation and mediates its chemopreventive actions are poorly understood. Specific binding proteins for 2 natural forms of vitamin A (retinol and retinoic acid) have been detected in the soluble fraction of a number of normal and pathological tissues (2, 10, 15), and these may play a role in the actions of vitamin A. This concept is supported by the finding that the abilities of a number of retinoids to promote growth in epithelial cell cultures correlated with their binding efficacy to the retinoic acid-binding protein (7).

In view of the findings that vitamin A is transported in the blood as the alcohol (11) and that cellular uptake is an active process, an investigation into the binding of retinol by human hyperplastic prostate seemed a logical approach. Furthermore, we have also described some of our findings on the characteristics of the retinoic acid-binding properties of the human hyperplastic prostate. Although in an earlier investigation Brandes (5) had detected a saturable retinoic acid-binding component in the soluble fraction of the benign and malignant prostate, no characterization of the specific retinoic acid binding protein has so far been undertaken.

MATERIALS AND METHODS

Radiochemicals

All-trans-11,12-[3H]retinoic acid (32 Ci/mmol) was a generous gift from Hoffmann LaRoche, Basel, Switzerland. All-trans-11,12-[3H]retinol (60 Ci/mmol) was purchased from Amersham International, Inc., High Wycombe, Buckinghamshire, United Kingdom. Radiochemicals were stored under nitrogen at -20° and the purity checked every 2 weeks by thin-layer chromatography using methylene chloride:ethanol (97:3, v/v) for the retinol (1) and benzene:chloroform:methanol (4:1:1, v/v/v) for retinoic acid (17).

Other Chemicals

The synthetic retinoids RO 10-16702 and RO 13-7410 and 13-cisretinoic acid were gifts from Hoffman LaRoche, Welwyn Garden City, Hertfordshire, United Kingdom. Retinol, retinal, retinoic acid (all-trans isomers), and various steroids were purchased from Sigma Chemicals, Poole, Dorset, United Kingdom. Solutions of natural and synthetic retinoids were stored in ethanol under nitrogen at -20°. All other chemicals were of analytical grade and purchased from either Sigma or BDH Chemicals, Ltd., Poole, Dorset, United Kingdom.

Tissue Preparation

Frozenly obtained transurethral resected or retropubic human hyperplastic prostates were transported to the laboratory in ice-cold 0.9% NaCl solution (saline). The tissue was either used fresh or snap-frozen in liquid nitrogen and stored at -20° until analysis. Fragments of each prostatic specimen included in the study were examined histologically.

Received May 15, 1984; accepted August 28, 1984.

2 The abbreviations used are: RO 10-1670, all-trans-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8-nonatetraenoic acid; RO 13-7410, p-[(E)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthyl)-1-propenyl]benzoic acid; TEDG buffer, 10 mM Tris·1.5 mM EDTA·1.0 mM dithiothreitol·glycerol, 10% (v/v), pH 7.4.
and in each case benign hyperplasia was confirmed. Unless specified, all subsequent procedures were carried out at 4°C.

The prostate tissue was washed in TEDG buffer, blotted dry, and minced finely with scissors. The tissue was pulverized for 20 sec on a Mikrodismembrator II (B. Braun Melsungen, A. G., Federal Republic of Germany) in a prechilled Teflon container and homogenized in 2 to 4 volumes of TEDG buffer with a Ystral GmbH homogenizer (three 15-sec bursts; speed 7 with 1-min intermittent cooling). The homogenate was filtered through one layer of nylon gauze (Nybolt 9-150; John Stainer & Co., Manchester, United Kingdom) and centrifuged to obtain the cytosol fraction (105,000 g supernatant) as described previously (6).

Blood obtained from patients with the benign disease was fractionated to give the serum component. The serum was diluted with an appropriate volume of TEDG buffer to give a protein concentration comparable with prostate cytosol.

Retinol Binding Assay

Retinol binding in the cytosol fraction was assessed as described previously (18). Briefly, aliquots of subcellular preparation were incubated at 4°C in the dark for 18 hr with [3H]retinol (100 nM) in the presence and absence of unlabeled ligand in a 100-fold excess. Preliminary studies indicated that pellets formed from dextran-coated charcoal suspension [activated charcoal (0.25%), dextran (0.025%), and gelatin (0.1%)] in 10 mM Tris, 1.5 mM EDTA, and 1.0 mM dithiothreitol buffer, pH 7.4, removed greater than 90% of retinol and retinoic acid from solution.

