Retinoid-binding Proteins in Retinoblastoma Tumors

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ABSTRACT

A combination of Western blot, Northern blot, and radiolabeled ligand-binding techniques was used to investigate retinoid-binding proteins in retinoblastoma (RB) cells from fresh tumors and from 19 RB tumor lines cultured in vitro. Using rabbit anti-bovine cellular retinol-binding protein (CRA1BP) antibodies, no CRA1BP could be detected. As determined by 3Hretinol binding, cellular retinol-binding protein was sometimes not detectable but averaged 2.3 ± 2.7 ± SD, n = 7 pmol [3H]retinol bound/mg protein, similar to adult retina cytosol. Using 3Hretinoic acid as ligand, cellular retinoic acid-binding protein was not detectable in some lines and averaged 1.0 ± 1.2 ± SD, n = 7 pmol [3H]retinoic acid bound/mg protein, well below the adult retina cytosol level of 94.4 ± 16.3 ± SD, n = 4 pmol [3H]retinoic acid bound/mg protein. Using rabbit anti-bovine interstitial retinol-binding protein (IRBP) antibodies, IRBP of the same molecular mass as human IRBP (135,000) was detected in the medium from all cultured RB cells and averaged 75.9 ± 19.2 pmol/mg cells (bovine IRBP immunochemical equivalents). Cytosol levels were less than 1% of the medium. Using a human IRBP complementary DNA probe, levels of IRBP RNA transcripts in 19 RB cell lines were comparable with the adult retina. The Y-79 RB cell line was different from the others; the amount of IRBP in the medium was only about 1% of the RB cell lines. Levels of cellular retinol-binding protein were comparable with the other lines, but cellular retinoic acid-binding protein was 9 times more abundant. IRBP RNA transcripts in Y-79 cells were below the limits of detectability but appeared at low levels after induction of differentiation of Y-79 by 10-8 M retinoic acid. Although this cell line has been in culture longer than the others, it may also have been initiated at an earlier stage of retinal development.

INTRODUCTION

Although the role of retinoids in cancer is still unclear, there have been many studies designed to investigate retinoid-binding proteins in tumor cells (1). In the case of RB, studies of retinoid-binding proteins (CRBP, CRABP, CRA1BP) have been mainly confined to the established Y-79 and WERI-Rb1 cell lines (2–4). Fresh RB tumors are able to express the gene for interstitial retinol-binding protein (IRBP; Ref. 5), an unexpected finding, because IRBP is synthesized and secreted by adult retina. The Y-79 RB tumor line was different from the others; in the experimental cultures, the cells were transferred to serum-free medium at a low concentration of 3 ± 105 cells/ml. The medium was collected over a 2- to 3-week period and then the cells were harvested. For differentiation studies, Y-79 (subclone 6) cultures were initiated with 5 ± 105 cells/ml in 10-4 M RA, 15% fetal calf serum, 10 µg/ml bovine insulin, and 5 x 10-5 M 2-mercaptoethanol and fed weekly.

The RB cells for determination of CRBP, CRABP, and IRBP were initially grown in the serum-containing medium. Because serum interfered with the determination of IRBP, the cells were then transferred to serum-free medium at an initial concentration of 3 x 105 cells/ml. The medium was collected over a 2- to 3-week period and then the cells were harvested. Control cultures without RA contained 0.1% ethanol, the carrier used for RA in the experiments. After 10 days treatment with RA in the presence of serum, the cells were transferred to serum-free medium containing the same concentration of RA for 2 days prior to harvesting the cells and medium for determination of CRBP, CRABP, and IRBP. However, because morphological studies suggested that more complete differentiation occurred in the presence of serum, RNA was prepared from cells maintained in medium containing 15% serum.

Tumors and Tissues Studied. Nineteen different RB cell lines were included in these studies. Eleven were from unilaterally affected children (RB412, RB383, RB355, RB369E, RB405, RB414, RB430, RB524, RB530, WERI-Rb1 (12), Y-79 (13), and eight were from bilaterally affected children (RB344, RB247C3, RB409, RB429R, RB517R, RB522A, RB529A, RB529C). RB529 A and C are separate tumors from each eye of one patient. Cells with 500 numbers were from fresh surgical specimens and all others were from cultured cell lines.

Human retinal samples of different gestational ages were obtained from postmortem pathology specimens. Two human adenovirus-transformed fetal retinal cells lines (AD12FR and AD5FR; Ref. 14) representing 18 wk gestation, and several nonretinal cell lines (TABI, primitive neural ectodermal tumor; HL-60, myelogenous leukemia; IMR32, neuroblastoma; and OS108 and OS234, osteosarcomas) were also studied on Northern blots.

