Quantitative Estimation of Cellular Retinoic Acid-binding Protein Activity in Normal, Dysplastic, and Neoplastic Human Breast Tissue

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ABSTRACT

A technique for reproducible and quantitative determination of human cellular retinoic acid-binding protein (CRABP) activity in breast tissue specimens is described. A multiphasic polyacrylamide disc gel electrophoresis system (operative at pH 10.2) was adapted for this purpose. This technique allows, after incubation with tritiated retinoic acid (RA) overnight, the separation of the specific CRABP activity from the nonspecific serum-originated binding activity and from the free RA. Previous purification of the tissue cytosols is therefore not necessary. The same assay method was also used for the determination of the molecular weight (Ferguson plot, m.w. 13,000) and the dissociation constant $K_d$ ($2.5 \times 10^{-7}$ M) of mammary CRABP. The activity in tissue cytosol, stored at $-70^\circ$, was found to be stable for at least 3 months. Results from 88 breast tissue specimens of different pathological degree are presented. CRABP activity was found in all tissue categories with progressively increasing amounts from normal tissue to breast cancer. The activity in the cancer tissues ($14.85 \pm 12.05$ pmoles RA bound per mg soluble protein; $N = 27$) was significantly different ($p < 0.001$) from the activity determined in tissue with simple dysplasia without epithelial proliferation [$4.3 \pm 2.2$ (S.D.) pmoles RA bound per mg protein; $N = 30$]. It is possible that in the cases where high amounts of CRABP activity are found in dysplastic and preneoplastic tissue, a high risk for breast cancer development exists. Therefore, CRABP is tentatively proposed as a dedifferentiation and/or proliferation marker.

INTRODUCTION

There is considerable evidence that in certain forms of dysplasias there exist risk factors for breast cancer (25). Whereas the histological examination of biopsies today provides conclusive information with respect to the diagnosis, the prognostic significance for the individual case is still uncertain. Therefore, it is necessary to find additional criteria not only to detect early breast cancer but also to discover the high-risk patients with established dysplasia. It is possible that biochemical parameters measured in mammary biopsy material (75% of which are different forms of mastopathias) can provide more information of prognostic value (4, 9).

The finding by Ong and Chytil that the specific CRABP activity increased during the perinatal development of rat tissues (14) and occurs in human breast and lung cancer tissue (16) raised the idea that CRABP is a tumor-associated protein and may therefore be useful as a marker for proliferation and/or differentiation processes. Our earlier study on human breast tissue (5) provided considerable evidence for the utility of CRABP measurement. Inasmuch as the method used at that time gave only qualitative results, a quantitative assay using PAGE was developed to measure the CRABP concentrations in individual small breast tissue specimens.

MATERIALS AND METHODS

Chemicals. $N,N^\prime$-Methylenebisacrylamide and acrylamide (twice crystallized) were obtained from Serva (Heidelberg, Germany). All-trans-11,12-$^3$H]RA (35.7 Ci/mmol; dissolved in toluol) was a generous gift from Dr. Liebman, Hoffman-La Roche Inc. (Nutley, N. J.). The grade of tracer purity was routinely checked on Merck Kieselgel 60 F$_{254}$ using cyclohexane:ethyl acetate (3:2) as a solvent system. Unlabeled RA (crystalline) was a gift from Hoffmann-La Roche (Basel) and was not further purified. All other reagents were analytical or reagent grade and were purchased mainly from Merck (Darmstadt, Germany).

Preparation of Human Tissue Cytosol. Human breast tissue was obtained from biopsies and mastectomies. After macroscopic inspection by the pathologist, one part was processed for histological examination (primary diagnosis). An additional histological preparation (cryotome section) was prepared from a representative portion of the other part in our laboratory. The rest of the tissue was freed of excess fat and kept frozen at $-20^\circ$ until pulverization in liquid nitrogen. The powdered tissue was kept at $-70^\circ$. One part of tissue powder was homogenized with bovine serum albumin as standard.

