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Retinoic Acid Binding Proteins and Steroid Receptor Levels in Human Breast Cancer*

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Abstract—*Samples of* 31 *human breast cancer tissues were examined for the presence or absence of cytosolic retinoic acid binding proteins (cRABP), estrogen receptors (ER) and progesterone receptors (PR). cRABP were detected as specifically sedimenting* 2*S components on sucrose density gradients. Specific binding under the* 2*S region for cRABP and under the* 8*S region for ER and PR were determined. The levels of cRABP ranged from less than* 1 pmol *to* 14 pmol *per* mg *of cytosol protein. Tumors containing less than* 3 pmol *cRABP were designated as* low *cRABP; tissues were considered ER⁻ and PR⁻ when the receptor levels were less than* 10 fmol/mg *protein. Approximately* 60% *of primary and metastatic tumors examined contained* high *cRABP. Tumors with well differentiated histopathology exhibited increased levels of cRABP. There was no significant correlation between the presence or absence of steroid receptors and cRABP in human mammary tumors.*

INTRODUCTION

IT HAS been reported that vitamin A deficiency can lead to an increased incidence of epithelial metaplasia and tumors in experimental animals and possibly in humans [1, 2]. Dietary supplementation with synthetic analogs of vitamin A (retinoids) prevents chemical carcinogenesis of many tissues in experimental animals [2–7]. We have previously reported that the addition of the retinoids retinyl acetate and *N*-(4-hydroxyphenyl)retinamide to the diet suppressed the induction of mammary carcinogenesis in rats by *N*-methyl-*N*-nitrosourea (MNU) or 7,12-dimethylbenz(a)anthracene (DMBA). Recent studies have indicated that retinoid in combination with ovariectomy reduced the occurrence of second tumor appearance when treatment was begun after excision of the first palpable tumor [8]. Although the mechanism by which these retinoids exert their effects is unknown, specific intracellular binding proteins for retinoids, retinoic acid-binding protein (cRABP) and retinol-binding protein have been detected in several experimental tumor systems, as well as in human breast carcinomas [9–14]. It is currently believed that the action of retinoids may be mediated by a specific retinoid-binding protein in a manner similar to that known for steroid hormones [15].

Analysis of estrogen and progesterone receptors in human tumor biopsies is currently employed in conjunction with other clinical criteria to determine the course of therapy of the patient with advanced breast cancer [16]. Previous studies have shown that 60–70% of all breast tumors exhibit estrogen receptors; approximately 60% of the patients with estrogen receptor-positive tumors respond to endocrine therapy [16–19]. Recently it has been established that determination of progesterone receptor content in addition to estrogen receptor concentration provides a more accurate criterion of responsiveness to hormonal therapy [16]. However, relevant biochemical markers which may predict response to possible retinoid treatment are currently unknown.

In this report we present quantitative measurements of retinoic acid-binding protein in human breast carcinomas and correlate them with the pathological classification of tumors, tumor stages and the presence or absence of estrogen and progesterone receptors.

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MATERIALS AND METHODS

Tissues

Human breast tumors were obtained through cooperation with surgeons and pathologists of the hospitals associated with Bowman Gray School of Medicine, Winston-Salem, North Carolina. After surgical removal, tumor tissue was immediately frozen in liquid nitrogen and stored at −80°C in an ultrafreezer. Shipment of the tissues from Winston-Salem to Chicago was made on dry ice; upon arrival tissues were stored at −80°C.

Preparation of cytosol

Cytosols were prepared by homogenizing individual tumors either in 10 mM Tris–HCl buffer (pH 7.4), containing 1.5 mM EDTA, 10 mM monothioglycerol and 10% glycerol for estradiol and progesterone-binding assays, or in 50 mM Tris–HCl buffer (pH 7.0 at 23°C) for retinoic acid-binding proteins. The homogenates were centrifuged for 30 min at 0–4°C at 40,000 r.p.m. in a Beckman Ti50 rotor. The supernatant fraction thus obtained was used for the receptor analysis.

