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Secretion of apolipoprotein B in serum-free cultures of human hepatoma cell line, HepG2

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We have developed a defined medium which can maintain efficient growth of HepG2 cells sustaining the synthesis of a variety of plasma proteins including apolipoprotein B. This defined system was used to investigate long-term effects of insulin, estrogen, triiodothyronine, cholesterol, and oleate on the growth pattern of HepG2 cells and secretion rate of apolipoprotein B. Oleate and triiodothyronine caused significant increases in secretion of apolipoprotein B. The stimulatory effect of triiodothyronine was only observed after long (6 days) exposure of cells to the hormone. In contrast, insulin caused up to a 4-fold decrease in the secretion rate of apolipoprotein B during the early growth periods. This inhibitory effect appeared to be partially abolished after 6 days. Our data suggest that some important questions on regulation of apolipoprotein B expression can be addressed by the long-term culture of HepG2 cells in defined medium.

Apolipoprotein B; Protein secretion; HepG2 cell; Serum-free medium

1. INTRODUCTION

Human hepatoma cell line, HepG2, synthesizes a broad spectrum of plasma proteins and secretes many of these into the medium [1,2]. The HepG2 system has been used extensively as a model to study the regulation of the synthesis and secretion of a number of human apolipoproteins including apolipoprotein B (apoB) [3–5]. ApoB is the major protein constituent of the human very low density lipoprotein (VLDL), comprising approximately 40% of the total protein [6]. A better understanding of the regulation of apoB synthesis is of considerable interest because of its essential role in the secretion of hepatic VLDL and in the interaction of low density lipoprotein (LDL) with its receptors. Many studies on the regulation of apoB synthesis and secretion have focused on the effects of various nutritional and hormonal factors in short-term cultures of HepG2 cells in serum-free medium [7–9]. In most studies, cells grown in medium supplemented with 10% FBS were transferred to serum-free medium and the effects of various factors were observed for a relatively short period of time (24–48 h). Long-term responses to these factors have not been rigorously examined, due to limited viability of the culture systems employed.

Our study was undertaken to examine the direct action of various factors on the synthesis of apoB in long-term cultures of HepG2 cells. To achieve this goal, we have developed a defined medium which can maintain efficient growth of HepG2 cells sustaining the synthesis of a variety of plasma proteins including apoB. This defined system has made it possible to test the direct effect of various hormonal and nutritional factors, because the cells do not require serum, hormones, or other macromolecular medium supplements for the synthesis of apoB. This report describes the characteristics of this serum-free culture system and the long-term effects of insulin, estrogen, triiodothyronine (T_3), cholesterol, and oleate on the growth pattern of HepG2 cells and secretion of apoB.

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Abbreviations: apoB, apolipoprotein B100; MEM, Eagle's minimum essential medium; α-MEM, alpha modification of Eagle's minimum essential medium; T_3, 3,3′,5-triiodo-L-thyronine; BSA, bovine serum albumin; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; FBS, fetal bovine serum

2. MATERIALS AND METHODS

2.1. *Materials*

HepG2 cells were obtained from ATCC (HB 8065). Cell culture media and reagents were from Gibco (Paisley, UK). Estrogen, T_3, oleic acid, BSA, cholesterol and other common laboratory reagents were from Sigma (St. Louis, MO). ApoB antibody was from Atlantic Antibodies (Stillwater, MN). Protein assay kit was from Bio-Rad (Richmond, CA).

2.2. *Cell culture*

2.2.1. Media

The defined media reported by Darlington et al. [10] was extensively modified to prepare medium A and medium B. Basal medium consisted of 3 parts MEM (basal medium A) or α-MEM (basal medium B) and one part Waymouth's MB 752/1. Medium A was prepared by

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supplementing basal medium A with 2 mM L-glutamine, 1 × antibiotic-antimycotic solution (100 × solution contains 10000 units penicillin, 10000 μg streptomycin, 25 μg amphotericin B/ml and Fungizone in 0.85% saline), 3×10^{-8} M sodium selenite, 1.0 mg/l *i*-inositol, 8.0 mg/l thymidine, 0.1 mM sodium pyruvate and trace elements (0.05 mg/l $CuSO_4.5H_2O$, 0.016 mg/l $MnSO_4.H_2O$, 0.03 mg/l $ZnSO_4.7H_2O$, 0.024 mg/l $Mo_7O_{24}.4H_2O$, 0.022 mg/l $CoCl_2.6H_2O$). Medium B was prepared by supplementing basal medium B as above except for sodium pyruvate.