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CRABP Assay with PAGE. [$^3$H]RA was prepared for incubation by blowing off the solvent under argon and replacing it by DMSO. A specific activity of 5 to 10 Ci/mmol was obtained by dilution with an appropriate solution of unlabeled RA in DMSO. Incubations of the thawed cytosols were carried out on ice in conical 1.5-ml polypropylene microtubes (Sarstedt, Sevlen SG, Switzerland). Ligand solution ($10 \mu$l) was added with disposable glass micropipets (Brand end-to-end 1-µl pipets; Brand, Wertheim, Germany) to 100 µl of tissue cytosol. After

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complete mixing, the probes were stored at 4° for 16 hr. Total labeled ligand concentrations varied between 2 and 3 \times 10^{-7} \text{ M}. In competition experiments, a 100- to 200-fold excess of unlabeled RA was added in an additional 1.0 \mu\text{L} of DMSO.

PAGE was performed in multiphasic zone Electrophoresis Buffer System B (19) consisting of Tris:chloride:phosphate buffer operative at pH 10.2. Polyacrylamide resolving gels (lower gels) were prepared 2% cross-linked with \text{N,N'}-methylenebisacrylamide at a total gel concentration of 7% \% of total gel concentration \(T = \text{acrylamide (g)} + \text{cross-linking agent (C) (g/100 ml)}\). Concentration gels (upper gels) were prepared using 8% \(T\) and 20% \(C\). The gels were prepared in 14-cm glass tubes (inner diameter, 0.5 cm) with lower gels 6 cm long and upper gels 1 cm long. Procedures of polymerization were those described by Chrambach et al. (1). Total volumes or aliquots of the incubated cytosols were loaded on the concentration gels. Bromophenol blue solution (8 to 10 \mu\text{L}; 0.1 mg/ml) in 50 mm Tris-HCl buffer, pH 7.4, containing 5% sucrose was layered on top followed by the upper buffer. The gels were run in a jacketed gel electrophoresis cell (12 cm) at a constant current of 1.0 ma/gel for 2.5 hr (separation length, 38 to 42 mm).

After electrophoresis, the gels were immediately removed from the glass tubes and frozen on dry ice. The frozen gels were cut into 1-mm sections on a Mickle gel-slicing apparatus (The Mickle Laboratory Engineering Co., Gomshall, Surrey, England). The gel sections were then extracted at 4° overnight in plastic scintillation vials containing 1.5 ml of distilled water.

The radioactivity in the peak fractions was calculated after subtraction of the nonspecific binding. The values are expressed as pmol specifically bound RA per mg soluble tissue protein according to the following expression:

\[
\text{pmol/mg} = \frac{\text{cpm/peak area} \times 4.505 \times 10^3}{E \times \text{S.A.} \times V \times \text{mg protein/mL} \times (100 - (D/h \times 0))}
\]

where \(E\) is \(^3\text{H}\) counting efficiency of scintillation spectrometers, S.A. is specific activity of retinoic acid (Ci/mmol), \(V\) is sample volume (\muL), \(D/h\) is dissociation rate per hr in \% (depends on RA concentration), and \(t\) is running time of electrophoresis. The factor 4.505 is derived from the specific activity, expressed in Ci/mmol. The calculation was based on the assumption that only intact RA is specifically bound to CRABP in a molar ratio of 1:1 (23).

**Estimation of the Molecular Weight of Human Mammary CRABP.** Human mammary cytosol was subjected to PAGE at different gel concentrations ranging from 5 to 10% total gel concentration \(T\). The gels (start-to-front lengths, 6 cm) were analyzed as described, and the total gel concentrations plotted against the corresponding log \(R_f\) values of the specific activity peak. The \(R_f\) values were determined from the ratio between the slice number at maximal activity and the slice number of the front, indicated by the tracking dye bromophenol blue and the radioactivity peak of free \[^3\text{H}]\text{RA}.\) Ferguson plots were constructed and evaluated in terms of their slopes (retardation coefficients, \(K_r\)) as described (19, 20). Molecular weight of CRABP was derived from \(K_r\), using data on standard globular proteins (3).