Binding of Retinoids to Cytosolic Proteins. Cells from eight RB tumors and cell lines were homogenized in phosphate-buffered saline, (150 mM NaCl, 5 mM sodium phosphate, pH 7.5) and centrifuged at 100,000 × g for 1 h to yield the cytosolic supernatant. The protein content was determined (15) and the volume adjusted to give 500 µg protein/ml. Human neural retinas were rinsed free of interphotoreceptor matrix (16), homogenized, and treated similarly. Two hundred µl volumes (100 µg protein) were incubated in replicate with 10 µl ethanol solutions of all-trans [11,12-3H] retinoic acid (4 x 109 dpm, 35.9 Ci/mmol, Hoffman La Roche (or all-trans [11,12-3H] retinol (2 x 108 dpm, 40 Ci/mmol, Amersham). Both ligands were initially purified by high-performance liquid chromatography (17). Controls were run by adding the organomercurial p-chloromercuribenzenesulfonate (4 mM) to abolish the specific binding to CRBP or CRABP (18). Mixtures were incubated in the dark at 4°C overnight, then vortexed with 80 µL of a dextran-coated charcoal suspension (see below), left for 10 min, and centrifuged for 4 variance from the other cell lines in that it expresses very low levels of IRBP. CRABP, however, was more abundant in the cytosol of Y-79 cells. Some of these findings have been reported in abstract form (9).

MATERIALS AND METHODS

RB Tumor Cell Culture. RB surgical specimens were obtained from enucleated eyes and grown initially as xenografts in the anterior chamber of athymic nude mouse eyes (10) and/or in tissue culture on Dexter (11) mouse bone marrowstromal feeder layers. Subsequently the RB cells were harvested from xenografts or weaned from the feeder layers and grown as suspension cell cultures in Dulbecco's medium, 15% fetal calf serum, 10 µg/ml bovine insulin, and 5 x 10-5 M 2-mercaptoethanol and fed weekly.

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min in a Fisher model 235 centrifuge. A 200-μl volume was then subjected to high-performance liquid chromatography on a 7.5 x 300 mm Bio-Sil TSK-250 column (Bio-Rad), mobile phase 0.1 mM NaSO₄, 0.02 mM sodium phosphate (pH 6.8), 1 ml/min. Molecular mass standards consisted of thyroglobulin (which eluted at the void volume, 669,000 daltons), IgG (150,000 daltons), chicken ovalbumin (43,000 daltons), myoglobin (17,000 daltons), and vitamin B₁₂ (which eluted at the included volume). Fractions were collected at 0.5-min intervals, mixed with 9 ml aqueous counting solution (Amersham), and counted in a Packard Tricarb liquid scintillation spectrometer. Authentic CRBP was prepared from dog liver (19) and a CRABP-containing preparation was obtained from bovine eyes (20). CRBP (1-10 μg) or the CRABP-containing samples (50 μg total protein) were dissolved in 200 μl phosphate-buffered saline and incubated with the corresponding labeled ligand as described above. Dextran-coated charcoal was prepared by adding 1.5 g washed Norit-A charcoal (Fisher) to 0.15 g Dextran 80 (Fluka) in 299 ml of 10 mM Tris-HCl (pH 7.5) containing 1 mM EDTA. The suspension was stored for up to 2 mo at 4°C.

Interstitial Retinol-Binding Protein and CRA1BP—Western Blot Analysis. Various amounts of cytosol or medium from eight RB cell lines were subjected to SDS-polyacrylamide gel electrophoresis (21, 22) and electrophoretically transferred to nitrocellulose paper (23, 24). Immunological visualization of IRBP was carried out by incubating the paper with rabbit antiserum IRBP IgG followed by peroxidase-conjugated goat antirabbit IgG (Bio-Rad; Refs. 6, 25). Samples containing 0.5-4.0 ng of purified bovine IRBP were also electrophoresed and transblotted. The intensities of the bovine bands were then matched against the samples. In the present work, retinoblastoma IRBP was calculated as immunochemically equivalent amounts of bovine IRBP.

The true quantities must be higher because human IRBP is not serologically identical with bovine IRBP (16, 26).

Rabbit antiserum CRA1BP (which we demonstrated to be immunologically cross-reactive with the human protein) was prepared and generously provided by J. C. Saari (27) and used to probe similar blots.

