Production and secretion of retinol-binding protein by a human hepatoma cell line, HepG2

Laura Marinari, Catherine M. Lenich, and A. Catharine Ross[1]

Department of Physiology and Biochemistry, The Medical College of Pennsylvania, Philadelphia, PA 19129

Abstract Retinol-binding protein (RBP) that is synthesized and secreted by the human hepatoma cell HepG2 has been measured using a sensitive radioimmunoassay in which RBP in media and hepatoma cell sonicates reacts identically to human serum RBP. RBP was synthesized and secreted when cells were grown in retinol-depleted as well as retinol-containing media. However, immunoreactive transthyretin (prealbumin) could not be detected in concentrated HepG2 medium. RBP secretion and accumulation per mg of cell protein could be modulated by the concentration of fetal calf serum in the growth medium: secreted RBP equaled 782 ± 123 ng/mg of cell protein per 8 hr after preincubation with 10% fetal calf serum versus 555 ± 86 ng/mg per 8 hr in the absence of serum, whereas RBP in cell sonicates decreased only slightly. When HepG2 cells were cultured for two or more passages in medium containing fetal calf serum depleted of retinol by ultraviolet irradiation, the amounts of RBP in the cells and released to the medium were both significantly increased. When vitamin A (90% as retinyl esters) in the form of chylomicron remnants was presented to cells, there was a significant, dose-dependent redistribution of RBP from cells to medium, both in cells grown in normal fetal calf serum and in retinol-depleted serum. These data indicate that the secretion of RBP by HepG2 can occur constitutively in the absence of retinol, but that secretion can be enhanced and regulated by retinol delivered by the chylomicron remnant.—**Marinari, L., C. M. Lenich, and A. C. Ross.** Production and secretion of retinol-binding protein by a human hepatoma cell line, HepG2. *J. Lipid Res.* 1987. **28:** 941–948.

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The specific transport protein for retinol, retinol-binding protein (RBP), is produced by and secreted from liver (1). This function has been shown to be maintained by primary rat hepatocytes in culture (2, 3) and by certain rat hepatoma cell lines (4, 5). In the intact animal the release of RBP is clearly regulated by the availability of the ligand, retinol. Smith et al. (6) showed that vitamin A deficiency results in an accumulation of RBP in liver, concomitant with a decrease in the concentration of circulating RBP and, more so, of retinol. Administration of vitamin A i the form of a retinol dispersion or of chylomicrons containing newly absorbed vitamin A rapidly produced a redistribution of RBP from liver into plasma in association with an increase in plasma retinol concentration (6). Further studies showed that the level of translatable and total messenger RNA for RBP was not reduced or elevated in the livers of vitamin A-deficient rats (7, 8), suggesting that regulation by vitamin A status differentially affects post-translational events.

Among several rat hepatoma cell lines studied by Goodman and colleagues, only some lines were observed to synthesize and secrete immunoreactive RBP (4, 5). Synthesis and secretion from the MH_1C_1 or H_4II EC_3 cell lines could be stimulated by addition of retinol to a vitamin A-free medium (4) and was also enhanced in an additive fashion by addition of dexamethasone to cell cultures (9). The human hepatoma cell HepG2 is one of two cell lines established by Knowles, Howe, and Aden (10) and Aden et al. (11) from liver biopsies of children with primary hepatoblastoma and hepatocellular carcinoma. This cell has been found to synthesize and secrete a considerable number of plasma proteins (10, 11) and apolipoproteins (12, 13) and to have retained various cell surface receptors (14–18) including the asialoglycoprotein receptor characteristic of hepatocytes (19–21). This cell is also able to bind and degrade chylomicron remnants and to metabolize remnant retinyl esters (22). Although the presence of immunoreactive RBP in normal culture medium has been demonstrated by the immunodiffusion technique (10), no further studies have been reported on the regulation of RBP synthesis or secretion by this human hepatoma cell line. We have used a sensitive radioimmunoassay to quantitate RBP in cells and medium in order to explore the regulation of RBP synthesis and release by this cell.

METHODS

Cell culture

HepG2 cells, obtained from Dr. Barbara Knowles of the Wistar Institute, Philadelphia, PA, were grown routinely

Abbreviations: FCS, fetal calf serum; MEM, Eagle's modified minimal essential medium; RBP, retinol-binding protein.