After incubation of cytosol with [3H]retinol, aliquots were transferred to tubes containing preformed charcoal pellets and mixed. After a 10-min incubation period, charcoal was sedimented at 1500 × g in a refrigerated Chlinspin centrifuge (MSE). The supernatant was then either overlaid in sucrose gradients (7.5 to 20%) or counted in 6 ml of scintillation cocktail (Triton X-100 based; Vickers Laboratories, Ltd., Burling-Whardale, West Yorkshire, United Kingdom).

To establish when the binding of [3H]retinol had reached equilibrium, cytosol was incubated with the radiolabeled compound at 4°C for varying lengths of time in the presence and absence of a 100-fold excess of unlabeled retinol. Free ligand was removed with charcoal, and the treated soluble fraction was counted in 6 ml of scintillation cocktail.

Saturation analysis was carried out by incubating the subcellular fraction with an increasing concentration (10 to 100 nM) of [3H]retinol at 4°C for 18 hr in the presence and absence of a 100-fold excess of unlabeled retinol. Unbound retinol was removed with charcoal and the treated supernatant counted for radioactivity in a Packard liquid scintillation spectrometer with a counting efficiency of 40%. The binding data were analyzed by the method of Scatchard (20).

Sucrose Density Gradients

Linear sucrose gradients (7.5 to 20%) were centrifuged at 400,000 × g for 2 hr in a Sorvall TV 865 vertical rotor (DuPont Instruments). Myoglobin (25S) and bovine serum albumin (4.6S) (Sigma Chemicals) were run on separate gradients as external standards. Fractions (200 μl × 20) were harvested by upward displacement using 40% sucrose as displacing agent.

Other Experiments

[3H]Retinoic Acid Binding Assay. The binding of trans-retinoic acid was assessed by the method of Brandes (5); cytosol was incubated with [3H]retinoic acid (100 nM) in the dark at 4°C for 18 hr in the presence and absence of a 25-fold excess of unlabeled ligand. Unbound ligand was removed by charcoal as described above and the treated subcellular preparation analyzed by sucrose density gradient centrifugation.

Serum Binding of Retinol and Retinoic Acid. Serum diluted in TEDG buffer was incubated with [3H]retinol or [3H]retinoic acid (100 nM) in the dark at 4°C overnight. Excess ligand was removed with charcoal and the treated preparation analyzed on sucrose gradients.

Protein Measurement. Cytosol protein concentration was measured by the method of Bradford (4) using bovine serum albumin as standard.

Effect of Heat and Trypsin. Prior to incubation with [3H]retinol or retinoic acid, cytosol was heated to 60°C for 10 min or treated with trypsin (0.05% bovine pancreas, type 1; Sigma Chemicals) at 37°C for 90 min. The binding of [3H]retinol and [3H]retinoic acid were assessed by sucrose gradient analysis after incubation with 100 nM of the radiolabeled compounds as described above.

Metabolism Studies. In order to ensure that retinol or retinoic acid were not metabolized under our receptor assay conditions, the cytosol fraction was incubated with 100 nM of either [3H]retinol or [3H]retinoic acid at 4°C for 18 hr. The cytosol was then extracted with 2 × 1 ml of diethyl ether to remove the radioactivity from the incubation medium. Following the drying down of the diethyl ether, the residue was reconstituted in 50 μl of ethanol and subjected to reversed-phase chromatography using Whatman KC 18 thin-layer chromatographic plates (Whatman, Maidstone, Kent, United Kingdom). The plates were run at 4°C in subdued lighting using an ethanol:water (8:2, v/v) solvent system. At the end of the run, the plates were dried down, markers were located, and the positions of the 3H-labeled retinoids were identified in relation to the reference standards. The appropriate zones containing the various retinoids were cut from the plates and inserted directly into the counting vials, and the radioactivity was measured.

RESULTS

Characterization of Retinol Binding

Specificity. Sucrose gradient analysis of cytosol labeled with [3H]retinol revealed a peak of activity coincident with the 2S external marker (Chart 1). The peak was abolished with a 25-fold excess of retinol (Chart 1a) but was unaffected by trans-retinoic acid (Chart 1a), the synthetic retinoids RO 10-1670 and RO 13-7410 (Chart 1b), testosterone, dihydrotestosterone, and 17β-estradiol (Chart 1c). In contrast, Chart 1, a and b, illustrates that retinol and cis-retinoic acids both have some affinity for the binding component.

