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Studies on the synthesis and secretion of transthyretin by the human hepatoma cell line Hep G2

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Transthyretin (TTR) is a circulatory protein which plays an important role in the transport of both thyroid hormone and retinol. Hep G2 cells, a human hepatoma-derived cell line, have been used extensively in studies of protein secretion by liver cells. The original description of this cell line indicated that this line, unlike primary hepatocytes, does not secrete TTR. We now report studies which reexamine the ability of Hep G2 cells to synthesize and secrete TTR. For this purpose, total RNA was isolated from Hep G2 cells grown on both uncoated and collagen-coated plastic plates and was examined for TTR expression by Northern blot analysis. TTR mRNA was found to be present in nearly equal amounts in Hep G2 cells cultured in either condition. When Hep G2 cells were cultured in [^{35}S]methionine-containing medium, the cells were found both to synthesize and to secrete immunoprecipitable [^{35}S]TTR. Hep G2 cells were found, by sensitive and specific radioimmunoassay, to contain 142 ± 91 ng TTR/10^6 cells and to secrete TTR into the medium at a nearly constant rate for at least 24 h after medium change. Our data demonstrate that Hep G2 cells do synthesize and secrete TTR and suggest that this cell line might be useful for studies of the secretion of TTR.

Liver cell; Protein secretion; Secretory protein; Retinol-binding protein

1. INTRODUCTION

Plasma transthyretin (TTR) circulates as a 54 980 dalton tetramer composed of 4 identical subunits [1,2]. TTR has high affinity binding sites for both thyroxine [1,3,4] and plasma retinol-binding protein (RBP) [4–6]; hence, TTR plays an important role in the transport of both thyroid hormone and retinol in the circulation. Synthesis of TTR occurs primarily in the liver and the choroid plexus of the brain [7–9], although other tissues have been reported to express TTR in much lesser amounts [7–11]. Additionally, neoplastic tissues, including choroid plexus papillomas, glucagonomas, and gut carcinomas have been reported to synthesize TTR [12,13].

TTR is synthesized with an N-terminal signal peptide which is cleaved from the nascent polypeptide in the endoplasmic reticulum [7]. At present however, little is known regarding how TTR is secreted. For instance, very little information is available regarding the possible assembly of the 4 TTR subunits within the secretory pathway. Docherty et al. [14] have reported studies exploring the subunit assembly of TTR in microinjected *Xenopus* oocytes. These authors concluded from their studies that liver- and choroid plexus-specific factors may be required for the correct subunit assembly of TTR. Other studies exploring the factors which influence TTR synthesis and secretion into body fluids are also limited. Dixon and Goodman, employing isolated and cultured primary rat hepatocytes, demonstrated that dexamethasone could maintain TTR secretion rates, whereas TTR secretion rates, otherwise, were observed to decline in untreated primary cultures [15,16]. Studies of TTR secretion by cells have been limited by the lack of a useful cell line able to synthesize and secrete TTR. Hep G2 cells, a human hepatoma-derived cell line [17], have been used extensively to study protein synthesis and secretion [18–20]; however, the original report, by Knowles et al. describing this line, indicated that Hep G2 cells do not secrete TTR into the culture medium [17]. While conducting other studies with Hep G2 cells, we observed that Hep G2 cells are indeed able to synthesize and secrete TTR. The present report describes our characterization of the synthesis and secretion of TTR by Hep G2 cells.

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2. MATERIALS AND METHODS

Hep G2 and Hep 3B cells were obtained from the American Type Culture Collection (Rockville, Maryland, USA). For all of our studies, cells were maintained in Dulbecco's Modified Eagle's Medium (DME) containing low glucose, 10% fetal calf serum, and penicillin/streptomycin (100 units each/ml) at 37°C in an atmosphere of 8% CO_2. All tissue culture reagents were obtained from Gibco Laboratories (Grand Island, New York, USA). Cultures of Hep G2

cells were routinely maintained on collagen-coated plates which were prepared as described by Dixon et al. [21].

The presence of TTR mRNA was ascertained by Northern blot analysis of total RNA prepared from confluent cultures of Hep G2 and Hep 3B cells. Total RNA was isolated from these cells using the method of Chomczynski and Sacchi [22]. Northern blot analysis was carried out exactly as described by Soprano et al. [7] using the 500-bp human TTR cDNA pHTT1 [7].

Cellular and media levels of TTR protein were measured by radioimmunoassay (RIA). The RIA was carried out exactly as described for the RIA of human RBP [23] except that a monospecific rabbit antiserum directed against human plasma TTR was used in the RIA incubations. This antiserum did not recognize bovine TTR. The Hep G2 cells and their culture media were treated exactly as described by Dixon and Goodman [15,16] for measurement of TTR levels in primary cultures of rat hepatocytes and in the culture media. For our studies, the culture medium was changed and at 2, 4, 6, and 24 h after this change the cells and media were taken for TTR determination.