[1]To whom correspondence should be addressed.

in T-75 flasks with 25 ml of Eagle's Minimum Essential Medium (MEM) (Flow Laboratories, Inc., McLean, VA) buffered with sodium bicarbonate and supplemented with gentamicin (50 μg/ml) and 10% fetal calf serum (FCS, Gibco Laboratories, Grand Island, NY). In some experiments, FCS was irradiated with ultraviolet light (two FS-20UV sunlamp bulbs for 13 hr or longer at a distance of 8 cm over a covered plastic Petri plate) to destroy all endogenous retinol. After treatment, sterile distilled water was added to compensate for any evaporation and the absence of retinol was confirmed by high performance liquid chromatography (23). Confluent cultures were trypsinized (0.25% trypsin), 2 ml of appropriate serum-supplemented medium was added, and cells were subcultured using a split ratio of 1:3 into T-75 flasks, after which 25 ml of medium with FCS was added. One passage is defined as one such 1:3 split of cells.

For experiments, cells were released with trypsin, diluted into appropriate media, plated into 35- or 60-mm plastic culture dishes, and incubated at 37°C in an atmosphere of 95% O_2, 5% CO_2 until nearly confluent. After each experiment, medium was removed and frozen until the time of assay. Cell monolayers were washed two times in phosphate-buffered saline, then scraped into this buffer. Cells were sonicated using a probe sonifier (Branson model 350) until no unbroken cells were observed by light microscopy; aliquots were removed immediately after sonification for immunoassay and protein measurement (24).

Immunoassays

The radioimmunoassay used for human serum RBP is a modification of that developed by Smith, Raz, and Goodman (25). Human serum RBP for standards and for immunization was purified from human plasma using ion exchange chromatography at pH 6 (26), gel filtration on Sephadex G-100 in 5 mM glycine–NaOH (pH 10), ion exchange chromatography at pH 8.3 (26), and gel filtration on Sephadex G-75 in 25 mM Tris buffer, pH 7.4. Fractions that produced single bands on polyacrylamide gel electrophoresis (PAGE) (27) were combined; these had an absorbance ratio, A330/A280, of 0.84. Portions of this material were stored in lyophilized form under nitrogen and in some cases were rechromatographed on a Waters I-125 Protein Analysis column (Milford, MA) in 0.05 M potassium phosphate buffer, pH 7.0, before iodination. ^{125}I-labeled RBP was prepared using 60–100 μg of RBP and a Radioiodination System kit (New England Nuclear, Boston, MA) utilizing lactoperoxidase. Labeled protein was separated from free iodine by chromatography on a Bio-Gel P-6DG column equilibrated with 0.07 M Tris–HCl, 0.07 M NaCl buffer, pH 8.6, and treated with 1 ml of 20% bovine serum albumin before use. Fractions were collected in buffer containing 20% BSA. Labeled RBP was dialyzed extensively; the mean percentage of remaining free iodine was 5.1 ± 1.4% for five preparations. When tested for homogeneity by gel electrophoresis (27), a single peak of ^{125}I comigrated with purified human serum RBP.

Antisera were raised in two rabbits immunized with 0.5 mg of purified human serum RBP in Freund's complete adjuvant and boosted twice with RBP in incomplete adjuvant, similar to the procedure of Muto and Goodman (28). Six weeks after the initial immunization, blood was collected and serum was prepared and stored at −70°C. Serum from the rabbit with the highest titer as determined by immunodiffusion was utilized in our assay. This antiserum produced a single arc after immunoelectrophoresis (29) and had a titer similar to commercially available antiserum to human RBP from Calbiochem-Behringwerke as determined by radial immunodiffusion. These antisera produced a line of identity after immunodiffusion when tested against human serum or concentrated medium from HepG2 cells. Antiserum was used at a dilution of 1:400 in the radioimmunoassay and precipitated approximately 45–70% of the ^{125}I-labeled RBP tracer. Duplicate samples of medium or of cell sonicates for immunoassay were incubated with diluted anti-RBP serum and ^{125}I-labeled RBP at 4°C for at least 20 hr. Longer incubations did not change the amount of bound labeled antigen. Antigen–antibody complexes were precipitated using 0.1 ml of goat anti-rabbit gamma globulin preparation (Calbiochem, San Diego, CA) as second antibody in the presence of bovine gamma globulin (200 μg/tube) and 2.25% polyethylene glycol (30). Pellets were counted for ^{125}I-labeled RBP for 5 min and data were calculated after the logit-log transformation of Rodbard, Bridson, and Rayford (31). Quality control samples consisted of dilutions of media from HepG2 cells that were frozen in small portions at −70°C and run in each assay. The interassay variability averaged 19%, whereas intraassay variability for ten replicates equaled 7.3%. The reactivity of FCS in this immunoassay was negligible. A comparison of radioimmunoassay results for HepG2 medium and cell sonicates treated with 1% Triton X-100 (32) indicated that no greater amount of RBP was released upon detergent treatment.