2.2.2. Serum-free growth experiments

Approximately 1×10^5 cells were plated in each well of several multi-well dishes in complete medium (α-MEM, 10% fetal bovine serum) and were grown for 24 h at 37°C, 5% CO_2. The medium was removed, cells were washed 3 × with Earle's balanced salt solution, and were incubated in serum-free medium B supplemented with one of the following factors: insulin (35 nM), estrogen (20 nM), cholesterol (50 μg/ml), oleic acid-albumin complexes (prepared according to [11]) (0.4 M oleate bound to 1.5% BSA), and T_3 (20 nM). Every two days, media were removed from duplicate wells and cells were lysed with 50 mM Tris-HCl, pH 7.5, 5 M guanidine isothiocyanate, 25 mM EDTA, 1% β-mercaptoethanol. Media were changed for the remaining wells at the same time and the experiment was continued. Time zero was defined as 24 h after replating.

2.3. *Isolation of total cellular RNA*

Total HepG2 RNA was extracted essentially as described by Han et al. [12].

2.4. *Determination of total protein, total RNA, and apoB concentration*

Total protein was determined in duplicate by the Bio-Rad dye-binding microassay according to the kit's instructions. BSA was used as standard to calibrate the assay. Total RNA was determined by measuring the absorbance of the RNA at 260 nm (1 A_{260} absorbance unit equals approximately 50 μg/ml [13]). All RNA samples had A_{260}/A_{280} ratios of higher than 1.8, indicating their purity. Media samples were concentrated about 10–15-fold by placing sucrose outside dialysis bags (M_r 3000 cutoff) and the apoB concentration was determined in triplicate by electroimmunoassay [14]. The apoB assay was calibrated by dilution of a set of apoB standards.

3. RESULTS AND DISCUSSION

We extensively modified the defined medium reported by Darlington et al. [10] and developed two types of defined media which could support growth of HepG2 cells. The growth patterns of cells in these serum-free media were compared with that in serum-containing complete medium. Fig. 1 shows the growth patterns of HepG2 cells in these three types of media as estimated by total protein production. Both serum-free media A and B were capable of sustaining cell growth and proliferation. The rates of growth in the defined media were similar to that in complete media. However, the final density of the cells in the complete medium was higher than those in medium A and medium B by about 20 and 9%, respectively. Cells grown in both medium A and medium B expressed and secreted a wide range of plasma proteins based on SDS-PAGE analysis of the cells and media (data not shown). Medium B was used in our subsequent growth experiments as it performed superior to medium A.

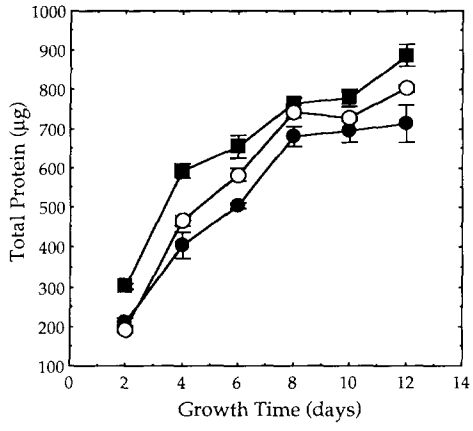

Fig. 1. Proliferation of HepG2 cells in complete medium and serum-free media. Growth was assessed by measuring cellular protein. (■) Cells grown in complete medium containing 10% FBS; (●) cells grown in defined, serum-free medium A; (○) cells grown in defined, serum-free medium B.

