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Cellular Binding Proteins for Vitamin A in the Normal Human Uterine Cervix and in Dysplasias[1]

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ABSTRACT

Cellular retinol-binding protein (CRBP) and cellular retinoic acid-binding protein are present in the cytosol of normal human uterine cervical tissues, as detected by ultracentrifugation analysis. Both binding proteins have characteristically high specificity for their respective ligands. In sucrose gradients, both proteins sediment in the 2S region and are of similar molecular weight (M.W. ~14,000). In blind analyses of cervical biopsies, obtained under direct vision by colposcopy of normal women (control) or from patients histopathologically diagnosed to have dysplasias or carcinoma *in situ* (study group), CRBP was not detectable by sucrose gradient analysis in 78.8% of the 33 abnormal biopsies, compared to 23.5% of the 34 controls. This difference was statistically significant ($p < 0.005$). In biopsies in which CRBP was detected, the mean levels were 2.76 and 0.72 pmol/mg protein in the cytosol for the control and study groups, respectively. In some subjects from each group, cellular retinoic acid-binding protein but not CRBP was detected in the biopsied tissue. The presence and role of these binding proteins in vitamin A metabolism, epithelial maturation and differentiation in cervical dysplasias, and *in situ* lesions remain to be investigated.

INTRODUCTION

There is an intensive and expanding interest in clarifying the role of vitamin A (retinol) in epithelial maturation, differentiation, and tumorigenesis. In addition, there is a concurrent opportunity to evaluate the pharmacological effectiveness of biologically active synthetic vitamin A analogs (known as retinoids) in inhibiting, preventing, or reversing tumor induction. The latter is being investigated in various animal model systems using well-established chemical carcinogens (5, 6, 29, 30).

The action of vitamin A in normal tissue may well be mediated by specific intracellular binding proteins (4, 22), perhaps in a manner similar to that known for steroid hormones (9, 12). CRBP[3] and/or cellular retinoic acid-binding protein have been detected in human myometrium (8), in human testis (1), and in different animal tissues (4, 13, 24, 25, 32). Each binding protein has been purified to homogeneity, and the homogeneous preparations have been partially characterized (15, 16, 20, 21, 33).

Retinoic acid-binding protein has been reported in human lung and breast carcinomas but was not detected in adjacent noninvolved normal tissues (18). In preliminary experiments, Bashor and Chytil (3) could not detect retinol-binding protein in extracts of Novikoff hepatoma, ascites hepatoma As-30D, mouse Ehrlich ascites tumor, or mouse pituitary tumor cell line AtT-20. In mouse skin papillomas, human adenocarcinoma HAD-1, Dunning leukemia, Walker carcinosarcoma 256, and mammary adenocarcinoma MAC-1, Ong and Chytil (14) reported finding both cellular binding proteins, while only cellular retinoic acid-binding protein was detected in chondrosarcoma and Sarcoma 180. Neither binding protein was detected in Ehrlich carcinoma and L1210 leukemia. Sani and Titus (26) identified the presence of retinoic acid-binding protein in 2 metastatic colon tumors, B16 melanoma, Lewis lung carcinoma, Ridgway osteogenic sarcoma and keratoacanthoma. Recently, Ong *et al.* (17) have reported that induced adenocarcinomas contain higher levels of CRBP than of the levels of CRBP found in adjacent mucosa of the same animal or colorectal mucosa from normal rats. The presence, relative distribution of CRBP and cellular retinoic acid-binding protein, and the significance of binding proteins in precursor and other neoplastic lesions require further investigation.

The identification of vitamin A-binding proteins in the normal human uterine cervix and its dysplasias or in cervical carcinoma has not been investigated previously. Here, we report the results of studies of retinol- and retinoic acid-binding proteins in crude extracts of cervix tissue samples obtained from normal women (control subjects) and from patients diagnosed histopathologically to have one of the spectrum of cervical lesions identified as mild, moderate, or severe dysplasia or carcinoma *in situ* of the cervix.

MATERIALS AND METHODS

Subject Selection. A total of 34 control and 33 study subjects constitutes the basis for this report.

The normal control (negative Papanicolaou smear) or study subjects (patients with suspicious or confirmed positive vaginal cytology) were initially identified by the routine cytological screening program available in our institution. All subjects were interviewed, and only those enrolled in the research program who after careful indoctrination as to the nature of the study and its protocol gave informed consent were accepted for the study. Women who did not wish to participate or did not understand the protocol were not recruited. The study group was matched with control subjects according to age, parity, socioeconomic group, and ethnicity by the nurse-coordinator. Each subject was assigned a code number that identified her participation in the biochemical analyses, as well as in an epidemiological and dietary-nutritional survey and an examination by colposcopy in order to permit blind analysis. The results of the epidemiological and nutritional surveys will be reported separately.