Receptor analyses

Aliquots of cytosols were incubated with 5 nM [^{3}H]-estradiol (New England Nuclear, Boston, MA) either alone or with a 100-fold excess of unlabeled diethylstilbestrol (Steraloids, Pawling, NY) at 0°C for 4–5 hr. For progesterone receptor analyses, the reactions were carried out in presence of 5 nM [^{3}H]-R5020 (17, 21-dimethyl-19-nor-pregna-4, 9-diene-3,20-dione, from New England Nuclear, Boston, MA) either alone or in the presence of a 100-fold excess of unlabeled R5020 for 4–5 hr at 0°C. After the incubation period, bound and unbound steroids were separated by the dextran-coated charcoal procedure as described previously [19, 20]. Quantitative determinations of steroid receptor content were made by computer analysis of the results [21]. For the retinoid receptors, aliquots of the cytosols were incubated with 1 μM all-*trans*-[^{3}H]-retinoic acid (sp. act. 1.23 Ci/mmol) either alone or in the presence of a 25-fold excess of unlabeled retinoic acid in the dark at 0°C for 16 hr. Bound and unbound retinoic acid were separated by using the dextran-coated charcoal procedure [22].

Sucrose density gradient centrifugation

Labeled steroid–receptor complexes were layered on preformed 10–40% (w/v) sucrose gradients prepared in Tris–EDTA buffer. Gradients were centrifuged at 56,000 r.p.m. for 16 hr in a Beckman SW 60.1 rotor at 0–4°C. Retinoic acid-binding proteins were separated on 5–20% (w/v) sucrose gradients by centrifugation at 65,000 r.p.m. for 2 hr in a Sorvall TV-865 vertical tube rotor at 0°C. Gradients were fractionated by collecting either 8-drop (for steroid receptors) or 10-drop fractions (for cRABP) from the bottom of the tube.

Miscellaneous procedures

Protein concentration in the cytosol was estimated by the use of u.v. spectrophotometric analysis as described by Waddell [23] and modified recently by Kute *et al.* [21]. Radioactivity in each fraction was measured by the addition of 4 ml liquid scintillation fluor (4 g Omnifluor from New England Nuclear, Boston, MA, in 700 ml toluene and 300 ml Triton X-100) followed by counting on a liquid scintillation spectrometer (Tracor Analytical Co., Mark III).

RESULTS

The presence or absence of specific 8S estrogen-binding protein is generally used as a criterion for the designation of estradiol receptor-positive (ER^+) or negative (ER^-) tumors respectively. A similar determination is made for progesterone receptors (PR) using [^{3}H]-R5020 as a ligand; tumors are designated as PR^+ or PR^-. Typical profiles for ER^+ and PR^+ are shown in Fig. 1. Unlabeled diethylstilbesterol at 100-fold excess concentration completely displaced the [^{3}H]-estradiol from the binding site, while unlabeled R5020 effectively competed for the progestin binding sites. Tumors with 10 fmol/mg cytosol protein or less of ER or PR in the 8S region were considered steroid receptor negative.

Human breast cancer extracts bound [^{3}H]-retinoic acid specifically and the retinoic acid-binding protein complexes sedimented as 2S components. The specific binding under the 2S peak ranged from 0.4 pmol to 14 pmol per mg cytosol protein. Tumors containing less than 3 pmol of cRABP per mg protein were designated arbitrarily as *low* retinoic acid binders. Typical profiles for the tumors containing *low* cRABP and *high* cRABP are shown in Fig. 2. Of 31 tumors examined, 19 had greater than 3 pmol of retinoic acid-binding protein per mg cytosol protein, while the remaining 12 exhibited less than 3 pmol.

Measurements of cRABP were made on metastatic as well as primary lesions. The

metastases studied were to the chest wall, lymph nodes and skin. Approximately 60% of both primary (15/24) and metastatic (4/7) tumors were found to contain *high* cRABP.

Since the clinical significance of the presence or absence of steroid receptors is well established, a comparison was made between the levels of steroid receptors and cRABP.

