Cellular Binding Proteins for Vitamin A in Colorectal Adenocarcinoma of Rat

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ABSTRACT

Rat colorectal mucosa was examined during the course of carcinogenesis, induced by chronic administration of 1,2-dimethylhydrazine (DMH), for the presence and amount of cellular retinol-binding protein (CRBP) and cellular retinoic acid-binding protein. These two binding proteins are implicated in the action of vitamin A in normal and neoplastic tissue. Induced adenocarcinomas were found to contain low levels of cellular retinoic acid-binding protein (10 pmol/g), similar to the levels found in adjacent mucosa of the same animal and also in colorectal mucosa from normal rats or rats chronically treated with DMH. However, the adenocarcinomas had high levels of CRBP (300 to 500 pmol/g), and these levels were dramatically higher than levels of CRBP in adjacent mucosa of the same animal (40 to 100 pmol/g), colorectal mucosa from normal rats (20 pmol/g), or colorectal mucosa from rats chronically treated with DMH (22 to 25 pmol/g). Consequently, the increase in CRBP occurred only with tumor appearance and not with the general hyperplasia of the crypts caused by DMH administration. The CRBP of the tumor was associated with endogenous retinol (77 to 100% saturation) and was similar to, if not identical with, CRBP of normal tissue, as judged by fluorescence spectra, sedimentation behavior, and elution position on Sephadex G-75.

INTRODUCTION

There is a considerable body of evidence that suggests that vitamin A and its analogs can influence the development of some epithelial tumors. In particular, vitamin A alcohol (retinol), esters and ethers of the alcohol, vitamin A acid (retinoic acid), and various synthetic analogs of the acid have shown promise as prophylactic and/or therapeutic agents against spontaneous and chemically induced tumors. These findings have been reviewed recently (23, 24). Because vitamin A is necessary for the control of proliferation and the direction of differentiation of many epithelial tissues (28, 29), its ability to act on some tumors of such tissues is perhaps not surprising.

The action of vitamin A in normal tissue may well be mediated by specific intracellular binding proteins, perhaps in a manner similar to that known for steroid hormones (2, 10). Two binding proteins have been described. The first, discovered in many rat tissues (2), binds retinol with high affinity and specificity. It is called CRBP (12). The second, also found in many rat tissues, binds retinoic acid with high affinity and specificity and is called cellular retinoic acid-binding protein (11, 14, 21). Each binding protein has been purified to homogeneity, and the homogeneous preparations have been partially characterized (15, 16).

The body of evidence that these 2 binding proteins, present in many species, mediate vitamin A action has been reviewed recently (4, 5). For example, CRBP and cellular retinoic acid-binding protein bind analogs of retinol and retinoic acid, respectively, with affinities that parallel the activity of these analogs in various test systems (3, 11, 22). It has been proposed that the binding proteins may also be mediating the reported antitumor activity of vitamin A and its analogs (3, 13, 17). Consequently, we believe that presence or absence of the binding proteins in various tumors is of interest in evaluating reported or potential effects of such compounds.

For this report we have examined the presence and properties of CRBP and cellular retinoic acid-binding protein in colorectal mucosa and adenocarcinomas during the course of carcinogenesis induced by DMH in rats (6).

MATERIALS AND METHODS

Treatment of Animals. Male Sprague-Dawley rats weighing 200 g were fed standard laboratory chow ad libitum. The rats were given weekly s.c. injections of 20 mg DMH per kg. Immediately before use DMH (Fluka AG, Buchs, Switzerland) was dissolved in 0.14 M NaCl (sterile) and neutralized.

Chemicals. All-trans-[15-3H]retinol (2.45 Ci/mmol) was purchased from New England Nuclear, Boston, Mass. All-trans-[11,12-3H]retinoic acid (11.1 Ci/mmol) was a generous gift from Hoffmann-LaRoche Inc., Nutley, N. J. The [3H]retinoic acid was purified routinely before use by partition chromatography in subdued light on Sephadex LH-20 with a solvent system of cyclohexane:chloroform (1:1) containing 1 mg butylated hydroxytoluene per ml. This system is similar to several described by Ito et al. (7).

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 cochromatographed with their respective unlabeled compounds.