The presence of human serum transthyretin (prealbumin) was examined using antiserum from Calbiochem-Beringwerke and medium from cells after incubation for 24 hr in MEM. A 6-ml portion of medium was dialyzed overnight versus 2 liters of water, lyophilized, and reconstituted with 0.1 M potassium phosphate buffer, pH 7.0, to produce a 60-fold concentrated sample. Portions (10 μl) of this sample and of dilutions (up to 1:9) were placed in wells in 1% agarose and allowed to diffuse against 20 μl of antiserum in the center well. Human serum was run as a control in parallel with HepG2 medium. The same set of dilutions was also treated using antiserum to human RBP.

Human serum albumin secreted into the medium was quantitated using the radial immunodiffusion technique of Mancini, Carbonara, and Heremans (33). Antiserum to human serum albumin was incorporated at a dilution of

1:250 into 1% agarose. Samples of medium (10 μl) from cells incubated for 24 hr in MEM containing 0 or 1000 nM dexamethasone (Hexadrol; Organon, Orange, NJ) or of human serum albumin standards were pipetted into wells and allowed to diffuse at room temperature for 3 days before ring diameters were determined.

Preparation of chylomicrons and remnants

After cannulating the mesenteric lymph duct of a female Sprague-Dawley rat, lymph was collected while infusing saline through a second cannula placed in the duodenum (22). To obtain chylomicrons that were relatively poor in vitamin A, safflower oil was intermittently infused through the duodenal cannula while maintaining saline flow at 2 ml/hr. Chylomicrons enriched in vitamin A were collected after supplementing the oil with retinyl palmitate (10 mg/ml) and infusing 0.4 ml over a 1-hr period. Lymph was collected on ice in tubes containing enough EDTA and sodium azide to give a final concentration of 2 mM and 0.02%, respectively. After declotting and filtering, the lymph was layered under an equal volume of buffer (0.73% NaCl, 0.14 M disodium phosphate, 0.01% EDTA, pH 7.0) and centrifuged in a Beckman No. 40 rotor for 30 min at 17,500 rpm (20,000 *g*) at 4°C (34). The centrifuge tubes were sliced below the chylomicron layer which was collected; aliquots were analyzed for triglyceride (22) and retinol.

Chylomicron remnants were prepared by incubating chylomicrons in medium from cultured cells that secrete lipoprotein lipase (35). This medium was assayed for lipase activity (35) and added at 1 U activity (1 μmol/hr) per mg of chylomicron-triglyceride. For this study, 30 mg of chylomicron-triglyceride, 30 U of lipoprotein lipase activity, 1 g of bovine serum albumin, and 15 mg (protein) of human high density lipoprotein, added as a source of apoprotein C-II for lipase activation, were incubated in 18 ml of 0.1 M Tris buffer, pH 8, for 2 hr at 37°C. To isolate chylomicron remnants from the incubation mixture, aliquots were placed in centrifuge tubes and overlayered with an equal volume of pH 7.0 buffer (see above) containing sodium azide. After centrifugation in a Beckman No. 60 rotor for 16 hr at 40,000 rpm (105,000 *g*) at 4°C (34), chylomicron remnants in the upper 1.5 cm of the tube were collected. After extensive dialysis against buffer (without sodium azide) and finally against MEM, the chylomicron remnants were incubated with cells. Lipid analysis indicated that vitamin A-poor chylomicron remnants contained 9 nmol of total retinol/mg triglyceride and vitamin A-enriched remnants contained 39 nmol of total retinol/mg triglyceride.