**Saturation Experiments.** Aliquots (100 \muL) of a cytosol pool from carcinoma biopsies were incubated as described with \[^3\text{H}]\text{RA}\) in concentrations ranging from \(5 \times 10^{-8}\) to \(1 \times 10^{-6}\) \text{M}. After incubation, 80 \muL of each solution were subjected to gel electrophoresis, whereas 10 \muL were counted directly to determine the respective total RA concentrations. The specific binding activity was calculated and then subtracted from the total activity to give the free ligand concentrations which were then corrected for tracer purity.

**RESULTS**

**Separation of Binding Proteins for RA in Human Mammary Cytosol by PAGE.** A typical result is shown in Chart 1. Unbound RA moved with the front, marked by bromophenol blue (\(R_f = 1.0\)). Human breast tissue cytosol incubated with \[^3\text{H}]\text{RA}\) revealed 2 peaks of radioactivity bound to proteins, one at \(R_f\) 0.70 to 0.75 and another, usually much smaller, at \(R_f\) 0.48 to 0.57. After simultaneous incubation of the cytosol with \[^3\text{H}]\text{RA}\) and a 200-fold excess of nonlabeled RA, the second peak is completely abolished, indicating that this peak represents the specific binding protein for RA (CRABP). A 100-fold excess of vitamin A (retinol) did not compete for this RA-binding site. No radioactivity, except a low background, was found in the concentrating gel.

In control experiments, serum, plasma, and erythrocyte lysates were also analyzed. The respective supernatants were diluted with cytosol buffer to give 3.5 to 5 mg of soluble protein per ml. One hundred \muL of each were incubated as above, and aliquots with 300 \mug of protein were run on gels as described. Besides the albumin peak (\(R_f\) 0.70), serum and plasma contained a binding constituent at \(R_f\) 0.56 inhibited by excess unlabeled RA. The peak fractions contained radioactivities between 2- and 3-fold above background counts. In a few samples, most probably due to contamination with high concentrations of serum proteins, this peak at RF 0.5 which could not be inhibited by excess RA. This

![Chart 1. Typical profile of radioactivity in polyacrylamide gel fractions after electrophoretic separation of binding components of RA in human breast cytosol without (■) and with a 200-fold excess on nonlabeled RA (○). The distribution of RA after the run without protein is also shown (▲). Specific binding is running with an \(R_f\) of 0.54. Total acrylamide concentrations of the gels were 7%](image-url)
binding peak does not substantially interfere with the analysis of CRABP from human mammary tissue.

Estimation of Molecular Weight of CRABP by PAGE (Ferguson Plot). Experiments were performed as described. An apparent molecular weight of 13,000 was found (data not shown).

Assay Linearity and Reproducibility. Increasing amounts of incubated mammary tissue cytosols were subjected to electrophoresis. The peak areas increased proportionally to the cytosol volumes analyzed (25 to 150 µl). Cytosols were also diluted with cytosol buffer (down to 1:4) and then incubated with $5 \times 10^{-7}$ M $[^{3}H]$RA. The resulting radioactivity in the peak fractions was linearly decreasing with the dilution factors. Results of duplicate CRABP analysis in cytosols were always in the 5% variability range.

Stability of CRABP in Cytosol Preparations. CRABP activity in cytosol stored at $-20^\circ$ was stable for more than 3 months. Results were in the 10% variability range. Cytosol stored at 2 to 4$^\circ$ showed no differences in CRABP activity at least over 2 weeks. When the cytosol was incubated at $25^\circ$ overnight, a slightly increased binding activity could be measured. Heating of the cytosol at $60^\circ$ for 1 hr destroyed the specific binding site completely.