If a cervical lesion was detected colposcopically and the

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[3] The abbreviation used is: CRBP, cellular retinol-binding protein.

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lesion was large enough, a representative biopsy was submitted for both histopathological and biochemical examination. If the lesion was small, the entire biopsy was used for histopathological diagnosis and determination of the appropriate therapy for the patient. If any of the subjects were hysterectomized, multiple tissue samples were obtained from the excised uterus. The histopathological diagnoses were established by staff pathologists. All tissue aliquots (study or control) were submitted and processed for sucrose gradient analysis under the assigned code number. Thus, all the cytosols were run as "unknowns."

Chemicals. All-*trans*-[15-^{3}H]retinol (2.66 Ci/mmol) was purchased from New England Nuclear, Boston, Mass. All-*trans*-[11,12-^{3}H]retinoic acid (1.11 Ci/mmol) was a generous gift from Hoffman-LaRoche Inc., Nutley, N. J. The purity of both tritiated compounds was evaluated by comparison with authentic all-*trans*-retinol and all-*trans*-retinoic acid (Sigma Chemical Co., St. Louis, Mo.) with the use of thin-layer chromatography on Baker-Flex silica gel sheets (J. T. Baker Chemical Co., Phillipsburg, N. J.) in 2 solvent systems, cyclohexane:ethyl acetate (6:4) and hexane:isopropyl alcohol (6:4). Greater than 97% of the applied radioactivity for both tritiated compounds was found in the respective R_F's when cochromatographed with their respective unlabeled compounds. Unlabeled retinal was also purchased from Sigma.

Preparation of Cytosols and Assay for Binding Proteins. All preparations were done at 0–4°. Tissue samples were washed, blood was removed, and any gross underlying fibrous stroma was dissected and discarded. The remaining epithelium was minced in a clean, nonsterile fashion and homogenized in 2 volumes of 0.05 M Tris-HCl:1 mM EDTA:1 mM dithiothreitol (pH 7.1). The cytosol fraction was prepared by centrifugation at 105,000 × *g* for 60 min as described previously (13) and stored at −80° for 2 weeks until assay. The sucrose density gradient sedimentation technique involved incubation of 0.3 ml cytosol in the dark at 4° for 4 hr with 60 pmol all-*trans*-[15-^{3}H]retinol in 10 μl of dimethyl sulfoxide or with 70 pmol all-*trans*-[11,12-^{3}H]retinoic acid in 10 μl of isopropyl alcohol. Parallel incubations were run containing a 200-fold molar excess of unlabeled retinol or retinoic acid over labeled material in the respective solvent. Immediately prior to sucrose gradient centrifugation, each incubation mixture was treated by adding 0.2 ml of dextran-coated charcoal suspension to remove free ligands. After incubation for 5 min at 4°, the mixtures were centrifuged at 3000 × *g* for 5 min. This did not decrease the amount of specific binding observed in the 2S region, but it did lower the background which made quantitation of binding easier (1, 13). A portion (0.2 ml) of the incubation mixture was layered on a linear 5 to 20% sucrose gradient of 5 ml and centrifuged at 145,000 × *g* for 18 to 19 hr. The gradients were fractionated from the bottom, and radioactivity in each fraction was determined. Myoglobin (2S) and rat serum albumin (4.6S) on separate gradients alone were used as external markers. The binding proteins were detected as a peak of radioactivity in the 2S region of the gradient. The peak was substantially reduced in the presence of unlabeled ligand. The radioactivity in this peak was used to quantitate the binding which is expressed as pmol of ligand bound per mg of protein in the cytosol. Protein concentration in the cytosol was determined by the method of Lowry *et al.* (11). Whenever the tissue sample size was limited, only the retinol-binding protein determination was carried out.

Molecular Weight Determination. To determine the molecular weights, cervical tissue extracts prepared as crude cytosols, previously labeled with [^{3}H]retinol or [^{3}H]retinoic acid, were submitted to gel filtration on a Sephadex G-75 column (2.0 x 48 cm) previously calibrated with standard proteins, following the method described by Andrews (2) and Bashor *et al.* (4). Fractions of 1 ml were collected, eluting with 0.05 M Tris-HCl (pH 7.5) containing 0.1 M KCl. The fractions containing the elution portion of the cellular binding proteins were determined by measurement of radioactivity.