RESULTS

Application of immunoassay for RBP

Our first goal was to determine whether RBP released by the HepG2 cell or in cell sonicates reacted identically to human serum RBP in our radioimmunoassay. As illustrated in **Fig. 1**, serial dilutions of media collected from HepG2 produced a dilution curve that could be superimposed on that for purified human serum RBP. Similar results were found when dilutions of cell sonicates were tested. It may also be noted that dilution curves were identical, whether cells had been cultured in the presence of normal FCS or irradiated FCS devoid of retinol (below). Thus, by this criterion, the antiserum to purified human serum RBP reacted in the same manner with our purified standard, with whole human serum (data not shown), and with RBP in HepG2 cell sonicates or after secretion from the cell.

The stability of RBP in the culture medium was tested in two experiments. In one, medium was collected from HepG2 cells after culture for 24 hr in serum-free MEM, a small portion was analyzed for RBP content, and a larger portion was transferred to cell-free plates for continued incubation at 37°C. After 72 hr, 88% of the original amount of RBP was measured in the medium by radioimmunoassay, indicating good stability of the protein after secretion. In a second experiment, ^{125}I-labeled RBP was added to the culture medium and incubated with HepG2 cells for 24 hr. Label that remained soluble after precipitation with trichloroacetic acid equaled 8.4% in the original ^{125}I-labeled RBP preparation, 8.5% after 24 hr of cell-free incubation at 37°C, and 9.5% after a similar incubation with cells. These experiments indicate that RBP is not readily degraded in the culture medium and, thus, that the accumu-

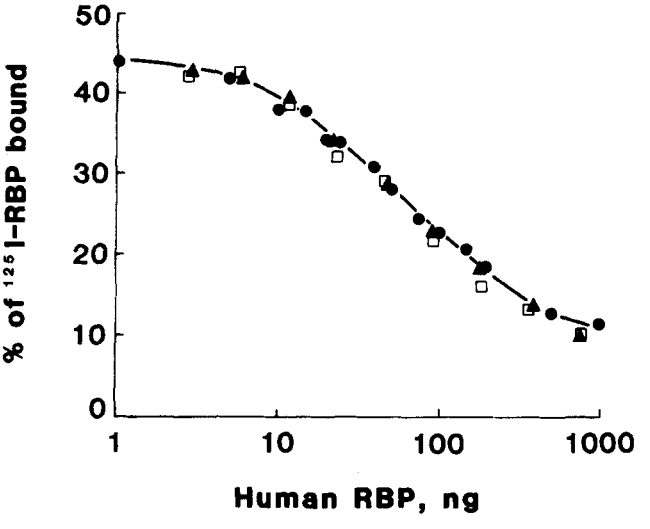

Fig. 1. Immunoreactivity of HepG2 medium from cells cultured in medium containing 10% normal or ultraviolet-treated FCS. Dilutions of the two media were compared with a standard curve generated using known concentrations of human RBP. Displacement curves for dilutions of cell sonicates and for human serum were also superimposable on the standard curve for human RBP. Closed circles (●), purified human serum RBP; closed triangles (▲), medium from cells grown with normal FCS; open squares (□), medium from cells grown with irradiated FCS.

lation of immunoreactive protein in this medium can be used to assess secretion from the HepG2 cell.

We also asked whether the HepG2 cells synthesize and secrete transthyretin, the protein with which RBP forms a non-covalent association in plasma. Media from HepG2 cells after 24 hr of incubation were concentrated 60-fold before immunodiffusion against appropriate antiserum for transthyretin or RBP. No reaction against anti-transthyretin was found for this concentrated medium, although human plasma reacted clearly. In contrast, concentrated medium or dilutions of medium (up to 1:8) as well as human plasma, formed visible precipitin lines with antiserum to RBP. Thus, these experiments using more concentrated media confirm those of Knowles et al. (10) and indicate that the HepG2 cell secretes very little, if any, immunoprecipitable transthyretin even though RBP, albumin, and other plasma proteins (10) are easily detected.