Saturation Experiments: Estimation of the Dissociation Constant $K_d$. Results of a representative experiment are shown in Chart 2. Dissociation of the ligand from CRABP during the electrophoresis was observed. The dissociation rate per hr was high at low ligand concentrations (14% at $5 \times 10^{-8}$ M free $[^{3}H]$RA) and decreased nonlinearly with increasing ligand concentrations (see Chart 2, inset). Therefore, an appropriate correction for specifically bound RA before electrophoresis was carried out resulting in a corrected binding curve and its parameters. Tracer purity was 86%. The apparent $K_d$ estimated with this method was $2.2$ to $2.7 \times 10^{-7}$ M.

CRABP Levels in Normal and Pathologically Altered Human Breast Tissue. Cytosols of human mammary tissues were analyzed for CRABP content. The values determined as described above were additionally corrected for maximal binding according to the saturation curve (Chart 2). The final data are shown in Chart 3. Except for the values of the MIII group, only data of tissues are presented where both diagnoses were in accord.

The average CRABP concentration per mg soluble protein in carcinomas was found to be significantly different ($p < 0.001$) from the groups MIF and MI. In fibroadenomas, which were included in this study because they represent a benign breast tumor type, considerably lower values than in carcinomas were observed. The great majority of the dysplasias with intraductal proliferation of epithelial cells (MII, regarded as possible preneoplasia) also showed relatively low levels of CRABP.

The data on CRABP activity presented in Chart 3 are the relative amounts per mg of soluble protein. It is conceivable, however, that CRABP activity increases from normal tissue to carcinomas due to the increasing cell density (4). Therefore, the cellular densities of the cryotome sections were estimated and compared with the data of CRABP concentrations by Spearman’s rank correlation test. The results (Table 1) show a significant dependence of CRABP-binding activity on cell density in tissue with simple dysplasia (MIF and MI groups), whereas for the carcinoma group no such correlation could be found.

Where data were available, the estrogen receptor concentrations of cancer tissue specimens (determined in the same cytosol in our laboratory) were compared with the respective CRABP content. No correlation between these 2 parameters could be found.

![](image)

Chart 2. Saturation of CRABP from human breast tissue by RA. Aliquots of a cytosol pool were incubated with increasing concentrations of RA, and the specific binding activity was analyzed as described. The results were corrected for zero dissociation based on the data of separate experiments. Inset, dissociation rate per hr of electrophoresis related to the concentration of free RA after incubation. The parameters of the saturation curve were estimated with the help of computerized least-square determination. The horizontal asymptote (---) indicates full saturation of CRABP ($8.7 \times 10^{-8}$ M RA specifically bound). The $K_d$ estimated is $2.72 \times 10^{-7}$ M (arrow).

![](image)

Chart 3. Binding activity for RA in normal and pathologically altered human mammary tissue. The age of the patients was between 18 and 75 years. Histological staging is from Ref. 24. bars, means. Carcinoma (CA) group was significantly different from the combined data of MIF plus MI ($p < 0.001$), determined by a nonparametrical test (10). MIF tissue was regarded as normal tissue.
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Table 1
Correlation between the relative CRABP binding activity per mg protein and the cellular density in normal, dysplastic, and neoplastic human mammary tissue

<table>
<thead>
<tr>
<th>CRABP activity pmol RA bound/mg protein</th>
<th>Cellular density</th>
<th>Spearman’s rank correlation coefficient</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1 2.52 ± 1.00</td>
<td>1.17 ± 0.41</td>
<td>6</td>
<td>0.403 p &lt; 0.025</td>
</tr>
<tr>
<td>MI 4.75 ± 2.2</td>
<td>2.42 ± 1.72</td>
<td>24</td>
<td>0.179 NS</td>
</tr>
<tr>
<td>MI 5.83 ± 3.93</td>
<td>3.76 ± 1.25</td>
<td>17</td>
<td>0.179 NS</td>
</tr>
<tr>
<td>CA 14.85 ± 12.05</td>
<td>6.67 ± 2.02</td>
<td>27</td>
<td>−0.019 NS</td>
</tr>
</tbody>
</table>

a Mean ± S.D.
b NS, not significant.