RESULTS

Age and Pathological Diagnoses. The age span of the women whose tissues were investigated was 18 to 53 years. The morphological spectrum associated with the tissue samples included normal cervix; mild, moderate, and severe dysplasias; and carcinoma *in situ* of the cervix. The age distribution and histological characterization of the tissue samples investigated are seen in Tables 1 and 2.

Determination of Levels of CRBP and Cellular Retinoic Acid-binding Protein. In a typical profile, after the crude cytosols from the normal cervix were labeled with [^{3}H]retinol and submitted to ultracentrifugation, 2 peaks of radioactivity were observed, one in the 2S region and one at the 4.6S region

Table 1

Cellular retinol- and retinoic acid-binding proteins in extracts of human uterine cervix clinically diagnosed as normal

Sample	Age (yr)	mg protein/ml cytosol	[^{3}H]Retinol bound (pmol/mg protein)	[^{3}H]Retinoic acid bound (pmol/mg protein)
A994	44	0.25	4.1	—[a]
F972	28	0.30	4.2	—
B765	21	0.44	1.6	2.47
S961	31	0.79	1.93	0.6
E813	32	2.72	2.6	2.6
M415	25	0.34	3.52	—
N548	21	0.18	2.44	—
L495	28	0.33	5.43	0
B245	33	0.25	0	2.16
S129	29	0.26	3.67	—
S604	41	0.50	3.2	3.8
A268	19	0.73	0	3.56
B127	23	0.83	0	3.0
H904	21	0.44	5.63	—
B953	26	0.55	2.40	—
A039	22	0.44	1.88	—
A591	25	0.53	0	6.11
A335	21	0.34	2.16	—
A169	29	0.50	2.6	0
A982	33	0.46	1.24	—
A504	52	0.33	2.1	—
A486	37	0.53	0	6.4
B885	37	0.55	0	7.8
B915	33	0.41	0	1.2
B153	35	0.41	0	2.56
A935	30	0.59	1.96	—
A802	43	0.24	3.58	—
C068	49	0.30	2.32	—
B703	27	0.50	0.62	3.76
A101	27	0.31	2.9	—
A278	47	0.39	1.3	6.66
A140	38	0.34	2.51	—
D306	21	0.32	1.29	—
C367	49	0.20	4.74	—
Total: 34			2.11 ± 1.63[b]	3.29 ± 2.39

[a] —, not determined; 0, not detectable by sucrose gradient centrifugation.
[b] Mean ± S.D.

Table 2

Cellular retinol- and retinoic acid-binding proteins in extracts of human dysplastic cervix

Sample	Age (yr)	mg protein/ml cytosol	[^{3}H]Retinol bound (pmol/mg protein)	[^{3}H]Retinoic acid bound (pmol/mg protein)	Clinical diagnosis
F676	39	2.08	0	0.4	Invasive carcinoma
F577	53	0.31	0	—[a]	Carcinoma *in situ*
H995	32	0.51	0	2.4	Carcinoma *in situ*
G233	25	0.54	0	—	Carcinoma *in situ*
H164	32	0.47	0	—	Carcinoma *in situ*
B919	37	0.23	0.33	—	Carcinoma *in situ*
B002	35	0.52	0	—	Carcinoma *in situ*
B430	33	0.44	0	—	Carcinoma *in situ*
B390	50	0.29	0	—	Carcinoma *in situ*
A463	33	0.34	0	—	Severe dysplasia
Y404	32	0.18	0	—	Severe dysplasia
S570	21	0.14	0	—	Severe dysplasia
L662	21	0.42	0.51	—	Severe dysplasia
M121	28	0.23	0	—	Severe dysplasia
B764	26	0.36	0	—	Severe dysplasia
A999	50	0.34	0	—	Severe dysplasia
A851	41	0.28	0	—	Severe dysplasia
A488	26	0.44	0	—	Severe dysplasia
A628	28	0.46	0	0	Moderate dysplasia
M955	26	0.23	0	—	Moderate dysplasia
R085	48	0.14	0.7	—	Moderate dysplasia
G237	31	0.31	0	—	Moderate dysplasia
G480	29	0.36	0	0	Moderate dysplasia
C647	33	0.39	0	—	Moderate dysplasia
C647	33	0.40[b]	0	—	Moderate dysplasia
A515	23	0.33	1.17	—	Moderate to severe dysplasia
A626	24	0.54	1.03	—	Mild dysplasia
B937	53	0.44	0	—	Moderate to severe dysplasia
B636	24	0.47	0	—	Mild dysplasia
A131	18	0.31	0	—	Mild to moderate dysplasia
B891	26	0.38	0	—	Mild dysplasia
M011	30	0.34	0.50	—	Mild dysplasia
M011	30	0.30[b]	0.74	—	Mild dysplasia
Total: 33			0.150[c]		

[a] —, not determined; 0, not detectable by sucrose gradient analysis.
[b] Second biopsy.
[c] Mean.