For metabolic labeling and immunoprecipitation studies, cultures of Hep G2 cells in 100 cm^2 plates (approximately 90% confluent) were washed twice with phosphate-buffered saline (PBS) and were incubated with 4 ml of methionine-free Minimal Eagle's Media supplemented with 200 μCi [^{35}S]methionine (specific activity 1000 Ci/mmol, Amersham UK). The cells were incubated in this medium for 5 h at 37°C. After incubation, the medium was collected, passed through a 45 μm filter to remove debris, and used directly for immunoprecipitation. Cells were washed twice with PBS, scraped from the plate, and concentrated by low speed centrifugation. The cells were resuspended in 1 ml potassium phosphate buffer, pH 7.5, and lysed by several passages through a 25-gauge syringe needle. Immunoprecipitations were performed as described by Adrian et al. [24]. Analysis of the immunoprecipitated proteins was performed by SDS-PAGE on 15% discontinuous gels [25]. Gels were fixed for 30 min in 10% trichloroacetic acid, 10% acetic acid, and 40% methanol, soaked in Amplify (Amersham Limited), dried and exposed to X-Ray film using intensifying screens.

3. RESULTS

The original description of Hep G2 and Hep 3B cells by Knowles et al. indicated that these two hepatoma-derived cell lines do not secrete TTR into the culture medium [17]. These observations were based on the inability of anti-human TTR antiserum to recognize TTR in the culture medium on Ouchterlony double-diffusion plates. While carrying out other studies of the anatomic sites of RBP expression in human placental tissue, we employed total RNA prepared from Hep G2 cells as the positive control for RBP expression in our Northern blots (since RBP is known to be synthesized by Hep G2 cells [17,20]). Upon stripping our blots and reprobing with a cDNA for human TTR, unexpectedly we observed a positive signal for TTR in the lanes containing Hep G2 total RNA. This observation led us to undertake a systematic characterization of the ability of Hep G2 cells to synthesize TTR.

Northern blot analysis of total RNA prepared from both Hep G2 and Hep 3B cultures indicates that TTR mRNA is present in both of these cell lines. Figure 1 shows Northern blots for TTR mRNA in total RNA preparations from 2 cultures each of both Hep G2 and Hep 3B cells maintained on uncoated plastic plates and from cultures of Hep G2 cells maintained on collagen-coated plastic plates. It is clear from Fig. 1, that the

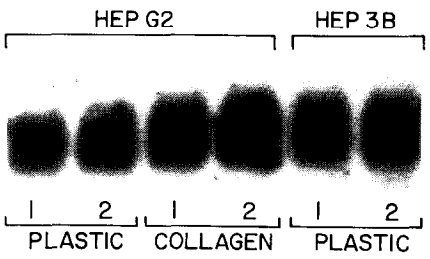

Fig. 1. Northern blot for TTR mRNA in total RNA prepared from Hep G2 and Hep 3B cells. Hep G2 cells were cultured in both uncoated and collagen-coated plastic plates. The Hep 3B cells were cultured solely in uncoated plastic plates. Each lane contains 20 μg of total RNA and the designations A and B indicate RNA preparations from duplicate cell preparations. This blot was exposed for 50 h.

mRNA is expressed by both Hep G2 and Hep 3B cells and that the culture of Hep G2 cells on collagen-coated plates does not markedly influence the level of TTR expression in these cells.

We next asked if Hep G2 cells are able to synthesize and secrete TTR. For this purpose, Hep G2 cells were metabolically labeled by culturing them in [^{35}S]methionine-containing medium. Newly synthesized TTR was immunoprecipitated with anti-human TTR antiserum. The results of these studies are presented in Fig. 2. This figure indicates that [^{35}S]TTR is both synthesized and secreted by Hep G2 cultures. Cellular levels of TTR were determined by specific RIA for human TTR from 8 plates of Hep G2 cells. The mean ($\pm$SD) level of TTR present in these 8 cultures was 142

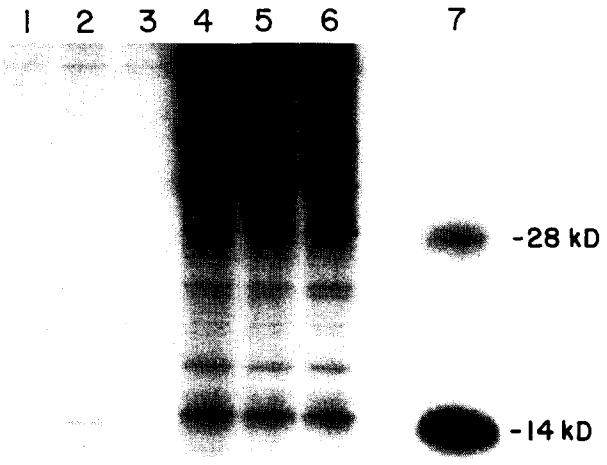

Fig. 2. SDS-PAGE of newly synthesized [^{35}S]TTR immunoprecipitated from Hep G2 cells and medium from over the cells. Lanes 1, 2, and 3 respectively, are Hep G2 media which have been immunoprecipitated with non-immune rabbit serum, specific anti-human TTR, and specific anti-human TTR plus an excess of unlabeled purified human TTR. Lanes 4, 5, and 6 respectively, are Hep G2 cell homogenates which have been immunoprecipitated with non-immune rabbit serum, specific anti-human TTR, and specific anti-human TTR plus an excess of unlabeled purified human TTR. Lane 7 is a standard of purified human [^{125}I]TTR.