Effects of culture conditions

In preliminary work, the amount of secreted RBP was measured in medium collected from cells from several hours to 6 days after plating into culture dishes. When cells were plated at a density of approximately 10^6 cells/35-mm plate, secreted RBP could be detected 2 hr after plating. Daily secretion per mg of cell was greatest during the first 24 hr, then decreased significantly to about 60–70% of day 1 values and remained essentially constant during the next 6 days. When cells were plated at a much lower density, 0.2×10^6 cells/plate, daily RBP secretion per cell protein remained constant for at least 2 days. These patterns were observed both for HepG2 cultured with 10% or normal FCS or irradiated FCS. Thus, these data indicate that cell density and/or time after plating can affect the amount of RBP released from the HepG2 cell. We have not pursued these growth effects further; however, care was taken to make all platings within an experiment at a uniform density. Generally, at least 1×10^6 cells were plated and experiments were conducted 2–4 days later.

The effects of serum concentration on RBP synthesis and release were investigated. HepG2 cells were cultured for 24 hr in 0 to 20% FCS or 1 or 10% rat serum, and then incubated for 8 hr in serum-free medium. Data in **Table 1** show that secreted RBP was increased significantly when cells were preincubated with 5, 10, or 20% of serum. RBP remaining in cell sonicates was not affected. Thus, both the total amount of RBP and its distribution between cells and medium were dependent on serum concentration during the 24-hr preincubation period.

Data were compared from HepG2 cells grown with normal FCS or irradiated FCS under several experimental conditions. A consistent finding was that secreted and cell-associated RBP (per mg of cell protein) were significantly greater in those cells grown in irradiated serum for four or eight passages prior to plating for growth studies. Slopes of the protein content per culture versus time were not significantly different, suggesting that prolonged growth in the absence of retinol had no adverse effect on cellular mass. To ask how soon such an increase in RBP output could be observed after change to irradiated serum, we cultured HepG2 cells for one to four passages in T-75 flasks containing medium with 10% of either normal or irradiated FCS. After each passage, cells were plated in their respective media, grown for 3 days, then cultured for 24 hr in serum-free medium, after which secreted RBP and cellular RBP were assayed. After one passage, no significant effect of serum treatment was observed. However, at passage number two, cells in control medium secreted an average of 390 ng of RBP/mg cell protein and contained 430 ng/mg in the cell monolayer, whereas cells grown with irradiated serum secreted 630 ng/mg cell protein and contained 630 ng/mg in the monolayer ($P < 0.005$). A further example is evident in Table 3 (see below), in which cells grown with irradiated media for five passages contained and secreted twice as much RBP as those grown on normal FCS medium ($P < 0.001$); nonetheless, the distribution between cells and medium was normal.

TABLE 1. Effect of serum concentration on RBP content and secretion

Serum Concentration	RBP (ng per mg cell protein per 8 hr)					
	In Medium	% Change from Control	In Cells	% Change from Control	% of Total RBP in Medium	Total in Cells and Medium
0	555 ± 86[a]		285 ± 56[a]		66	840 ± 134[a]
1% FCS	610 ± 22[a,b]	+10	296 ± 19[a]	+4	67	905 ± 34[a,b]
5% FCS	742 ± 81[b,c]	+34	228 ± 23[a]	−20	77	971 ± 97[a,b]
10% FCS	782 ± 123[c]	+41	244 ± 36[a]	−14	76	1026 ± 159[a,b]
20% FCS	841 ± 82[c]	+52	230 ± 32[a]	−19	79	1071 ± 105[b]
1% Rat serum	567 ± 79[a]	−2	246 ± 34[a]	−14	70	813 ± 106[a]
10% Rat serum	839 ± 115[b]	+51	253 ± 33[a]	−11	77	1092 ± 120[b]

HepG2 cells were incubated for 24 hr with MEM containing the indicated percentage of FCS, after which serum-free MEM was added for 8 hr. Values represent RBP found in media and cell sonicates after this 8-hr incubation. Values are given as means ± SD for five plates except for 5% FCS where n = 4.

[a,b]Means in the same column bearing different letter superscripts are statistically different ($P < 0.05$) when tested by Tukey's test following a one-way analysis of variance.

