Presence of Cellular Retinol and Retinoic Acid-Binding Proteins in Epidermoid Carcinoma of the Oral Cavity and Oropharynx

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Epidermoid carcinomas of the oral cavity and oropharynx from six patients were examined for the presence and amount of cellular retinol (CRBP) and cellular retinoic acid-binding (CRABP) proteins. In all cases adjacent, grossly normal tissue was similarly examined. For each example CRBP levels were significantly higher in tumor tissue compared to adjacent tissue. In four cases CRABP was significantly higher. This is of interest because retinol, retinoic acid and their analogs have been shown to inhibit the development of various epithelial tumors, and this inhibition is possibly mediated by these binding proteins.

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THERE IS A CONSIDERABLE BODY of evidence which demonstrates that vitamin A and its analogs (retinoids) can inhibit 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. This includes success with leukoplakia[1,2] and basal cell carcinoma in humans.[3,4] These findings have been reviewed recently.[5,6] Because vitamin A is necessary for the control of the direction of differentiation and the rate of proliferation of many epithelial tissues, its ability to effect 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. Two binding proteins have been described. The first, discovered in many rat tissues[7] as well as human tissues[7,8] binds retinol with high affinity and specificity. It is called cellular retinol-binding protein (CRBP).[9] The second, also found in many tissues,[10] binds retinoic acid with high affinity and specificity and is called cellular retinoic acid-binding protein (CRABP).[11]

The evidence that these two binding proteins, present in many species, mediate vitamin A action has been reviewed recently.[12] For example, CRBP and CRABP bind analogs of retinol and retinoic acid, respectively, with affinities that parallel the activity of these analogs in various test systems. We have proposed that the binding proteins may also be mediating the reported antitumor activity of vitamin A and its analogs.[13] Consequently, the presence or absence of the binding proteins in various tumors may well be of interest in evaluating reported or potential effects of such compounds on those tumors.

Here we have determined the presence and amount of CRBP and CRABP in human epidermoid carcinomas of the head and neck, compared to that found in adjacent, grossly normal tissue.

Materials and Methods

All-*trans*-[11,12-^{3}H]retinoic acid (10 Ci/mm) was a generous gift of Hoffmann-LaRoche, Nutley, New Jersey. Radioactive retinol was prepared by reduction of retinal by the following procedure. All-*trans*-retinal (2 μmole, from Sigma) was dissolved in 3 ml ethanol and added directly to 0.5 μmole tritiated $NaBH_4$ (48 Ci/mm from New England Nuclear). After a 30 min incubation, 10 μmole formaldehyde was added to react with any remaining $NaBH_4$. Three ml 0.5 *M* NaCl was

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TABLE 1. Quantitation of Binding Proteins in Tissue Samples

Patient	CRBP		CRABP	
	Tumor	Adjacent tissue	Tumor	Adjacent tissue
	pmol/g	pmol/g	pmol/g	pmol/g
A	65	*	270	60
B†	175	*	210	95
C	100	*	190	*
D	40	*	180	220
E	65	10	320	290
F	45	*	140	*

* Below detection by method used.

† Secondary tumor from lymph node of this patient had levels of 180 pmol/g, CRABP; 120 pmol/g, CRBP.

then added and the solution was extracted with 3 ml petroleum ether, done twice. The extract was taken to dryness under N_2 and the residue dissolved in a small volume of cyclohexane: chloroform (1:1) containing 50 μg butylated hydroxytoluene (BTH) per ml. The solution was applied to a 1.5 × 10 cm column of LH-20 (Pharmacia) equilibrated and then developed in the solvent mix above. This separated [^{3}H]retinol from traces of retinal present. Fractions containing [^{3}H]retinol were pooled and taken to dryness under N_2. The retinol was then dissolved in isopropyl alcohol (1 mg BTH/ml) and stored at −70° in sealed ampules until use. The specific activity of the [^{3}H]retinol was 12 Ci/mmole.