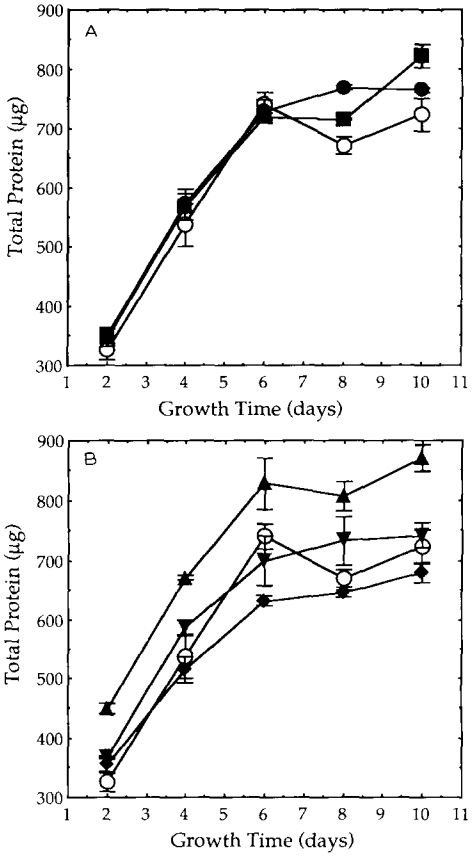

Fig. 2. Effects of various hormonal and nutritional factors on proliferation of HepG2 cells grown in defined medium. Growth was assessed by measuring cellular protein. (A) Effects of cholesterol and oleate. (○) Control, no supplementation; (●) cholesterol; (■) oleate. (B) Effects of insulin, estrogen and T_3. (○) Control; (▲) insulin; (▼) estrogen; (♦) T_3.

HepG2 cells were grown in medium B and medium B supplemented with insulin, estrogen, T_3, cholesterol, and oleate. Fig. 2 shows the proliferation patterns of cells grown in various media as estimated by total protein content. No significant changes in total protein were observed when cells were grown in medium B supplemented with cholesterol, estrogen, or T_3 compared to control. However, supplementation with insulin and oleate caused significant increases in total protein production. Increased proliferation was observed at all stages with insulin, but was only noticed at later stages (8 and 10 days) with oleate. Analysis of total RNA from the same cultures resulted in similar observations for insulin, oleate, cholesterol, and T_3 (Fig. 3). Total RNA was, however, increased in the presence of estrogen. The extent of the increase in total RNA did not correlate well with that in total protein. An interesting finding was that increased RNA levels, induced by insulin, estrogen and oleate, returned to normal levels at 9 days of growth. This rapid decline may be due to overpopulation of cells at this stage and lack of adequate nutrients to sustain continued RNA synthesis.

The production of apoB by HepG2 cells was monitored by measuring the concentration of apoB in the media. ApoB secretion rates were expressed as μg/mg of total protein per h. Fig. 4 shows the pattern of apoB production in defined medium B supplemented with hormonal and nutritional factors. HepG2 cells grown in defined medium B secreted apoB after 2 days at a rate of 0.253 ± 0.025 μg/mg per h, comparable to that previously reported in short-term cultures [9]. However, after 2 days, the rate was considerably increased so that after 8 days apoB was being accumulated at 0.963 ± 0.050 μg/mg per h. This increase clearly indicates the viability of HepG2 cells in the defined medium. The apoB secretion rate was unaffected by cholesterol and estrogen, but was significantly stimulated by T_3 (37% elevation at 10 days) and oleate (20% elevation at 10 days) (Fig. 4). The stimulatory effect of oleate on apoB production has been previously reported by several groups [5,8]. Our data confirm these previous observations and provide evidence for continued stimulation over a 10-day period in culture. The considerable stimulatory effect of T_3 on apoB production has not been previously reported and is quite interesting. T_3 increased apoB production despite the fact that no increases in RNA or total protein were observed, suggesting that the hor-

