Cellular Retinol-Binding Proteins in Head and Neck Tumors and Their Adjacent Tissues

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The levels of cellular retinol-binding protein (CRBP) were examined by Scatchard analysis in 17 surgically resected human head and neck tumors and their adjacent normal tissues (normal tissues corresponded to muscle tissue). The center area and the marginal area of tumors were tested for their levels of CRBP in 17 cases, and the adjacent tissue was tested in 13 of the 17 cases. Only a small difference (if any) in the dissociation constant (Kd values) was seen in some cases between tumor and adjacent tissue; in most cases the Kd values in both areas were similar, and these values were also similar between the central and marginal areas of tumors. The average level of CRBP in the marginal area of the tumors was higher than that in the center area of the tumors in all the 17 cases. Among the 13 tumor cases whose adjacent tissues were tested, 12 showed CRBP levels more than two times higher in the marginal area of tumors; whereas in only one case was the level in the marginal or the center area of tumor similar to that in the adjacent tissues.

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TWO DIFFERENT vitamin A-binding proteins—Cellular retinol binding protein (CRBP) and cellular retinoic acid binding protein (CRABP)—are found in mammalian cells.[1,2] CRBP and CRABP specifically bind to retinol and retinoic acid respectively, but the biological roles of these vitamin A-binding proteins are not well understood. Retinoids appear to exert their roles in the control of differentiation through interacting with the binding proteins.[3–6] Actively growing tumor cells have been reported to contain different levels of CRBP or CRABP from normal cells or tissues.[7–11]

Retinol or retinol palmitate has been applied to cancer treatment when combined with radiation or anticancer agents.[12–16] We have applied retinol palmitate in combination with cobalt radiation and 5-fluorouracil (FAR therapy) to head and neck tumors.[17,18] The factors that determine tumor sensitivity to retinol or retinol palmitate are unknown; but the sensitivity or resistance of mammalian cells is often discussed in relation to the presence or absence of CRBP or CRABP.[19–23] There is a possibility, then, of a close correlation of levels of CRBP (or CRABP) and tumor sensitivity to retinoids. In addition, if CRBP or CRABP were a marker for rapidly proliferating tumor cells, the levels of such binding proteins might be diagnostically useful for head and neck tumors. For these reasons, we have tested the levels of CRBP in head and neck tumors and their adjacent normal tissues.

Materials and Methods

Surgical Specimens

The study group included 17 head and neck cancer cases. Tissue samples were obtained at the time of surgical excision. Specimens were taken from the center of the viable tumor (Fig. 1,A), the margin of tumor (Fig. 1,B) and the adjacent normal tissue (Fig. 1,C). Normal tissue corresponded to muscle tissue. There was no detectable change in the binding activities for CRBP after storage of sample at −80°C for varying periods in our current study (Yanagita, T., unpublished data).

Detection of CRBP

All steps were carried out at 4°C. Tissue samples were homogenized in 50 mM TRIS-HCl, pH 7.5 containing 1 mM EDTA, 10 mM KCl and 1 mM dithiothreitol. The supernatant after centrifugation at ×105,000 *g* for 1 hour

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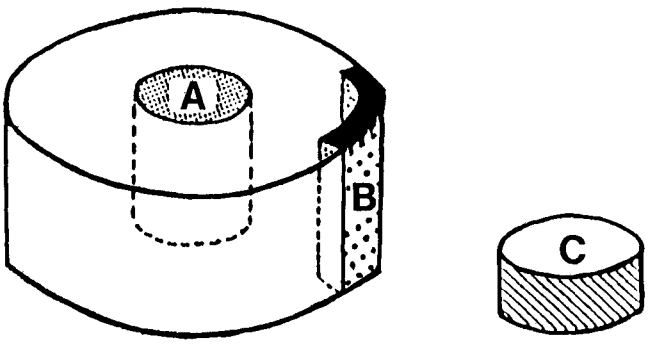

FIG. 1. Location of sampling of specimens from tumors and their adjacent normal tissues in 17 cases. Area A corresponds to cancer tissue from the center area and area B to marginal cancer tissue. Area C corresponds to adjacent normal tissues, which corresponds to muscle tissue.

was adjusted to pH 5.0 by the addition of suspension of dextran-coated charcoal. The clarified solution obtained by centrifugation was then titrated back to pH 7.5. Protein binding was determined by radioreceptor assay and Scatchard analysis as described previously.[8,19,23] Briefly, extracts were incubated for 3 hours at 4°C in the dark with 500, 250, 125, 31.3, or 7.8 pmol/ml of ^{3}H-retinol. After incubation, free ligand was removed with dextran-coated charcoal. The radioactivity in the supernatant was then measured. Nonspecific binding was assessed at each concentration by parallel incubations in the presence of a 400-fold excess of unlabeled retinol. Specific binding at each concentration was assumed to be the difference between the total and nonspecific values. The amount of free ligand at each concentration was also assumed to be the difference between total input and total binding. Protein concentration in each cytosol fraction was determined by the Lowry method.[24]