TABLE 2. Effect of dexamethasone on RBP production and albumin secretion

Concentration of Dexamethasone	Cells Grown with Normal Serum				Cells Grown with Irradiated Serum			
	Cell Protein	RBP in Medium	Total RBP	Serum Albumin	Cell Protein	RBP in Medium	Total RBP	Serum Albumin
	mg	*ng/mg protein*		*μg/mg protein*	*mg*	*ng/mg protein*		*μg/mg protein*
0	1.39 ± 0.04	804 ± 22	1817 ± 33	14.5 ± 1.2	1.24 ± 0.08	983 ± 64	2202 ± 134	18.3 ± 0.9
10 nM	1.41 ± 0.04	782 ± 22	1763 ± 31		1.30 ± 0.09	934 ± 46	2075 ± 99	
100 nM	1.51 ± 0.31	751 ± 69	1737 ± 121		1.30 ± 0.06	916 ± 56	2109 ± 111	
1000 nM	1.60 ± 0.24	712 ± 123	1642 ± 236	16.6 ± 2.4	1.31 ± 0.09	914 ± 66	2069 ± 119	22.2 ± 1.9

Values are the mean ± SD. Conditions were tested in quadruplicate. Measurements were made after a 24-hr incubation with dexamethasone (see text). Each condition was tested for statistical significance by a one-way analysis of variance followed by Tukey's multiple range test. Cells incubated in medium containing irradiated serum at all concentrations of dexamethasone had significantly higher amounts of RBP in medium ($P < 0.001$), RBP in cells ($P < 0.001$), and total RBP ($P < 0.001$). Serum albumin secretion was slightly greater in cultures grown on irradiated FCS as compared to normal FCS ($P < 0.05$). No significant differences in RBP due to dexamethasone treatment were found.

Experiments were also conducted in which HepG2 cells were incubated for 24 hr in the presence of an exogenous glucocorticoid, dexamethasone, which had been found by others (4, 5) to stimulate RBP accumulation and secretion from rat hepatoma cell lines and to prevent the decline in RBP output from primary hepatocytes in culture (3). HepG2 cells that had been grown continuously in medium with 10% normal FCS or grown in medium containing 10% ultraviolet-irradiated FCS for two passages were cultured for 24 hr in serum-free media containing 0–1000 nM of dexamethasone. As shown in **Table 2**, we observed no stimulation of RBP secretion into media nor any increase of RBP in cells. Secretion of albumin was increased slightly as was total cell protein in control cells. Thus, this and a similar experiment failed to reveal a significant effect of dexamethasone on RBP output from the HepG2 cell.

Effects of retinol added in chylomicron remnants

Having observed that the HepG2 cell secretes RBP in the absence of retinol, we asked whether addition of retinol to the normal or retinol-depleted culture medium has any regulatory effect on this process. We have reported previously that the HepG2 cell can bind and internalize chylomicron remnants and hydrolyze retinyl esters (22) and, hence, in this study we asked whether remnant vitamin A also modulates RBP secretion. Varying concentrations of remnant vitamin A (> 90% as retinyl esters) were added to HepG2 cells grown previously with normal or irradiated serum. Remnants were prepared from lymph collected after vitamin A dosing so that both vitamin A-poor and vitamin A-enriched remnants could be obtained. Remnants were added to provide 1 to 10 μM total retinol in the medium, and equal amounts of remnant triglyceride from vitamin A-poor remnants were added as controls. As shown in **Table 3**, addition of retinol-enriched remnants during an 8-hr incubation produced a significant redistribution of RBP from cells into media. No effect of equal amounts of vitamin A-poor remnants was observed nor, in a separate experiment, was there any effect of vitamin A-containing remnants after their exposure to ultraviolet irradiation. The

TABLE 3. Effect of Vitamin A-enriched chylomicron remnants on secretion of RBP

Addition to Medium	Cells Grown with Normal Serum				Cells Grown with Irradiated Serum			
		In Medium	In Cells	% of Total Medium		In Medium	In Cells	% of Total Medium
		ng/mg protein				*ng/mg protein*		
None (MEM)	(4)	244 ± 19	440 ± 36	36	(4)	647 ± 106	831 ± 164	44
Remnant, 1 μM retinol	(4)	410 ± 59	213 ± 7	66	(4)	870 ± 75	489 ± 230	64
Remnant, control	(4)	295 ± 49	404 ± 25	42	(4)	587 ± 109	761 ± 46	44
Remnant, 2 μM retinol	(4)	391 ± 39	208 ± 25	65	(3)	867 ± 15	414 ± 74	68
Remnant, control	(4)	239 ± 39	425 ± 25	36	(3)	586 ± 90	767 ± 44	43
Remnant, 10 μM retinol	(3)	475 ± 115	183 ± 47	72				
Remnant, control	(3)	259 ± 10	399 ± 13	39				