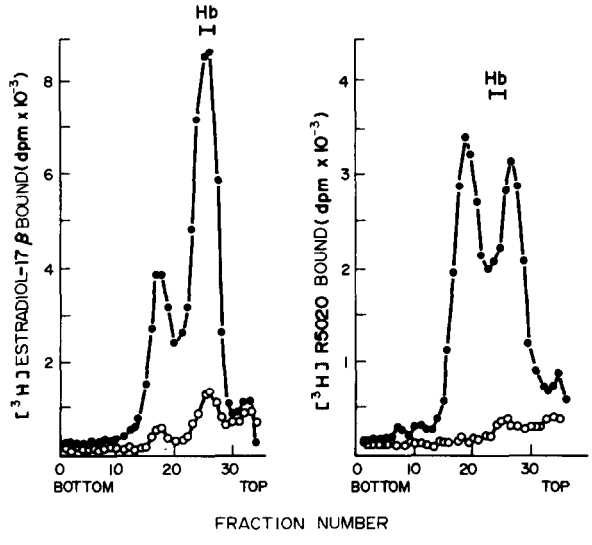

Fig. 1. Sucrose density gradient analysis of steroid receptors in human breast cancer. Cytosol from a human breast carcinoma was reacted either with [³H]*-estradiol (left) or with* [³H]*-R5020 (right) for 4 hr at 0°C in the presence (○) and absence (●) of a 100-fold excess competitor. Unlabeled diethylstilbestrol was used for the measurements of estrogen receptors, while R5020 was used for progesterone receptors. Each reaction was treated with a pellet of dextran-coated charcoal prior to separation by sucrose gradients.*

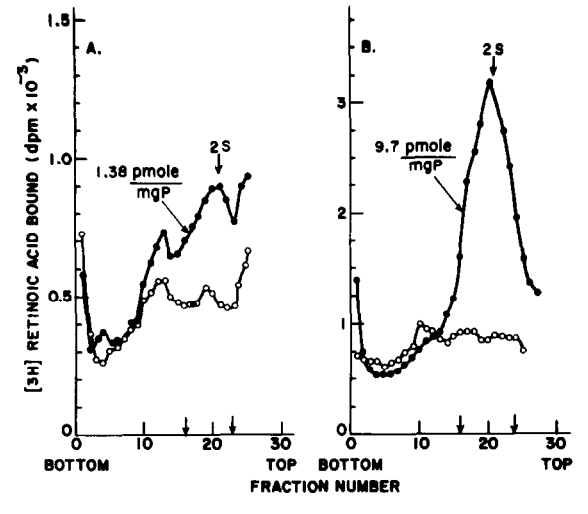

Fig. 2. Sucrose density gradient analysis of retinoic acid binding proteins in the cytosol of human breast cancer. Cytosols of tumors containing low (A) and high (B) levels of cRABP were reacted with [³H]*-retinoic acid (1 μM) either alone (●) or in the presence of unlabeled retinoic acid (25 μM) (○) for 16 hr at 0°C in the dark. Each reaction was treated with a dextran-coated charcoal pellet prior to separation by sucrose gradients. Arrows indicate the area used for quantitation of the 2S peak.*

Mean levels of cRABP were uniformly distributed among ER^+, ER^-, PR^+ or PR^- tumors. No significant difference was observed between cRABP levels of ER^+PR^+ tumors compared to ER^-PR^- (Table 1). Numerical comparison between ER or PR and cRABP resulted in correlation coefficients of 0.24 and 0.22 respectively.

There was an increased level of cRABP observed in histologically well-differentiated tumors. Poorly differentiated tumors had significantly less specific binding (ranging from 0.5 to 2.2 pmol/mg protein) than did well-differentiated tumors (range: 4.57–14.2 pmol/mg protein); moderately differentiated lesions exhibited intermediate values (Table 2).

DISCUSSION

Previous studies have provided evidence for the effectiveness of retinoids in suppression of chemically-induced mammary carcinogenesis of rat [4–7]. Moreover, recent studies from our laboratory have indicated that the retinoid in combination with ovariectomy was highly effective in inhibiting mammary tumor recurrence when animals were ovariectomized and fed diet supplemented with retinoid following the removal of the first palpable mammary tumor from a carcinogen-treated rat [8]. Thus, retinoid treatment for the prevention of recurrence of some human cancers may be a rational therapeutic modality. Since the presence or absence of steroid receptors is successfully used to determine the therapeutic strategy employed in the attempt to prevent the recurrence of human breast cancers, a similar approach was taken to measure cRABP in human breast cancer and to correlate that with other parameters.