Effects of Heat and Trypsin. It is apparent from Chart 2 that protease and heat pretreatment inactivate the 2S binding of [3H]retinol. These data suggest that [3H]retinol is bound by a protein macromolecule derived from the soluble fraction of human prostate.

Serum Binding of [3H]Retinol and [3H]Retinoic Acid. Chart 3 illustrates the results of sucrose gradient analysis of serum labeled with [3H]retinol. A peak of activity was observed in the 4.6S but not in the 2S region of the gradient. These findings confirm those of other workers (2) who were unable to detect any retinol binding in the 2S region of the sucrose gradient for serum specimens treated with radiolabeled retinol.

Affinity and Binding Capacity. Prior to saturation analysis of the soluble component, it was important to establish that the binding of [3H]retinol was in equilibrium under conditions of time and temperature. It is apparent from Chart 4 that the specific binding of [3H]retinol is at equilibrium after 4 hr and extends to 24 hr of incubation at 4°C.

Scatchard analysis of the saturation data determined under equilibrium conditions of exchange revealed a dissociation constant of 35 nM with a binding capacity of 3.5 pmol/mg cytosol protein (Chart 4).

Metabolism Studies. Chart 5, a and b, illustrates the absence of [3H]retinol and [3H]retinoic acid metabolism in vitro. The cho-
VITAMIN A BINDING IN THE HUMAN PROSTATE

Chart 1. Specificity of [3H]retinol binding in cytosol from benign human prostate: sucrose gradient analysis. Aliquots of prostate cytosol were incubated for 18 hr at 4° with either 100 nM [3H]retinol alone (A) or in the presence of 25-fold excess of unlabeled (a) retinol (●), retinal (●), trans-retinoic acid (○), (b) RO 10-1670 (●), RO 13-7410 (○), cis-retinoic acid (●); (c) dihydrotestosterone (●), testosterone (●), 17β-estradiol (○). The binding of [3H]retinol was assessed by sucrose gradient centrifugation as described in "Materials and Methods." Each experiment was repeated 3 times.

Matographic profiles obtained were identical to those for [3H]retinol and [3H]retinoic acid extracted from buffer alone (data not shown).

Chart 2. Inactivation of [3H]retinol binding by heat and trypsin treatments: sucrose gradient analysis. The soluble fraction of human prostate was treated with either 0.05% trypsin or briefly heated to 60°. Untreated (A), heated (○), trypsin (●) and 100-fold excess unlabeled retinol (●)-treated cytosols were then incubated with 100 nM [3H]retinol and overlayed on sucrose gradients. All studies were performed in triplicate.

Chart 3. Binding of [3H]retinol and [3H]retinoic acid in serum from patients with benign prostate hypertrophy. Serum from patients with benign prostate hyperplasia was diluted with TEDG buffer to give protein concentrations of 5 to 10 mg/ml. The diluted serum preparations were incubated with either 100 nM [3H]retinol (A) or [3H]retinoic acid (○) at 4° for 18 hr. The binding of radiolabeled ligands was analyzed by sucrose gradient centrifugation as described in "Materials and Methods." These measurements were repeated at least 3 times.

Radiolabeled Retinoic Acid Binding

Sucrose gradient centrifugation of cytosol labeled with [3H]retinoic acid revealed peaks of activity in the 2S and 4.6S regions (Chart 6). While the 2S peak was abolished by a 25-fold excess of retinoic acid (all trans- and cis-isomers; Chart 6, a and b), RO 10-1670 and RO 13-7410 (Chart 6b), dihydrotestosterone, and testosterone were with little effect (Chart 6c). However, it is apparent from Chart 6a that both retinol and retinal possess some affinity for the 2S binding component. In contrast to the binding profile of the 2S peak, the secondary peak of activity in the 4.6S region was unaffected by any competitor present in excess, suggesting a nonspecific association.
VITAMIN A BINDING IN THE HUMAN PROSTATE

Chart 4. Scatchard analysis of \(^{3}H\)retinol binding in prostate cytosol. Cytosol was incubated with 100 nM \(^{3}H\)retinol at 4° for varying times in the presence and absence of unlabeled retinol (2.5 \textmu M). The specific binding of \(^{3}H\)retinol was determined after removal of free ligand by charcoal (inset). Saturation analysis was performed by incubating the soluble fraction with a range of \(^{3}H\)retinol concentrations for 4 hr at 4° (10 to 100 nM), and the data were analyzed by the Scatchard method to yield the \(K_d\) and the number of specific binding sites. Analysis was performed on 3 separate sets of tissues.