Vitamin A-rich chylomicron remnants which provided the total retinol concentration indicated or an equivalent mass (based on triglyceride) of vitamin A-poor remnants (indicated as 'Remnant, control' for each remnant retinol concentration) were added to HepG2 cells that had been maintained in MEM containing 10% FCS or 10% ultraviolet-irradiated FCS for five passages. RBP levels were measured in media and cell sonicates after an 8-hr incubation of cells in MEM. Values are means ± SD of the number of plates shown in parentheses. The amounts of RBP in medium and cells after addition of vitamin A-enriched remnants differed significantly from the amounts in respective controls ($P < 0.005$) at each vitamin A concentration tested. Total RBP amounts did not differ within groups of cells previously grown in normal FCS or ultraviolet-irradiated FCS. However, those cells grown in irradiated FCS for five passages had significantly greater ($P < 0.0001$) amounts of RBP in both medium and cells as compared to cells maintained with normal FCS. The RBP contents of medium and cells was statistically equivalent for the three concentrations of control (vitamin A-poor) remnants added.

dependency of RBP secretion on the dose of remnant retinol was also determined (**Fig. 2**). In this experiment, as little as 0.05 μM retinol significantly reduced the cellular content of RBP whereas RBP in the medium increased by 16%. Thus in cell cultures without remnants, 44% of total RBP was found in cells after 8 hrs, whereas less than 32% remained cell-associated when 0.05 μM retinol was added in the form of remnants ($P < 0.05$). When remnant concentration was increased up to approximately 1 μM of total retinol, an even greater redistribution of RBP from cells to medium was observed so that only 14% of total RBP remained cell-associated after 8 hr.

DISCUSSION

The availability of the HepG2 cell line has presented an opportunity to explore regulation of RBP secretion in a liver cell of human origin. Although transformed, HepG2 has retained a number of properties associated with normal hepatocytes including the ability to bind and internalize asialoglycoproteins (19–21). The original report of Knowles et al. (10) indicated that immunoreactive RBP is secreted by HepG2, but no further studies of RBP secretion have been reported. We have found that RBP is released from HepG2 both in the absence and presence of retinol. Indeed, a significant increase in RBP in both cells and medium was measured after growth of HepG2 with ultraviolet-irradiated FCS for two to eight passages. The distribution between cells and medium was essentially normal. In spite of its ability to secrete RBP in the absence of retinol, HepG2 responds to direct addition of retinoid by a rapid secretion of RBP into the medium. In these studies, we added vitamin A in the form of chylomicron remnants, the lipoprotein that transports newly assimilated vitamin A from the intestine to liver. In previous studies, we have shown that this cell can bind chylomicron remnants in a saturable manner and that binding of the remnant to the HepG2 surface has several features of normal hepatic uptake including stimulation by apolipoprotein E and discrimination between remnants and intact chylomicrons (22). In the present study, addition of retinoid (retinyl esters and retinol) carried by chylomicron remnants produced a concentration-dependent release of RBP from cells to medium, as shown in Table 3 and Fig. 2. Together, these results indicate that HepG2 synthesizes and secretes RBP both constitutively in the absence of retinol and at an enhanced rate after retinoid addition. These results are consistent with those reported by Goodman and colleagues (4, 5, 9) who used rat hepatomas positive for RBP production; in these cells release of RBP was measurable in serum-free, retinol-free medium and increased significantly after addition of a dispersion of retinol. Primary hepatocytes of retinol-depleted rats (2, 3) have also responded to addition of retinol with an increased output of RBP; however, it has not yet been reported whether some secretion of RBP, presumably as apoRBP, also occurs constitutively from these cells or from the intact liver.