Results

Specimens were obtained from the center area (A) of surgically resected head and neck tumors, the marginal area (B) of the tumors and their adjacent normal tissues (C) as seen in Figure 1. Figure 2 shows an example of the histologic features of the tumors: areas A and B are respectively indicated. All 17 tumors were squamous cell carcinomas, and the center areas were found to be nonnecrotic, both histologically and macroscopically. Figure 3 shows sample assays for CRBP activity in the cell homogenates of areas A, B and C (Fig. 1) of Case 16 (Table 1). Saturation kinetics for ^{3}H-retinol binding are presented in Figure 3a. Scatchard analysis showed 2.6 (A), 4.5 (B), and 0.6 (C) pmol/mg protein respectively (Fig. 3b). Kd values for CRBP in areas A, B, and C were estimated to be 32.1, 33.7 and 19.5 $\times$ 10^{-9} M, respectively. The CRBP

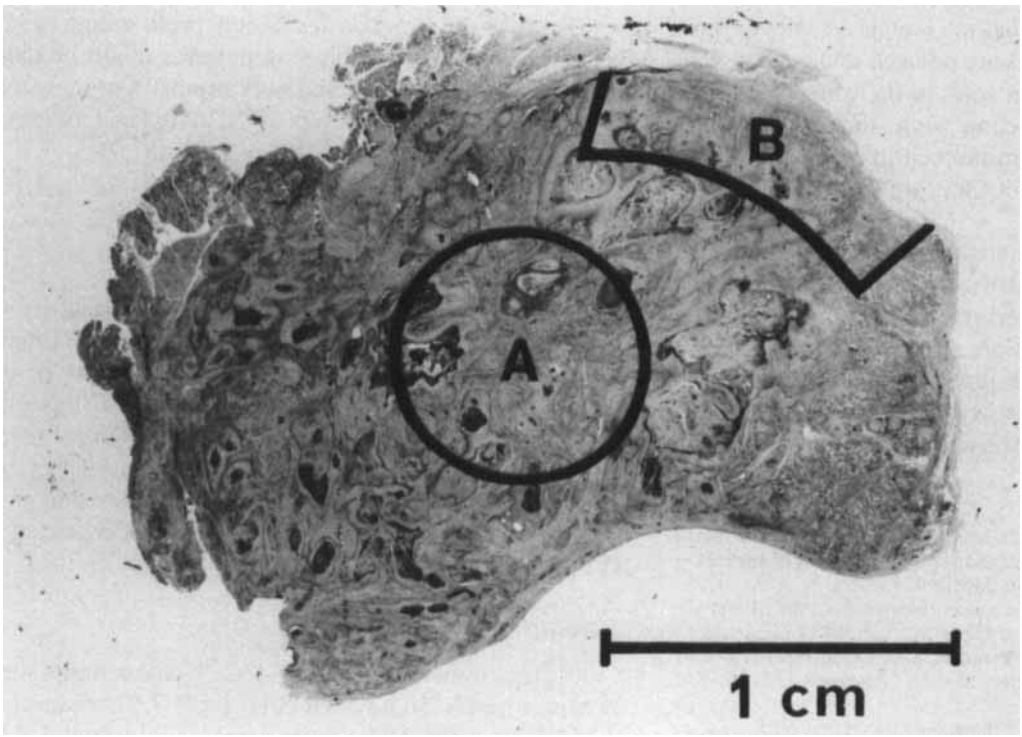

FIG. 2. A specimen of the surgically resected tumor. (A, center area; B, marginal area).