(Chart 1*A*). The peak in the 2S region was greatly reduced in the presence of a 200-fold molar excess of unlabeled retinol but was not affected by a 200-fold molar excess of retinoic acid or retinal. This is highly suggestive that the binding protein, sedimenting in this region, is specific for retinol. Excess unlabeled retinol did not affect the peak at 4.6S, which may represent nonspecific serum albumin binding that unavoidably contaminates tissue extracts. This 4.6S peak has not been further characterized by the immunoprecipitation method of interfering serum albumin (27, 32) because these initial studies have focused on investigating the specific CRBP peak (2S).

When normal cervix cytosol was labeled with [^{3}H]retinoic acid, a similar profile of radioactivity was observed on sucrose gradient centrifugation (Chart 1*B*). In the latter case, however, the 2S binding was substantially reduced only by unlabeled retinoic acid and not by retinol or retinal. This evidence indicates the presence of a second cellular binding protein, with high specificity for retinoic acid. Again, nonspecific binding was observed in the 4.6S region and presumed to represent serum albumin. Serum albumin has been shown to bind retinoic acid administered to the rat (28).

The data in Tables 1 and 2 reveal the presence of detectable amounts of cellular retinol- and retinoic acid-binding protein in the cytosols of cervical tissue extracts obtained from control and study subjects. In the study group (dysplasias and *in situ* disease), either CRBP is undetectable or [^{3}H]retinol is bound in very minute amounts (Table 2). In 18 of 34 control subjects, because of the small size of the lesion and the primary need to

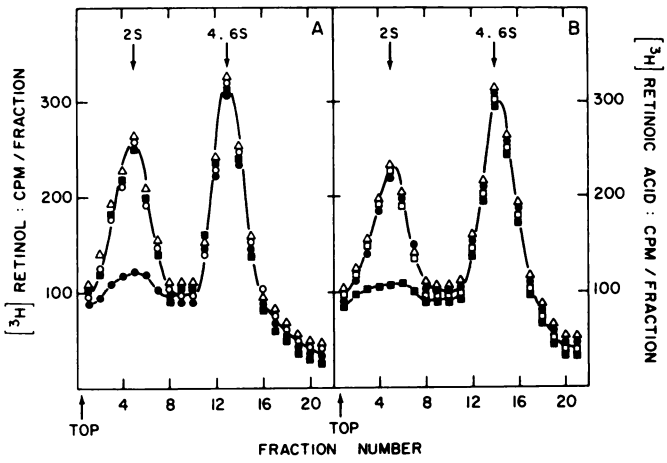

Chart 1. The binding specificity of retinol- and retinoic acid-binding proteins in cytosols of normal human uterine cervix. *A*, cytosols incubated with 60 pmol [^{3}H]retinol (○) and with an additional 12 nmol unlabeled retinol (●), retinoic acid (■), or retinal (△). *B*, cytosols incubated with 70 pmol [^{3}H]retinoic acid (□) and with an additional 14 nmol unlabeled retinoic acid (■), retinol (●), or retinal (△). Aliquots of the incubation mixture were submitted to sucrose gradient centrifugation for 18 to 19 hr at 145,000 × *g*.

submit the entire biopsy for histopathological diagnosis, the tissue sample which was homogenized was not large enough to permit investigation for the presence of both retinol- and retinoic acid-binding proteins. In the control group, retinoic acid-binding protein was present in varying amounts in normal cervical tissue, and retinol-binding protein was easily detected in the majority of samples (Table 1).

In these initial data derived from the cervical tissue samples, in 78.8% of the 33 study women, compared to 23.5% of the 34 control women, CRBP was not detectable by sucrose gradient analysis. This difference is statistically significant by χ^2 test ($p < 0.005$). The mean for the total control group was 2.11 pmol/mg protein. The mean for the 26 control women in whom cellular retinol-binding protein was detectable was 2.76 pmol/mg protein, compared to a mean of 0.72 pmol/protein for the 7 study group women in whom CRBP was detectable. The mean for the 16 control women in whom cellular retinoic acid-binding protein was detectable was 3.29 pmol/mg protein. In 2 of 16 control subjects (13%), the amount of cellular retinoic acid-binding protein was nil or undetectable.

Gel Filtration Studies. Estimation of the molecular weights of the 2 binding proteins was accomplished by gel filtration of normal uterine cervical extracts labeled with [^{3}H]retinol or [^{3}H]retinoic acid on a previously calibrated Sephadex G-75 column. The molecular weights of the 2 binding proteins were found to be similar to that of RNase A, which is about 14,000.