Huber *et al.* [9] originally reported the presence of cRABP in 60% of primary breast carcinomas and proliferative dysplasias; a more recent report by the same investigators found cRABP in all the breast tissues examined. This difference in results was ascribed to the use of more sensitive techniques in the later study. The trend, however, in both studies was similar; simple dysplasias contained nondetectable, or detectable but very low, levels of cRABP, while cancers showed relatively increased levels of cRABP. It appears from these studies as well as our results, in which we detected cRABP in all the tumors, that the utilization of an arbitrary cut-off limit for distinguishing between *high* cRABP and *low*

Table 1. Comparison between steroid and retinoid receptors in human breast cancers

Steroid receptor status*	Number of tumors analyzed	cRABP† (pmol/mg protein)
ER^+	17	5.3 ± 0.9
ER^-	14	4.0 ± 1.1
PR^+	16	5.4 ± 0.9
PR^-	15	4.0 ± 1.1
ER^+PR^+	15	5.5 ± 0.9‡
ER^-PR^-	13	4.3 ± 1.2‡

*The levels of estradiol (E) and progesterone (P) receptors (R) were measured using sucrose density gradient analysis; less than 10 fmol/mg protein of ER and PR were considered negative.
†Mean cRABP ± standard error. Retinoic acid-binding protein (RABP) were measured using sucrose density gradient analysis. Area under the 2S region was calculated, specific binding was determined as a difference between the total and nonspecific binding in the 2S region.
‡These values are not significantly different, $P < 0.125$.

Table 2. Distribution of cRABP and pathology of breast tumors

Histologic classification of carcinomas	Number of* tumors	cRABP (pmol/mg protein)
Poorly differentiated	6	1.55 ± 0.59 (*a*)
Moderately differentiated	15	3.89 ± 0.64 (*b*)
Well-differentiated	6	8.0 ± 1.7 (*c*)

*Pathological classification was available only for 27/31 tumors.
a is significantly different from *b*, $P < 0.05$.
a is significantly different from *c*, $P < 0.01$
b is significantly different from *c*, $P < 0.01$.

cRABP is necessary and could be useful in the interpretation of the results. Such an arbitrary 'cut-off' level is currently used for quantitation of steroid receptors [16–19]. In this study we have selected 3 pmol/mg protein as an arbitrary number to distinguish *high* cRABP from *low* cRABP. Using this criterion, we observed that 61% of the primary carcinomas could be designated as *high* cRABP.

A correlation between the *high* and *low* cRABP and the degree of differentiation has been reported previously. For example, relatively low levels of cRABP in simple ductal dysplasia with fibrosis have been compared with the elevated levels of cRABP found in proliferative dysplasia and carcinomas [9]. Consistent with these results, the present study shows that poorly differentiated human mammary tumors contained lower levels of cRABP than did the well-differentiated tumors.

Although both ovariectomy and dietary supplementation of retinoid are effective inhibitors of mammary carcinogenesis of rat, recent results have shown that combination of ovariectomy and retinoid supplementation enhanced the chemopreventive effect [24]. Additionally, ovarian hormone-independent tumors contained increased levels of cRABP, and the occurrence of these tumors was selectively affected as a result of retinoid treatment [25]. A comparison, therefore, was made between the levels of retinoic acid-binding proteins and steroid receptors to investigate whether the presence of cRABP can be used as a biochemical marker for ovarian hormone-dependence of human breast cancer. It would be of considerable importance if a significant difference in the levels of cRABP could be detected between ER^+PR^+ and ER^-PR^- human breast cancers. The results, however, indicated that there was no significant correlation between steroid receptors and retinoid receptors. A possible reason for such a lack of correlation may be heterogeneity of the cell population of human breast cancers; a tumor containing both estrogen and progesterone receptors may also contain a significant number of ovarian hormone-independent (ER^-PR^-) cells. The failure

of approximately 40% of steroid receptor-positive human tumors to respond to endocrine therapy could result from such heterogeneity of cell populations. Although the results presented in this report indicate that presence or absence of cRABP may not serve as a possible biochemical marker for ovarian hormone-dependence of human breast cancer, it remains to be determined whether homogeneous populations of ovarian hormone-independent breast cancer cells contain significantly altered levels of cRABP compared to ovarian hormone dependent ER^+PR^+ breast cancer cells.

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