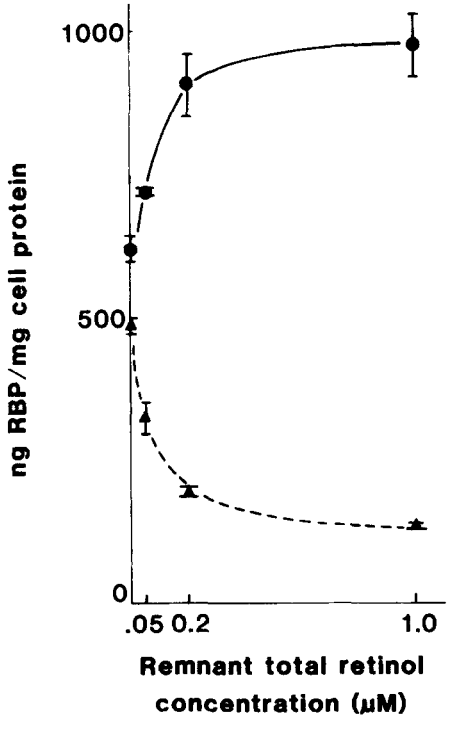

Fig. 2. Dose-dependent response of RBP secretion with increasing concentrations of total retinol added in chylomicron remnants. Remnants were added to HepG2 cells in serum-free medium for 8 hr. Cells had previously been maintained in medium with 10% normal FCS. Closed circles show RBP in medium and closed triangles show RBP remaining in cells. Each point is the mean ± 1 SEM of four plates.

Certain quantitative comparisons can be made of the relative rates of retinol and RBP release from HepG2, and of the release of RBP from this cell as compared to other hepatoma cell lines. In the present study, RBP secretion during 8 hr following remnant addition resulted in release of approximately 400 ng (20 pmol) of RBP per mg of HepG2 cell protein (Table 2). In an 8-hr time course following remnant binding, Lenich and Ross (22) found that approximately 6–15 pmol of chylomicron-derived retinoid per mg of protein was released to the medium as retinol. Thus, the amount of RBP released equals or exceeds that of retinol even when remnant retinoid is available, suggesting that at least a portion of the RBP secreted in the presence of remnants is apoRBP. It is also possible to compare roughly the rates of secretion of RBP from HepG2 to that observed for other hepatoma cell lines. Such a comparison indicates that synthesis and secretion rates for HepG2 are relatively high. When RBP was measured in cells and medium from HepG2 and the human hepatoma Hep3B (10) grown with 10% FCS under similar conditions, HepG2 contained and secreted approximately 10-fold more RBP

than did Hep3B (A. C. Ross, unpublished observation). It also appears that HepG2 secretes RBP at a rate some 10–20 times greater than the rat hepatoma H_4II EC_3.[2] Primary rat hepatocytes also secreted RBP at relatively high initial rates (98–113 ng per 2 × 10^6 cells/hr), but this rate was observed to decrease rapidly in culture unless dexamethasone (10 nM) was added (3). In the HepG2 system, RBP output was continuously high and addition of dexamethasone to MEM did not increase the total amount of RBP or the portion secreted into medium. It seems likely that further direct comparisons of these various hepatoma lines and hepatocytes could prove useful to define the genetic, hormonal, or other regulatory mechanisms that control expression of RBP synthesis and secretion.

A notable difference is that the rat hepatoma lines examined (9) secrete immunoreactive transthyretin as well as RBP, although retinol was found to regulate only the secretion of RBP (9). In the original report of Knowles et al. (10), no transthyretin (prealbumin) was detected in 10-fold concentrated medium from the HepG2 cell and we have failed to detect this protein even after concentrating medium 60-fold. These results with HepG2 provide an additional line of evidence that secretion of RBP can occur independently, and is likely to be regulated independently from that of transthyretin.

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[2]After growth with 10% FCS, HepG2 secreted an average of 782 ng RBP/mg cell protein in 8 hr (Table 1). Assuming 10^6 cells equal 0.49 mg of HepG2 protein (19), this equals 380 ng RBP/10^6 cells in 8 hr. The rat hepatoma H_4II EC_3 was found by Smith et al. (ref. 4, Fig. 4) to secrete approximately 15 pmol RBP/6 × 10^6 cells in 24 hr during culture in 10% FCS, equal to approximately 300 ng/6 × 10^6 cells in 24 hr, or about 17 ng/10^6 cells per 8 hr.

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