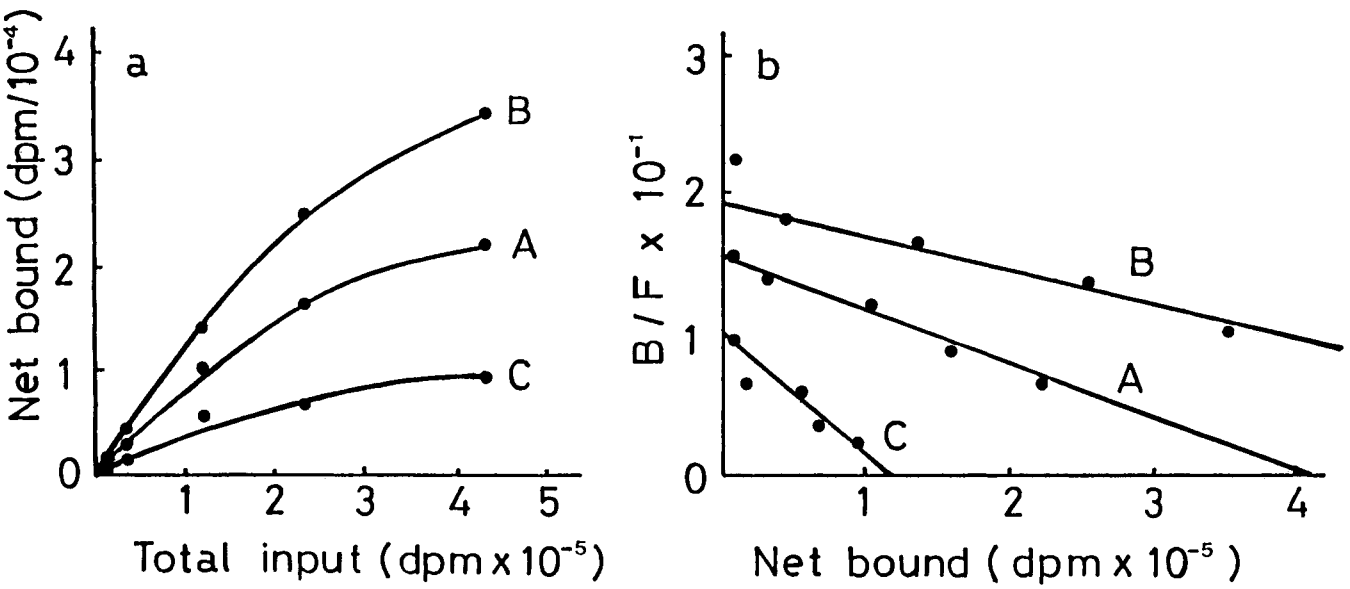

FIG. 3. (a, left) Binding of ^{3}H-retinol to CRBP in area A and B in head and neck tumor and in adjacent normal tissue (area C) of Case 16. Cytoplasmic extracts were incubated with various doses of ^{3}H-retinol and unbound ^{3}H-labels were removed to measure total binding activity. Nonspecific binding at each concentration was measured by incubation in the presence of 400-fold excess of unlabeled retinol. Specific bound was determined by subtracting nonspecific binding from total binding. (b, right) Scatchard analysis of the data in "a" was presented on each area. B/F: bound/free; total bound or net bound is expressed as dpm/tube.

levels in both areas A and B in the tumor were higher than the levels in the adjacent area C in Case 16.

Saturation kinetics of CRBP were examined for all 17 cases, and are presented in Table 1. Of the 13 cases, the adjacent normal tissues (muscle) could be examined for their CRBP levels. In comparison with CRBP levels in the adjacent normal tissues of the 13 cases, CRBP levels in area A and/or B of the tumors were significantly higher in all cases except one (Case 2) and the CRBP level in area C was similar to that in area A or B of the case 2 (Table 1). Mean values for CRBP in area A or B in all 17 cases were found to be higher than those in area C (Table 2).

Comparison of the CRBP levels between areas A and B in the tumors showed more than a 50% increase of the levels in the marginal tumor area B than the center area A in 14 cases (*i.e.*, in all cases except 6, 7, and 11; in these three cases, increase of the CRBP levels in the area B was less than 50% compared to those in the area A). Average values for CRBP levels in all 17 cases increased significantly ($P < 0.001$) in the marginal tumor area in comparison with those in the center area of the tumors (Table 2). We also tested another cellular vitamin A-binding protein, CRABP, in all 17 cases, but found no significant difference in the levels of CRABP between the tumor and adjacent normal tissues (unpublished data).

The Kd values of CRBP in tumors (area A and B) varied from 16.4×10^{-9} M to 84.1×10^{-9} M, whereas those of CRBP in the adjacent normal tissues (area C) ranged from 18.1×10^{-9} M to 57.7×10^{-9} M (Table 1). These values in our current study are roughly comparable to those reported for rat liver[10] and human hepatoma.[8] Although there were some differences in the Kd values in areas A and B in the tumors in seven cases (Cases 3, 5, 8, 9, 11, 15, and 17), the Kd values in the remaining ten cases were generally comparable for the two areas (Table 1). There was no change as great as a factor of 10 in Kd values among the three different areas, suggesting similar affinity constants of CRBP for tumors and normal tissues.

Discussion

A significant difference in CRBP levels was seen between tumor and normal tissue (Table 1 and 2). A relevant study by Ong *et al.*[25] (1982) showed that the levels of CRBP in tumors of the oral cavity in six patients were much higher than those in the adjacent tissues. CRBP is present in most normal tissues as well as in tumors of the kidney, breast, liver, cervix endometrium, ovary, and oral cavity[26]; and CRABP levels and the growth inhibitory effect of retinoic acid have been suggested to be closely correlated.[9] However, it remains unknown whether the cellular sensitivity to retinol or its analogs is mediated through CRBP.