The study group consisted of 6 patients (5 male, 1 female) with previously untreated epidermoid carcinoma of the oral cavity or orophaynx seen by members of the Department of Head and Neck Surgery at M. D. Anderson Hospital and Tumor Institute, Houston, Texas. Tissue samples were obtained at the time of surgical excision. One of us (W.J.G.) immediately reviewed the excized specimen with the responsible pathologist and 2 g samples were taken from the center of viable tumor and from the adjacent grossly normal mucous membrane. In Patient B, a sample was also taken from an excized lymph node, which was subsequently shown to contain metastatic cancer. All tissues were frozen and stored for 1-3 months prior to shipping to Nashville.

Tissue samples were homogenized in 4 volumes 0.05 M Tris, pH 7.5 (w/v) using a Polytron (Kinematica GMBH., Luzerne, Switzerland) homogenizer. Cell debris was removed by centrifugation at 10,000 × g for 15 min. The supernatant liquid was taken to pH 5 by the dropwise addition of 1 *M* aceticacid. Precipitated material was removed by centrifugation as above. The supernatant liquid was collected and adjusted to pH 7.5 with 1 N NaOH. All steps were carried out at 4°.

Determination of binding proteins was by sucrose gradient centrifugation essentially as previously described.[14] Briefly, extracts were incubated for 4 hours at 4° with 25 pmol [^{3}H]retinoic acid or retinol in the presence or absence of a 100-fold excess of the corresponding unlabeled compound. After treatment of the incubations with dextran-coated charcoal, an aliquot of each was submitted to centrifugation on linear 5–20% (w/v) sucrose gradients in 0.05 *M* Tris HCl pH 7.5. The binding proteins were revealed as peaks of radioactivity in the 2S region of the fractionated gradients. The peaks were abolished in the presence of excess unlabeled ligand. The difference in the amount of radioactivity observed in the 2S region between gradients with no competition and those with competition (considered specific binding) was used to quantitate the amount of binding protein present.

Results

The results for all samples are shown in Table 1. All tumor samples contained abundant amounts of CRBP and CRABP. This includes the metastatic lymph node studied in Patient B. In contrast, levels of CRBP in the epithelial tissue adjacent to the tumor were undetectable in five of six patients. Comparing CRABP levels, two patients (D and E) had comparable quantities of CRABP in adjacent tissue, but the remaining four had significantly lower or no detectable CRABP in the adjacent epithelial tissue.

The most dramatic example is shown in Figure 1. As can be seen, CRBP and CRABP were not detectable in the adjacent tissue, but both were clearly evident in the tumor tissue as revealed by the prominent peaks of bound radioactivity at 2S (equivalent to about 15,000 daltons). These peaks are absent in the presence of excess unlabelled compound (retinol or retinoic acid). For the retinoic acid gradients the second peak represents binding to serum albumin which unavoidably contaminates the tissue extracts. Both binding proteins are specific for their respective ligands as a 100-fold excess of retinol does not inhibit the binding of [^{3}H]retinoic acid to CRABP; a 100-fold excess of retinoic acid does not inhibit the binding of [^{3}H]retinol to CRBP.

Discussion

Elevated levels for both CRABP and CRBP in tumor tissue compared to adjacent, apparently normal tissue have been observed before for rodents and humans. Examples in humans of elevated levels of CRABP include breast,[15,16] lung,[15] skin and stomach carcinomas.[12] Human colon carcinomas frequently contain CRABP[17] but adjacent tissue was not examined in that study.

CRBP is frequently not assayed in such studies but its levels do show dramatic changes in some cases. These include colon adenocarcinomas induced in rat by chronic

administration of dimethylhydrazine[18] and papillomas induced on the skin of mice by administration of dimethylbenzanthracene and croton oil.[19] The examples presented in this report clearly show an elevation of CRBP in tumor versus adjacent tissue in all cases and an elevation of CRABP in four of six cases. The elevation observed is essentially the same whether the data is expressed as pmole bound per gram tissue or per milligram soluble protein in the tissue extracts.