Northern Blot Analysis of IRBP mRNA. Total cellular RNA was prepared from fresh surgical specimens or from cultured cells by the guanidine thiocyanate/cesium chloride gradient method (28). Polyadenylated RNA was obtained by oligo(deoxy)thymidylate-cellulose chromatography (29), separated by electrophoresis on a 1% agarose gel (30), and transferred overnight by capillary action to GeneScreen in 0.025 M NaHPO₄, pH 7.0. The RNA was then covalently linked to the nylon membrane by UV irradiation at a dose of 2000 J/m².

A human IRBP cDNA clone (H-4 IRBP; Ref. 31) and the ED1 fragment of ESD (32) were used as probes. In one instance (RB412) a bovine IRBP cDNA clone was used instead (B-23; Ref. 33). The homology of human and bovine IRBP cDNAs is 85% (31). The inserts were gel purified from the pUC13 after digestion with EcoRI and were labeled with 32P-dCTP by random-primer labeling (34) to a specific activity of 5 x 10⁶-10⁷ cpm/μg. The nylon membranes were prehybridized in 0.5 M NaHPO₄, pH 7.2, 1% crystalline grade bovine serum albumin, 1 mM EDTA, 7% SDS at 65°C for 10 min and then hybridized overnight in fresh pre-hybridization buffer 2 x 10⁶ cpm of the IRBP and ESD probes using high stringency conditions (65°C). The membrane was then rinsed once in 0.04 M NaHPO₄, pH 7.2, 0.5% fraction V bovine serum albumin, 1 mM EDTA, 5% SDS at 65°C for 5 min, followed by three similar 10-min washes in 0.04 M NaHPO₄, pH 7.2, 1 mM EDTA, 1% SDS at 65°C. Filters were exposed to XAR-5 X-ray film at -70°C with an intensifier screen. The IRBP and ESD probes were used either simultaneously or sequentially on the same nylon membranes.

RESULTS

CRBP and CRABP Levels Determined by Radiolabeled Ligand Binding. There was a linear relationship between the amount of protein and the amount of ligand bound to CRBP or CRABP in preparations of retina cytosol containing up to 2.5 mg protein/ml, 5 times the level used in the present assays. The radioactivity profiles of a selection of [³H]retinol-incubated cytosols subjected to high-performance liquid chromatography on a TSK size-exclusion column are illustrated in Fig. 1. The large vertical arrows mark the elution position of authentic dog liver CRBP (19), which corresponds to 16,000 daltons, close to the reported values for CRBP from various sources (1). The peaks of radioactivity in this position were attributed to CRBP because they were abolished by p-chloromercuribenzenesulfonate (18), as shown in A and B. The peaks indicated by the small curved arrows in A coincide with the void (thyroglobulin) and included (vitamin B₁₂) volumes, respectively. The magnitude of these peaks, which presumably correspond to free ligand, some of it in aggregates, was minimized by the use of dextran-coated charcoal (see "Materials and Methods"). They were unaffected by the presence of p-chloromercuribenzenesulfonate (see A and B). It is clear that the abundance of CRBP was variable. About 7.8 pmol retinol/mg protein were bound to CRBP in RB344 (A), but none was detectable in RB247C3 (D).

When samples containing CRABP were incubated with [³H]retinoic acid and subjected to high-performance size-exclusion chromatography, a peak of radioactivity emerged at a molecular weight of 16,000–17,000 daltons. This value is close to those reported for CRABP by others (1) and is indicated by the vertical arrows in Fig. 2. A prominent peak of [³H]retinoic acid binding is evident in the retinal cytosol in E, and smaller peaks are present in the Y-79 cytosols in D and F. Hardly any was detectable in the other lines examined, as exemplified in A (RB344), B (RB429), and C (RB355). As in the [³H]retinol experiments, p-chloromercuribenzenesulfonate was used to abolish specific [³H]retinoic acid binding, as illustrated in F.

Results for all of the cells examined are summarized in Table 1. As determined by the specific binding of [³H]retinol, the
levels of CRBP in the RB cells were 2.3 ± 2.7 (mean ± SD, n = 7) pmol/mg cytosol protein. The values for Y-79, Y-79, retinoic acid-treated, and adult retina cytosol were within 1 SD of this figure.

Levels of CRABP, judged by the specific binding of [3H]retinoic acid, averaged 1.0 ± 1.2 (mean ± SD, n = 7) pmol/mg cytosol protein in the RB cell lines. This represents 11% of the Y-79 cytosol and only 1% of the adult retina cytosol, which amounted to 94.4 ± 16.3 (mean ± SD, n = 4) pmol/mg protein.