Preparation of Extracts and Assay for Binding Proteins. Preparation was done at 0-4°. Large bowels were washed in 0.9% NaCl solution containing 1% neomycin (The Upjohn Co., Kalamazoo, Mich.). The mucosa was then collected by...
scraping. Tissue was homogenized with 2 to 4 volumes of 0.05 M Tris-HCl, pH 7.5, and the supernatant liquid was collected after centrifugation at 30,000 × g. The recovered supernatant liquid was adjusted to pH 5 with 1 N acetic acid, and the precipitated protein was removed by centrifugation at 30,000 × g. The solution was then adjusted to pH 7.5 with 1 N NaOH to provide the extract for testing. Samples of extracts were diluted to 0.3 ml with Tris-HCl buffer, pH 7.5, and then incubated at 4°C for 4 hr with 100 pmol [3H]retinol or 20 pmol [3H]retinoic acid. Parallel incubations contained a 100-fold excess of unlabeled retinol or retinoic acid. Immediately prior to sucrose gradient centrifugation, the incubations were treated with 0.2 ml of dextran-coated charcoal suspension to remove free ligand, as previously described (14). Aliquots (0.2 ml) of the incubation mixtures were submitted to centrifugation on linear 5 to 20% (w/v) sucrose gradients in 0.05 M Tris-HCl, pH 7.5. The binding proteins were revealed in the radioactivity profile of the fractionated gradient as peaks of radioactivity in the 25 region which were abolished in the presence of excess unlabeled ligand. The radioactivity in the 25 peak (specific binding) was used to quantitate the amount of binding protein present, assuming one binding site per molecule of binding protein.

**Gel Filtration.** Aliquots (1 to 2 ml) of the extracts of the adenocarcinomas were submitted to gel filtration on a small (1.6 × 25 cm) column of Sephadex G-75, equilibrated, and run with 0.2 M NaCl in 0.05 M Tris-HCl, pH 7.6. The elution position of CRBP had been determined previously by running an extract of testis cytosol and monitoring for the presence and amount of each binding protein by sucrose gradient centrifugation. The incubations were treated with 0.2 ml of dextran-coated charcoal suspension to remove free ligand, as previously described (14). Aliquots (0.2 ml) of the incubation mixtures were submitted to centrifugation on linear 5 to 20% (w/v) sucrose gradients in 0.05 M Tris-HCl, pH 7.5. The binding proteins were revealed in the radioactivity profile of the fractionated gradient as peaks of radioactivity in the 25 region which were abolished in the presence of excess unlabeled ligand. The radioactivity in the 25 peak (specific binding) was used to quantitate the amount of binding protein present, assuming one binding site per molecule of binding protein.

**Measurement of Retinol:CRBP Complex by Fluorescence.** After gel filtration as described above, the fluorescence of each fraction was determined with excitation at 350 nm and emission at 485 nm in an Aminco-Bowman spectrophotofluorometer equipped with a photon counter (American Instrument Co., Silver Spring, Md.) Each fraction was then exposed to a Blak-Ray Model xx-15 broad-spectrum UV lamp (Ultraviolet Products, Inc., San Gabriel, Calif.) for about 30 min at an intensity of 0.15 to 0.20 microwatts/sq cm, which was sufficient, from our experience, to destroy retinol bound to CRBP or in solution. The fluorescence was then redetermined. From the decrease in fluorescence and knowing the fluorescent yield of pure retinol:CRBP under similar conditions, the amount of retinol:CRBP complex was calculated for each fraction. This method was verified several times by labeling of CRBP with [3H]retinol prior to gel filtration and determining the amount of retinol:CRBP present in each fraction from the recovered radioactivity. The amount of retinol:CRBP determined by this method corresponded to the amount determined by fluorescence measurements.

**RESULTS**

**Induction of Carcinomas.** During the course of carcinogenesis, the large bowel showed all stages described earlier by other investigators: cell loss and inflammation 6 to 24 hr after injection (8, 25, 30); hyperplastic proliferation, lengthening of the crypts, and focal atypia, and higher vascularization during Week 4 to Week 16 (1, 19, 27); finally, foci of malignant cells in the crypts of Lieberkühn and macroscopically multiple cancers starting with Week 18 (1). The tumors are mostly adenocarcinomas of both the sessile and polypoid type, invasive but usually not metastasizing, the latter being the only pronounced difference to the human disease. As a very rare case, we found only one metastatic tumor (of >100) that invaded the peritoneum. This well-differentiated mucinous adenocarcinoma closely resembled the colorectal tumors histologically; necrosis could not be detected.