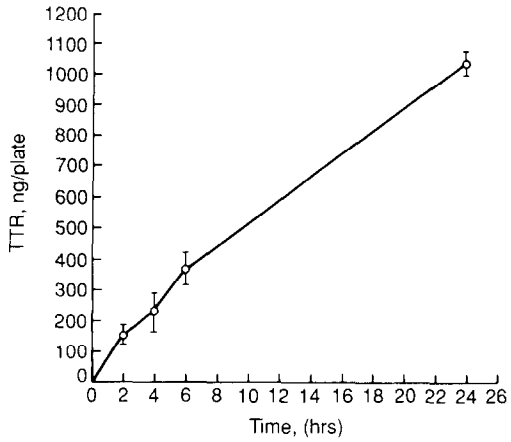

Fig. 3. Time course of TTR accumulation in Hep G2 culture media. Fresh media was applied to nearly confluent Hep G2 cells (approximately 1.0×10^6 cells/plate) at 0 h. Media was taken 2, 4, 6, and 24 h after this change of media for measurement of human TTR levels by RIA. The values are plotted as the mean ± SD with $n = 4$ for each time point.

(±91) ng TTR/10^6 cells. The time course of TTR accumulation in the Hep G2 culture medium is given in Fig. 3. This figure demonstrates that Hep G2 cells secrete TTR at a constant rate for at least 24 h after change of medium.

4. DISCUSSION

The data presented above demonstrate that Hep G2 cells synthesize and secrete TTR at levels which are comparable to those of primary rat hepatocytes in culture. Dixon and Goodman reported that primary rat hepatocytes contain approximately 150 ng TTR/10^6 cells [15,16]. In our studies the mean cellular level of TTR in Hep G2 cells was found to be 142 ng TTR/10^6 cells. Thus, the cellular levels of TTR in primary rat hepatocytes and in Hep G2 cells are very similar. Primary rat hepatocytes have been reported to secrete TTR into the culture medium at a rate of 1.0–1.2 μg TTR/24 h/10^6 cells [15,16]. In our studies of TTR secretion by Hep G2 cells, the average rate of accumulation of TTR in the medium was found to be 1.0 μg TTR/24 h/10^6 cells. Considering these very similar cellular TTR levels and secretion rates and the relatively simple procedures needed for the culture of Hep G2 cells, it would appear that Hep G2 cells might be very useful for studies of TTR synthesis and secretion by hepatic cells.

Hep G2 cells have been employed in studies addressing the transcriptional control of the mouse TTR gene [26]. If this cell line were to be used in studies of the regulation of the human gene, the presence of endogenous TTR mRNA will need to be taken into consideration. In addition, Hep G2 cells have been employed to demonstrate receptor mediated uptake of TTR [27]. Since the liver is in fact the major organ for the catabolism of TTR [28], this cell line may additionally be useful for studies addressing both the synthesis and the degradation of TTR.

It is not fully clear to us why previous investigators have not detected TTR synthesis in Hep G2 cells. The original work by Knowles et al. [17] describing this cell line and the lack of TTR accumulation in the culture medium employed an insensitive technique (radial immunodiffusion) for detecting TTR presence in the culture medium. Later work by Marinari et al. [20], using the same radial immunodiffusion techniques, confirmed that TTR could not be detected in the medium from Hep G2 cells. We feel it was very likely that these radial immunodiffucion assays were too insensitive to detect the levels of TTR present in the medium. Neither Knowles et al. [17] nor Marinari et al. [20] provide information regarding their lower limit of detection for TTR in Hep G2 conditioned media. We are not aware of any previous studies which examined Hep G2 cells for the presence of TTR mRNA.

Alternatively, it may be possible that we were working with an altered variant of the original Hep G2 cells described by Knowles et al. [17]. Our cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and would be representative of the Hep G2 cells in this collection. To further pursue the possibility that our Hep G2 cells were not a variant Hep G2 subclone, we obtained Hep G2 cells from several laboratories in the United States and Europe and screened them by RIA for the presence of TTR in the cells and culture media. All of these different laboratory stocks were found both to synthesize and to secrete TTR. Although it is not possible to be certain that the cells which we examined were not variants of the originally described Hep G2 cells, it is clear that the Hep G2 cells used in many laboratories throughout the world today, for a variety of studies, synthesize and secrete TTR.

In summary, the studies reported above indicate that Hep G2 cells can be used in studies exploring the TTR secretory pathway and the factors which influence this pathway. Little information is presently available regarding the biochemical factors which regulate and direct TTR secretion from the liver. Through the use of Hep G2 cultures, studies exploring hepatic secretion of TTR can be readily undertaken. In addition, our data would indicate that studies exploring possible linkages between the TTR and RBP secretory pathways also can be undertaken with Hep G2 cells. Such studies might provide much useful information regarding both TTR secretion and the factors important for RBP secretion from the liver.

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