It is also evident from Chart 7 that the retinoic acid binding component is degraded by both protease and heat treatment, indicating that the component is a heat-labile protein.

DISCUSSION

A binding component for retinol has been detected in benign human hyperplastic prostate. The component exhibits similar characteristics to the cellular retinol binding protein present in a number of other Vitamin A target organs (18, 22). In common with cellular retinoic acid binding protein (Chart 6), the prostate binding component has a sedimentation coefficient of 2S (Chart 1) and was inactivated by protease and heat treatments (Chart 2). Although this binding site failed to recognize trans-retinoic acid (Chart 1a), the profile illustrated in Chart 1b indicates some competition by the cis-isomer; this may reflect the different stereochemistries of the 2 isomers. Furthermore, this site binds retinol avidly with a dissociation constant of 35 nM (Chart 4); this value is not inconsistent with the 16 nM reported for purified cellular retinol binding protein (14).

The present results also suggest that human hyperplastic prostate is able to recognize the acid form of Vitamin A as a distinct entity. Although the binding sites for retinol and retinoic acid are similar in that they are both heat-labile proteins with sedimentation coefficients of 2S, it is unlikely that they are identical. In support of this, trans-retinoic acid was not recognized by the retinol-labeled peak (Chart 1a). Conversely, retinol displayed little affinity for the retinol acid-binding site (Chart 6). Furthermore, while the retinoic acid-binding site bound the synthetic analogues RO 10-1670 and RO 1-7410 (Chart 6), these compounds did not associate with the retinol recognizing structures (Chart 1). The data on the retinoic acid binding confirm and extend the findings of Brandes (5) who detected the 2S saturable component for retinoic acid in the cytosol of benign and malignant human prostate; this author, however, did not attempt to check the specificity of his binding component. Furthermore, our findings that retinol showed some affinity for \(^{3}H\)retinoic acid binding are at variance with other reports on the specificity of retinoic acid binding in prostate cytosol.
VITAMIN A BINDING IN THE HUMAN PROSTATE

Chart 6. Specificity of [3H]retinoic acid binding in cytosol from benign human prostate: sucrose gradient analysis. Aliquots of prostate cytosol were incubated for 18 hr at 4°C with either 100 nM [3H]retinoic acid alone (△) or in the presence of 2.5 μM unlabeled (a) trans-retinoic acid (C), retinol (β), retinal (γ); (b) RO 10-1670 (β); RO 13-7410 (C), cis-retinoic acid (α); (c) dihydrotestosterone (β), testosterone (α), trans-retinoic acid (C). The binding of [3H]retinoic acid was assessed by sucrose gradient centrifugation as described in "Materials and Methods." Each experiment was repeated 3 times.

Chart 7. Inactivation of [3H]retinoic acid binding by heat and trypsin treatments: sucrose gradient analysis. Cytosol was treated with either 0.05% trypsin or briefly heated to 60°C. Untreated (△), heated (○), trypsin (●), and 25-fold excess unlabeled retinoic acid (□)-subjected cytosols were then incubated with 100 nM [3H]retinoic acid and overlayed on sucrose gradients. Each experiment was performed in triplicate.

Acknowledgments

We wish to thank Hoffmann LaRoche, Basel, Switzerland, for their generous supply of [3H]retinoic acids.

References

VITAMIN A BINDING IN THE HUMAN PROSTATE

Characterization of the Retinol and Retinoic Acid Binding Proteins in the Human Prostate

Douglas Boyd, Leo Beynon, Geoffrey D. Chisholm, et al.

*Cancer Res* 1984;44:5532-5537.

Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/44/12_Part_1/5532

**E-mail alerts**
Sign up to receive free email-alerts related to this article or journal.

**Reprints and Subscriptions**
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

**Permissions**
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.