The presence and elevated levels of CRBP and CRABP may be of significance because the binding proteins may mediate not only the effects of retinoids in normal tissue but also the ability of retinoids to affect neoplastic tissue.[12] For example, several analogs of retinoic acid, as well as retinoic acid itself have been shown to be effective in prophylaxis or treatment of chemically induced or spontaneous tumors, and also have the ability of binding to CRABP of rodent as well as human origin.[20] No effective compound has yet been reported which, as itself or its major metabolite, does not bind to CRABP. This correlation has been extended to compounds effective in the inhibition of growth of cultured S91 melanoma cells.[21]

Such compounds have been shown to inhibit: lingual carcinogenesis in the Syrian Hamster,[22] skin carcinogenesis in the mouse[23] and bladder carcinogenesis in the rat.[24] They have been effective in the treatment of basal cell carcinoma[3,4] and leukoplakia[1,2] in humans.

The concept of chemoprevention of carcinogenesis by inhibitory chemical compounds is particularly apropos to head and neck cancer control. Tobacco and alcohol have been clearly implicated in causation[25] and this exposure is difficult to modify. "Field Cancerization," the diffuse abnormality of the mucous membranes of the upper aerodigestive trace, is the rule in patients with chronic exposure to these agents.[26] This explains the high incidence of second primary tumors and probably also accounts for some late local recurrences, which are major causes of failure in our present treatment regimens. In a study of failures in treatment of cancer of the oropharynx, Jesse and Sugarbaker[27] found a 20% incidence of local recurrence, and of the patients who survived five years, 37% developed second primary cancers in the embryonic foregut. Similarly, Moore[28] found a 40% incidence of second primary tumors in patients who were unable to stop smoking following initial treatment for their first head and neck primary cancer. Clearly, systemic or topical agents capable of inhibiting this occurrence would be a valuable adjunct to the presently utilized local therapy, be it surgery or irradiation.

The presence of CRBP and CRABP in the carcinomas examined here suggests that retinol, retinoic acid or analogs of these compounds might be considered as candidates for such inhibitory agents.

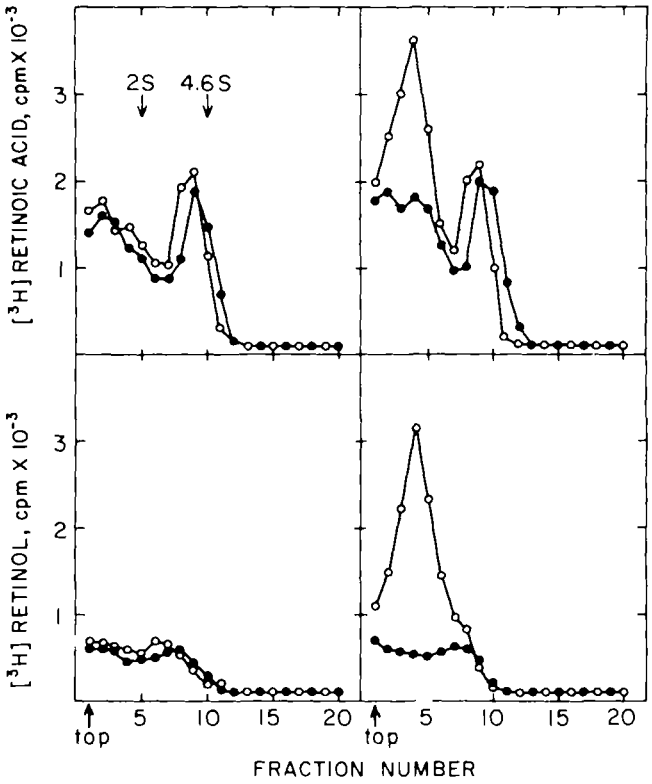

FIG. 1. Detection of CRABP and CRBP in tumor tissue. After incubation with [^{3}H]retinoic acid or [^{3}H]retinol aliquots of extracts of tumor tissue (right panels) or adjacent tissue (left panels) were submitted to sucrose gradient centrifugation. Fractions were collected and radioactivity determined for each fraction. Incubations were done with radioactive ligand along (○) or in the presence of a 100 fold excess of unlabeled ligand (●) to demonstrate competable binding, revealed as a peak in the 2S region of the gradient.

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