If the sensitivity of cancer cells to retinoids is mediated through CRBP, combination therapy with retinol palmitate might show a preferable affinity for cancer cells

TABLE 1. Comparison of CRBP in A, B, and C* of 17 Patients with Head and Neck Cancer

Patients			CRBP†			Kd‡		
No.	Age (yr)/sex	Site	A	B	C	A	B	C
1	61/F	Mesopharynx	4.6	7.9	0.6	84.1	71.7	28.9
2	70/M	Mesopharynx	0.4	1.0	0.7	36.6	31.5	38.3
3	62/F	Cervical esophagus	0.4	2.9	0.7	22.7	41.6	29.7
4	47/M	Maxilla	0.7	2.1	—§	32.6	26.6	—
5	46/F	Maxilla	2.0	5.5	—	16.4	66.8	—
6	78/M	Maxilla	10.1	12.1	2.3	23.2	26.6	28.3
7	43/M	Oral cavity	1.0	1.4	—	34.6	28.5	—
8	66/M	Larynx	1.3	7.4	1.9	21.4	54.2	57.7
9	53/M	Maxilla	2.5	4.9	0.9	48.5	64.6	24.3
10	50/M	Larynx	0.5	5.0	0.3	17.0	18.3	18.1
11	78/M	Larynx	4.5	6.2	0.7	52.9	81.1	48.8
12	76/M	Hypopharynx	3.1	5.8	1.6	44.7	55.6	47.1
13	70/M	Oral cavity	1.1	2.3	0.2	23.0	35.0	16.8
14	54/M	Oral cavity	0.4	0.8	0.1	50.0	44.7	38.4
15	65/F	Oral cavity	1.0	2.5	0.1	33.4	60.6	24.9
16	37/M	Oral cavity	2.6	4.5	0.6	32.1	33.7	19.5
17	60/M	Oral cavity	0.4	3.5	—	20.3	44.4	—

* Area A, B, and C correspond to center of the cancer tissue, marginal cancerous tissue, and adjacent normal tissue. All tumors are histologically squamous cell carcinoma.

† The amount of CRBP was presented as pmol/mg cytosol protein assayed by Scatchard analysis.

‡ These Kd values ($\times 10^{-9}$ M) were obtained from the data by Scatchard analysis.

§ Not tested because of failure of sampling.

with high levels of CRBP. We have tried FAR therapy clinically against head and neck tumors in some patients with indications of improved therapeutic effects.[17,18] Possibly an even greater therapeutic effect might be seen in head and neck tumors with high levels of CRBP.

In this study, we also examined whether the location of cancer cells in the center area or the marginal area of the tumors affects the levels of CRBP. The levels of CRBP were found to be significantly higher in the marginal area than in the center area in most cases tested (Table 1 and 2), supporting the hypothesis of a tumor-tissue specific increase in activity. Cancer cells in the marginal area of the tumors would be expected to be actively proliferating compared to those in the center area, consistent with the findings by Chytil and Ong[27] (1978) of elevated levels of CRBP in actively proliferating tissues and tumors. However, in contrast to their findings, we saw no significant difference in CRABP levels between head and neck tumors and their adjacent tissues (unpublished data).

TABLE 2. Summary of CRBP Levels in Head and Neck Cancer

Area	CRBP levels*
A	2.1 ± 2.5
B	4.5 ± 2.9
C	0.8 ± 0.7

* The amount of CRBP was presented as pmol/mg cytosol protein of average values from 17 cases as seen in Table 1 with mean ± ISD (C; 13 cases).

A: center of cancer tissue; B: marginal cancerous tissue; C: adjacent normal tissue.

Independent studies *in vitro* on variants of teratocarcinoma cells with altered levels of CRABP also suggest CRABP as a possible mediator for differentiation caused by retinoic acid.[4-6,28] Selection of variants with altered retinoic acid receptors from melanoma cells[22] or from mammary cancer cells[21,23] hints that cellular sensitivity to retinoic acid is partly due to CRABP. Only one variant with altered response to retinol or its analogs has been reported. In that case, the retinol acetate-sensitive variant derived from a Chinese hamster cell line did not show as much CRBP and CRABP as the parental V79.[19] Isolation of suitable variants with altered sensitivity to an alcohol type of vitamin A might give us further insights into the relation between cellular sensitivity to retinol or its analogs and the levels of CRBP.

Although the data from various sources do not suggest a simple correlation of CRBP levels and the sensitivity to retinol or its analogs, the increased level of CRBP in tumors might be a diagnostic marker for malignant tumor. However, a statistical survey with a greater number of cases will be necessary to confirm whether the increase of CRBP is closely correlated with tumor malignancy.

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