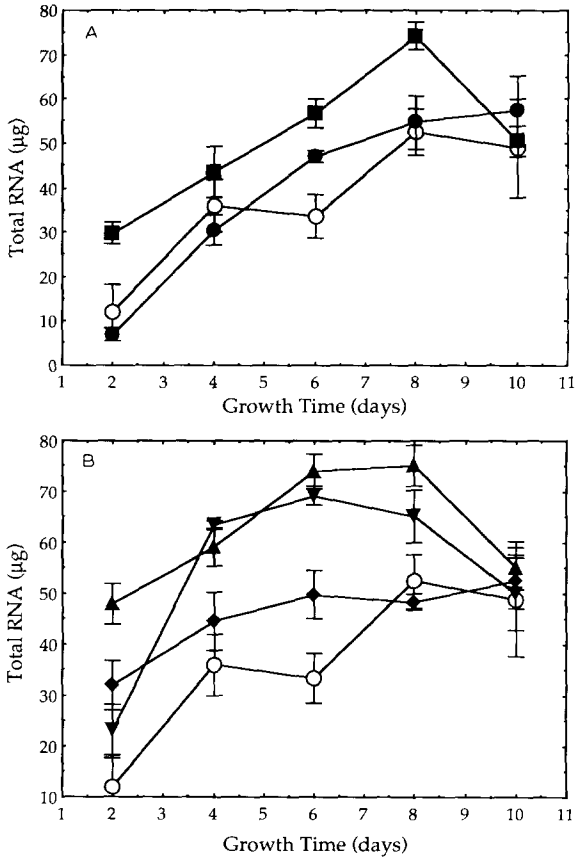

Fig. 3. Effects of various hormonal and nutritional factors on total RNA production by HepG2 cells grown in defined medium. (A) Effects of cholesterol and oleate. (○) Control, no supplementation; (●) cholesterol; (■) oleate. (B) Effects of insulin, estrogen and T_3. (○) Control; (▲) insulin; (▼) estrogen; (◆) T_3.

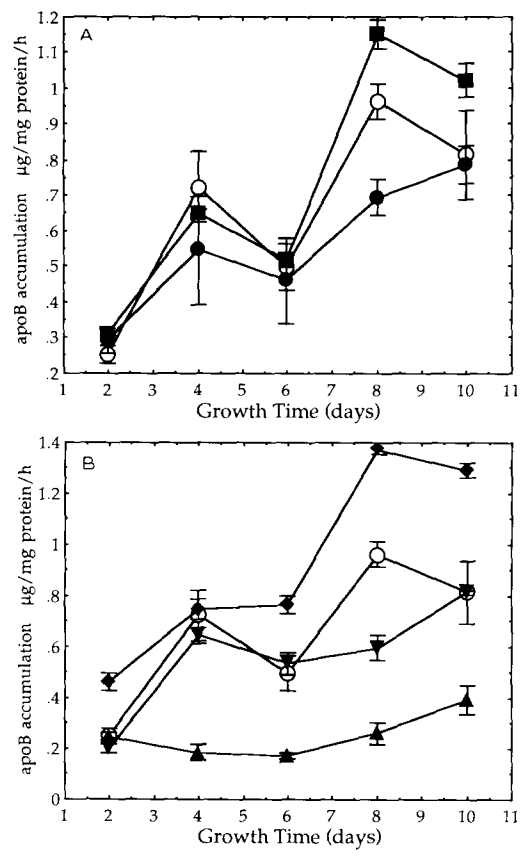

Fig. 4. Effects of various hormonal and nutritional factors on apoB production by HepG2 cells grown in defined medium. (A) Effects of cholesterol and oleate. (○) Control, no supplementation; (●) cholesterol; (■) oleate. (B) Effects of insulin, estrogen and T_3. (○) Control; (▲) insulin; (▼) estrogen; (◆) T_3.

mone specifically stimulated apoB expression. Our data on the effect of cholesterol on apoB accumulation did not agree with a recent report [15] which indicated a stimulatory effect. This contradiction may be due to the fact that we did not use high concentrations of cholesterol. At a concentration of 50 μg/ml cholesterol, used in our study, Fuki et al. [15] observed only about 25–30% increase in apoB. In contrast to oleate and T_3, insulin caused a significant decline in production rate of apoB by HepG2 cells. By the 4th day, insulin had caused about a 4-fold decrease in apoB production. However, after the 6th day, cells appeared to increase their production of apoB but the apoB level was still about 52% lower than the control at the 10th day. This partial reversal of insulin effect was unexpected. The data supported observations in short-term cultures of HepG2 cells [7–9] indicating that insulin inhibits apoB secretion despite an increase in total protein mass.

In conclusion, the development of a long-term culture system has permitted us to address a number of important questions regarding regulation of apoB gene expression. Our data suggest that in long-term cultures, apoB production can be significantly stimulated by oleate and T_3, but inhibited by insulin. The inhibitory effect of insulin appears to be partially lost after long exposure of cells to this hormone. Further investigations are underway to investigate the role of these factors in regulating apoB expression, especially at the translational and posttranslational levels.

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