Interstitial Retinol-Binding Protein and CRA1BP Levels by Western Blot Analysis. Rabbit anti-bovine IRBP antibodies (20) were used to probe nitrocellulose blot transfers of medium and cytosol proteins. As Fig. 3 shows, this procedure was able to detect 0.5 ng of bovine IRBP, although the sensitivity for human IRBP was probably lower (26). This figure illustrates that IRBP was present in the serum-free media in which the RB cells had been cultured (lanes 1–7). Its apparent molecular mass is 135,000 daltons, a value lower than that of bovine IRBP (i.e., 144,000; Ref. 20) but identical with that previously reported for human IRBP (16) and for IRBP from two fresh RB tumors (5). IRBP was also detected when cells were cultured in serum-containing medium but could not be reliably quantitated because of interference from the high levels of serum albumin.

From Fig. 3, it is apparent that the Y-79 medium (lanes 8–10) contained much less IRBP. The protein could only be visualized when the loading levels were increased by a factor of 40 over those used for the media from the other RB cell lines (lanes 8′–10′). In terms of bovine IRBP immunochemical equivalents, the Y-79 medium contained only 1.0 ± 0.7 pmol/106 cells (mean ± SD, n = 4) compared with 75.9 ± 19.2 pmol/106 cells (mean ± SD, n = 7) for the other RB media (Table 1).

Fig. 3B shows that IRBP was not detectable in the cytosol protein at loading levels that were equivalent to Fig. 3A, namely 2.7 x 105 cells (2.5 µg/soluble protein) in lanes 1–10 and 1.1 x 106 cells (100 µg soluble protein) in lanes 8′–10′. If much larger quantities of cytosol proteins were immunoblotted, traces of IRBP could be detected (data not shown), but the amounts were less than 1% of those present in the medium.

Similar immunoblots with rabbit anti-bovine CRA1BP antibodies failed to provide any evidence for the presence of CRA1BP in RB or Y-79 cytosols.

Levels of IRBP Expression by Northern Blot Analysis. Since ESD is ubiquitously expressed, we used the ESD signal on the Northern blots as a measure of the amount of RNA loaded. By comparing the signal intensity of ESD and IRBP on the same blot we estimated the relative expression of the two genes in each lane and assigned a +/− rating, given in Table 2.

Eighteen of the 19 RB tumor cell lines studied for IRBP expression using Northern blot analysis showed a positive signal (Table 2 and Fig. 4). Insufficient RNA was available from five RB tumors to isolate polyadenylated RNA, so the total RNA was tested instead.

In Fig. 4 the IRBP signal generated by RB414 total RNA is very close in size to the 28S RNA band (5.2 and 5.1 kilobases, respectively), resulting in a less clear signal than in the other lanes in which polyadenylated RNA was used.

Nonretinal cell lines (osteosarcomas, neuroblastoma, myelo-
Retinoid-binding proteins in RB tumors

Table 2 Relative IRBP:ESD signal on Northern blots

<table>
<thead>
<tr>
<th>Cells</th>
<th>Relative IRBP:ESD signal</th>
</tr>
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<tbody>
<tr>
<td>RB tumors</td>
<td></td>
</tr>
<tr>
<td>RB522A*, RB529C, RB530</td>
<td>++++</td>
</tr>
<tr>
<td>RB517R, RB525A, RB396E</td>
<td>+++</td>
</tr>
<tr>
<td>RB409, RB355, WERI-RB1</td>
<td>++</td>
</tr>
<tr>
<td>RB247C3, RB383, RB405, RB414, RB429R, Y-79, RB524, RB412, and RB340</td>
<td>+/−</td>
</tr>
<tr>
<td>Y-79, RA-treated</td>
<td>−</td>
</tr>
<tr>
<td>Retina</td>
<td></td>
</tr>
<tr>
<td>Fetal, 6–12 wk</td>
<td>−</td>
</tr>
<tr>
<td>Adenovirus 5 cell line (AD5FR), 18 wk</td>
<td>−</td>
</tr>
<tr>
<td>Adenovirus 12 cell line (AD12FR), 18 wk</td>
<td>++</td>
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<tr>
<td>Fetal, 22 wk</td>
<td>++</td>
</tr>
<tr>
<td>Newborn</td>
<td>++</td>
</tr>
<tr>
<td>Adult</td>
<td>+++</td>
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<tr>
<td>Other tumors</td>
<td></td>
</tr>
<tr>
<td>OS234, OS108, OS5, OS7</td>
<td>−</td>
</tr>
<tr>
<td>TAB1, HL-60, IMR32</td>
<td>−</td>
</tr>
<tr>
<td>Normal tissues</td>
<td></td>
</tr>
<tr>
<td>Newborn spleen, liver, kidney, brain</td>
<td>−</td>
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<tr>
<td>Adult brain, testis</td>
<td>−</td>
</tr>
<tr>
<td>6 EBV cell lines</td>
<td>−</td>
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</table>

* RNA was collected from the fresh tumor and from tumor cells that had been cultured for 8 mo. No difference in IRBP expression could be detected.