**Determination of Levels of CRBP and Cellular Retinoic Acid-binding Protein.** Extracts of tissues were examined for the presence and amount of each binding protein by sucrose gradient centrifugation after incubation with either [3H]retinol or [3H]retinoic acid. Typical assays for CRBP are shown in Chart 1. The CRBP is detected as a peak of radioactivity (bound [3H]retinol) in the 2S region of the gradient. The peak is absent in the presence of excess unlabeled retinol. As shown in the chart, the extract of the colon adenocarcinoma contained considerably more CRBP than did an extract prepared from adjacent mucosa from the same tumor-bearing animal.

Comparison of tumor tissue with other mucosa is shown in Table 1. In all cases examined the amount of CRBP in the adenocarcinomas was considerably elevated over either adjacent colorectal mucosa from the same animal or mucosa from a nontreated (normal) animal or a DMH-treated animal before the appearance of tumors. The highest amount of CRBP was observed in the metastatic adenocarcinoma found growing throughout the peritoneal cavity.

In contrast to CRBP no dramatic changes were observed in the content of cellular retinoic acid-binding protein in the samples examined; all had detectable cellular retinoic acid-binding protein. However, the amount of cellular retinoic acid-binding protein present was near or under the amount

![Chart 1. Sucrose gradient centrifugation of extracts of a DMH-induced colorectal adenocarcinoma (A, equivalent to 20 mg of tissue) and of colon mucosa from the same animal (B, equivalent to 40 mg of tissue) after incubation with [3H]retinol in the absence (●) or presence (○) of a 100-fold excess of unlabeled retinol. CRBP was revealed as the peak at the 2S position of the gradient.](chart.png)
Table 1
Quantitation of cellular retinoic acid-binding protein and CRBP in single specimens of colorectal mucosa and adenocarcinoma from representative animals during the course of carcinogenesis induced by DMH

<table>
<thead>
<tr>
<th>Tissue specimen</th>
<th>Treatment</th>
<th>Cellular retinoic acid-binding protein (pmol/g)</th>
<th>CRBP (pmol/g)</th>
<th>% saturation CRBP*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colorectal mucosa</td>
<td>None</td>
<td>&lt;100</td>
<td>20</td>
<td>ND</td>
</tr>
<tr>
<td>Colorectal mucosa</td>
<td>6 wk DMH</td>
<td>&lt;10</td>
<td>25</td>
<td>ND</td>
</tr>
<tr>
<td>Colorectal mucosa</td>
<td>19 wk DMH</td>
<td>&lt;10</td>
<td>22</td>
<td>ND</td>
</tr>
<tr>
<td>Induced adenocarcinoma</td>
<td>24 wk DMH</td>
<td>10</td>
<td>335</td>
<td>95</td>
</tr>
<tr>
<td>Adjacent mucosa (same animal)</td>
<td>24 wk DMH</td>
<td>&lt;10</td>
<td>100</td>
<td>ND</td>
</tr>
<tr>
<td>Induced adenocarcinoma</td>
<td>31 wk DMH</td>
<td>10</td>
<td>340</td>
<td>77</td>
</tr>
<tr>
<td>Adjacent mucosa (same animal)</td>
<td>31 wk DMH</td>
<td>10</td>
<td>43</td>
<td>ND</td>
</tr>
<tr>
<td>Metastatic adenocarcinoma found growing i.p.</td>
<td>28 wk DMH</td>
<td>10</td>
<td>540</td>
<td>100</td>
</tr>
</tbody>
</table>

* Saturation of CRBP with endogenous retinol.
* ‹<10, binding protein present at a level less than 10 pmol/g and too low for accurate quantitation by the assay method used.
* ND, not determined.

required for quantitation with the assay system used, and it is possible that small but significant increases in cellular retinoic acid-binding protein content occurred in the tumor tissue.

**Saturation of Cellular Retinol-binding Protein with Endogenous Retinol.** It was of interest to determine whether endogenous retinol was bound to CRBP. This was evaluated by submitting aliquots of extracts to gel filtration on Sephadex G-75 (Chart 2) and monitoring for the characteristic fluorescence of retinol bound to CRBP. As can be seen, a prominent fluorescent peak was observed at the elution position expected for CRBP. The fluorescent peak at the void volume is not retinol-like and is due to the high protein concentration in these fractions. This was established by examining fluorescence excitation and emission spectra and by exposing a sample from each peak to broad-spectrum UV for 30 min, which degrades retinol. As shown in Chart 2, the void volume fluorescence was stable to this treatment, while the fluorescence in the CRBP peak decreased. From this loss of fluorescence and knowledge of the fluorescence value for pure retinol:CRBP, it was possible to calculate the amount of retinol:CRBP in the extract. Coupled with the sucrose gradient assay for total CRBP, the degree of saturation of binding protein was calculated (Table 1). Only the adenocarcinomas had sufficient levels of CRBP to carry out this determination, and all showed a high degree of saturation of CRBP with endogenous retinol. The percentage of saturation of CRBP with endogenous retinol in normal tissues, such as lung and testis, varied from 50 to 70% (D. E. Ong, unpublished data).