DISCUSSION

This study presents results on the quantitative estimation of CRABP in normal and pathologically altered human breast tissue. Besides the higher sensitivity, the possibility of quantification of the CRABP content is another important feature of the PAGE method described here. The methods used to date to examine for presence of CRABP were sucrose density gradient centrifugation (14) and agarose gel electrophoresis (5). We tried several other methods to separate the specific CRABP from the serum retinol-binding protein (m.w. 20,000) (2).

CRABP analysis on PAGE System B can successfully be used also for cytosols of human uterus and the human mammary breast cell lines BT-20 and MCF-7. CRABP of rat origin cannot be measured with this method in crude cytosol because specific and unspecific binding are not separated.4

The Kd estimated (2.2 to 2.7 × 10−7 M for RA; Chart 2) differs from the data for CRABP of rat and root origin published by other workers (7, 15, 27). It is possible that the methods used for the determination of free and specifically bound RA are influenced by different adsorption of RA to the surfaces of the reaction vessels. In addition, tissue specificity and purification grade of CRABP may be important.

In contrast to our earlier study (5) where we used a much less sensitive method, CRABP was now found in all categories of mammary tissues studied. The mean value of CRABP binding activity increased progressively from MIF (regarded as normal tissue) to MI, MI, and the possibly precancerous stage MII (lobular carcinoma in situ) (25) to the carcinomas. As the cell densities also increase going from the stage MIF to carcinoma (4), it was of importance to show whether a correlation between these parameters can be found. Our results (Table 1) suggest the existence of such a correlation for tissues with simple dysplasia (MIF and MI), whereas this cannot be claimed for tissue classified MII or carcinoma. This finding indicates at best a partial dependence of the CRABP concentration on cell number in the stage MI and neoplastic tissue. The presence of elevated amounts of CRABP in MI and MII as well as in carcinoma tissue may then be associated with the transformation process. The large differences within the tissue groups are probably due to the highly complex morphological patterns (18).

The discrepancies between the results presented here and those published formerly (5) can be explained at least partially by differences in the sensitivity of the assays. Since the PAGE assay is more sensitive, it is conceivable that most if not all of the samples analyzed with agarose gel electrophoresis also contained CRABP which was then not detectable in MIF and MI tissue and found only in 43 and 52% of MII and carcinoma tissue, respectively. Our new results show that CRABP is a normal constituent of human mammary tissue, tending toward a higher concentration in pathologically altered proliferation and/or differentiation state. It is clear, however, that CRABP cannot be regarded as a highly specific marker for cancer in the strict sense, fulfilling rigorous criteria (28), since it has been known for a long time that CRABP occurs in normal human tissue (2, 17).

The functions of CRABP (as well as of the cellular retinol-binding protein) are not yet known. It is possible that CRABP serves mainly as an intracellular storage and transport protein. It has been shown, however, that in certain experimental systems the CRABP:RA complex potentially can enter the nucleus (22, 27). Therefore, it may be that CRABP mediates the effect of RA and the retinoids at a nuclear target analogous to the steroid receptors (2). There is evidence that the antitumor activity of retinoids is linked to the occurrence of CRABP in tumors because of some correlation of CRABP occurrence with the sensitivity of these tumors to retinoids (2) and with the potency of retinoids for stimulation of differentiation (7). In addition, the affinities of the retinoids for CRABP parallel the therapeutic activities on chemically induced epithelial tumors (26).

In spite of the many attempts to elucidate the specific actions of retinoids on growth and differentiation of epithelial tissue (12), it has not yet been possible to unequivocally demonstrate the underlying molecular mechanism. However, new aspects of RA actions on the biochemical level have been detected (6, 8, 13).

The data on CRABP activity in 88 human mammary tissue specimens presented here suggest that this parameter may be a useful marker for the detection of high-risk cases for breast cancer. To establish the prognostic value, this study is being continued and a future retrospective study is planned.

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4 W. M. Küng, E. Geyer, and P. R. Huber, unpublished work.

curve parameters. For reading the manuscript and helpful comments, we thank K. W. Talmadge.

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