* This blot was probed with a bovine IRBP cDNA probe (B-23; Ref. 33).

Genus leukemia, newborn spleen, liver, kidney, and brain, adult brain and testis, and Epstein-Barr virus lymphoblastoid cell lines) showed no IRBP signal. Fetal retina from 6- to 12-wk gestation and the two adenovirally-transformed 18-wk gestation human fetal retinal cell lines were also negative. However, human fetal retina (22-wk gestation), newborn retina, and adult retina all showed a strong signal. This finding is consistent with previous studies on the appearance of IRBP during human retinal development (35). With one exception, the RB cell lines were comparable to newborn and adult retina (+ to ++++). The only RB cell line in which we repeatedly failed to detect expression of human IRBP under the usual culture conditions for RB tumors (i.e., serum-containing medium) was Y-79. This cell line has been shown in our laboratory to differentiate morphologically in the presence of retinoic acid. In three experiments where Y-79 was exposed to retinoic acid for 7 days, low levels of IRBP mRNA were detected (Table 2), while control cultures failed to manifest any detectable signal.

Discussion

RB tumor cells have features in common with cells of the embryonic retina (36, 37); the oncogene N-myc, for example, is not expressed in adult retina but is expressed in RB tumors and in fetal retina (38). It would therefore be of interest to compare levels of retinoid-binding protein expression with those found in fetal and adult retinas. Unfortunately, the developmental expression of cellular retinoid-binding proteins has not been as extensively studied in the eye as in some other tissues (1). In rat retinas at P1 (the earliest age investigated), cellular differentiation is already well advanced, and CRBP and CRAIP are easily demonstrated by immunohistochemistry (39). It has not been determined whether CRABP is present at this time.

The RB cells examined had variable CRBP levels, but the average was within 1 SD of those found in the cytosol from mature human retinas. The Y-79 cells contained CRBP at about the same level as the other RB cells [Saari et al. (2) report higher levels in their Y-79 subclone, but this difference may not be significant].

RB cells were also variable in their expression of CRABP. Its average abundance, however, was only 1% of that in mature retina. Although the Y-79 cells were unusual in that their CRABP was about 9 times higher than in the other RB cells, our values (which were within 1 SD of those reported by Saari et al. (2) were still less than 10% of those we observed in adult retinas.

Two of our cell lines (RB247, RB383) did not have detectable amounts of either CRBP or CRABP, but none resembled WERI-Rb1 (2), which lacks CRBP and has CRABP that amounts to 40% of our value for mature retinas.

In contrast to the cellular retinoid-binding proteins, IRBP expression has been investigated during fetal and postnatal retinal development. In the mouse, the protein is detected when the photoreceptor inner segments start to develop (8). Similar results have been obtained with human retinas (35). These observations are consistent with the suggestion that IRBP is synthesized and secreted by retinal photoreceptor neurons (6, 7). It was therefore surprising to find that IRBP was secreted by nearly all of the RB cells examined. This result is concordant with our previous observations on cells from two fresh tumors which secreted IRBP with molecular mass and concanavalin A-
binding properties identical with the IRBP secreted by normal retina samples from the same eyes (5).

The expression of IRBP in RB tumor cells, except Y-79, was similar to the levels in normal retina at 22-wk gestation, suggesting that the tumors did not arise at an earlier developmental stage. Although the present evidence indicates that the cell line Y-79 may have been initiated prior to IRBP expression, it is possible that the long duration of Y-79 in culture has resulted in a decrease in IRBP expression. Other cell lines, however, do not suggest a correlation of IRBP expression and duration in vitro (40).

Several agents that promote differentiation of Y-79, retinoic acid, and butyrate (4) induce low levels of IRBP. Because retinal photoreceptors and Müller glial cells can share a single progenitor (41), it is interesting that we were unable to detect CRA1BP in the cytosol proteins from any RB cell line, including Y-79. This protein, which binds 11-cis retinal and 11-cis retinol, is restricted in adult retinas to the Müller cells (27).

In sum, the expression of retinoid-binding proteins in RB cells is variable. These findings are consistent with the postulated embryonic origin of these cells. With the notable exception of the Y-79 line, the only retinoid-binding protein that was consistently expressed by fresh RB tumors and cultured RB cells was IRBP, an extracellular glycoprotein normally associated with the maturing retina.

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