**Fluorescence Spectra of CRBP of the Adenocarcinoma.** The fluorescence excitation and emission spectra were determined for CRBP of the adenocarcinomas (Chart 3) after passage over the Sephadex G-75 column. The spectra obtained were quite similar to those determined for pure retinol:CRBP complex (15). Again, after exposure to broad-spectrum UV, the characteristic spectra were lost, as would be expected.

**DISCUSSION**

Previously reported examples of changes in binding protein content with spontaneous or chemically induced tumors have shown dramatic increases in the level of cellular retinoic acid-binding protein in tumor versus normal tissue (3, 17). In contrast, in our examination of colon adenocarcinomas induced by DMH we did not find significant increases in cellular retinoic acid-binding protein when compared to adjacent, apparently normal mucosal tissue or when compared to colorectal mucosa from untreated animals. However, when the same tissues were assayed for CRBP, the adenocarcinomas contained much higher amounts than did these other tissues. When mucosa was
excitation spectrum. For example, CRBP-bound retinol has an excitation maximum at 350 nm compared with 325 nm. The endogenous retinol:CRBP complexes from the adenocarcinomas examined during the course of treatment with DMH but before the appearance of tumors, no increase in CRBP levels over normal (untreated) mucosa was observed. Somewhat higher levels of CRBP were observed in apparently normal mucosa from tumor-bearing animals. This may be due to the presence of foci of tumors too small to be determined macroscopically. In general, the results suggest that the rise in CRBP is not a gradual process during DMH treatment and does not precede the appearance of the tumor but occurs only in the tumor tissue. In particular, no increase occurred in association with the general hyperplasia of the crypts caused by DMH administration.

In normal tissue CRBP carries endogenous retinol (11, 18). If the animal is made deficient in vitamin A, the binding protein is depleted of the retinol although the level of the binding protein does not change (18). This implies that the active form in normal tissue is the retinol:CRBP complex rather than CRBP alone. Several investigators have reported that the retinol content of DMH-induced colon adenocarcinomas (9) or transplanted jejunal adenocarcinoma (26) was low when compared with normal mucosal tissue. This raised the possibility that the tumor tissue may not have sufficient retinol to maintain appropriate levels of the presumed active form retinol:CRBP. However, we found that the CRBP recovered from the induced adenocarcinomas was almost fully saturated with endogenous retinol and comparable, if not higher in saturation, to CRBP in normal tissues.

When retinol binds to CRBP, the protein imposes constraints on the conformation of the retinol which leads to a considerably altered absorption spectrum and fluorescence excitation spectrum. For example, CRBP-bound retinol has an excitation maximum at 350 nm compared with 325 nm for retinol in ethanol solution (15, 18). The endogenous retinol:CRBP complexes from the adenocarcinomas examined here showed this same spectrum as reported for purified CRBP with all-trans-retinol (15). This would suggest that CRBP from the adenocarcinomas is quite similar to, if not identical with, CRBP from normal tissue and capable of normal function.

Rogers et al. (20) found that high levels of retinyl palmitate in the diet (sufficient to raise serum levels of vitamin A and inhibit growth of the animal) provided little or no protective effect against DMH induction of colon adenocarcinoma compared with normally nourished rats. If protective effects, found in other systems for high doses of retinol or retinyl esters, are mediated by CRBP, the lack of such protection in this system may be due to the fact that the CRBP in these tumors is already nearly saturated with retinol in the normally nourished animal. Consequently, the addition of pharmacological amounts of retinol or its esters to the diet would not significantly affect the degree of saturation of CRBP with retinol. Of course, this point applies only if the antitumor effects are mediated by CRBP.

This does not address the possibility that retinoic acid or its analogs might show some protective effect. Newberne and Suphakarn (9) have presented preliminary evidence that dietary supplementation with 13-cis-retinoic acid reduced the incidence of DMH-induced colon tumors in rat. Since cellular retinoic acid-binding protein was found to be present in the DMH-induced adenocarcinomas of this study, it may be mediating the antitumor effect of the 13-cis-retinoic acid, known to bind well to cellular retinoic acid-binding protein (5).

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