DISCUSSION

This study reports the presence of both CRBP and cellular retinoic acid-binding protein in the normal human uterine cervix. In the spectrum of lesions identified histopathologically as dysplasias, these binding proteins were not always detectable by standard ultracentrifugation techniques. The presence and amount of both binding proteins in the normal cervix, when contrasted with dysplastic cervical tissues, are significantly different and have not been previously reported. Variations in the specific binding activity for retinol and retinoic acid in uterine cervical cytosol, observed in the 2S region of the sucrose gradient, were noted in the blind analyses of all samples obtained from randomly selected subgroups of women screened initially by vaginal cytology. No attempt was made to determine the influence of such variables as age, parity, the phase of the menstrual cycle, or nutrition on the protein binding of retinol or retinoic acid. Such studies are currently being pursued.

It should be noted that the activity of the specific radioactive ligands was the limiting factor in the detectability of either retinol- or retinoic acid-binding proteins. Furthermore, the binding specificity and the molecular weights (about 14,000) of both of the cervical cytosol binding proteins are distinct from those of human serum retinol-binding protein (M.W. ~20,400) (10).

In other studies, we have observed that human cervix cytosol retinol- and retinoic acid-binding proteins are separable from a supernatant prepared from pooled cervical tissue obtained from surgically removed normal uteri by gel filtration and chromatography on DEAE-cellulose.[4] These findings confirm the presence of 2 specific binding proteins for retinol and retinoic acid in the normally diffferentiated epithelium of the human uterine cervix. The binding protein for retinol is not always present or can be only minimally detected in dysplasic lesions. These proteins have been reported previously in human myometrium, in human testis, and in diverse organs of several laboratory animals. This evidence of intracellular proteins in the crude cytosol with an affinity for retinol and retinoic acid in the normal cervix suggests that these binding proteins may be involved in the mechanism of action of vitamin A in the maturation and differentiation of cervical epithelium.

While the cellular homogeneity of the tissue samples in the study and control groups may be questioned, the biopsies were obtained and processed in a standard, uniform fashion. Moreover, all the analyses were completed in a blind fashion. A reasonable assumption is that comparable cellular elements exist in all of the homogenates investigated. The failure to detect retinol-binding protein in the cytosol of the tissues biopsied directly from sites determined to be colposcopically abnormal would appear to represent a significant biochemical difference between dysplastic and normal cells.

Previously reported changes in the content of binding protein(s) in spontaneous or chemically induced tumors have shown dramatic increases in the levels of CRBP and/or cellular retinoic acid-binding protein compared to those for normal tissue (7, 17, 18, 23). Other proteins are known to be present in transformed cells and malignant tissues that are not detected in normal tissues and cells (19, 31). In this study of dysplastic cervical tissues, a significant decrease in the level of CRBP compared to that in the normal control tissue has been observed. The absence or the diminished amount of the binding protein noted in the dysplasias suggests that a deficiency in this binding protein may influence the nature and extent of epithelial maturation and differentiation.

These findings emphasize the importance of the presence and interaction of vitamin A and its metabolites in the frequently encountered clinical entities involving the human uterine cervix identified as metaplasia and dysplasia. The presence of CRBP and cellular retinoic acid-binding protein in invasive carcinomas of the cervix is currently being investigated. The availability and screening capability of the Papanicolaou test to monitor large groups of women and the accessibility of the human uterine cervix to colposcopic examination and biopsy provide an unusual opportunity to investigate the possible nutrient influence of vitamin A in the pathogenesis of cervical precursor lesions.

Moreover, several current reports constitute a background for believing that synthetic retinoids which are not cytotoxic may be useful in cancer prevention by inhibiting, arresting, or reversing the biological processes that promote cancer (5, 6, 29, 30). The identification of intracellular binding proteins in normal human uterine cervical epithelium and the reduction or absence of retinol-binding protein in dysplastic lesions represent a model system for a prospective clinical trial focusing on the reversibility of early abnormal lesions. The availability of a vaginal suppository containing a biologically active retinoid would permit evaluation of the pharmacological effectiveness of the compound. Such topical use would have the advantage of avoiding or minimizing any toxicity related to systemic therapy. Compared to natural retinoids, current evidence suggests that the synthetic retinoids are less cytotoxic at higher dosage levels (30). Such a double-blind clinical trial could be closely

[4] P. R. Palan and S. L. Romney, unpublished data.

monitored by cytological, colposcopic, and biopsy observations. The histopathological findings could additionally be correlated with sucrose gradient ultracentrifugation analyses of the retinoid-binding proteins.

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