Thyroxine-binding Globulin Synthesis by Hepatocarcinoma Cells in Continuous Culture: Effect of Physiological Concentrations of Thyroxine

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SUMMARY: Thyroxine-binding globulin (TBG) synthesis and secretion were demonstrated in a continuous cell culture line, NCLP-6-E, of Rhesus monkey hepatocarcinoma cells. The cells were shown to survive and grow normally for up to 5 days in the absence of serum, thus permitting study of TBG production in chemically defined media. TBG was identified by its ability to bind thyroxine (T_4) and by immunoelectrophoresis, and quantitated by radioimmunoassay. TBG accumulation in the media was linear for up to 48 hours. Physiological concentrations of T_4 induced a biphasic response in TBG secretion. There was a progressive increase in TBG accumulation from 10^{-14}M to 10^{-11}M T_4. TBG accumulation decreased from the maximum at T_4 greater than 10^{-10}M, and was depressed below control at T_4 greater than 10^{-8}M. These results indicate that T_4 regulates the synthesis and secretion of TBG in hepatocarcinoma cells in culture.

We have recently demonstrated synthesis and secretion of thyroxine-binding globulin (TBG), the major plasma transport protein for thyroid hormones, by normal hepatocytes isolated from Rhesus monkeys (1). However, because of their brief survival *in vitro* (less than 24 hours), normal hepatocytes are not an ideal model to study the mechanism of control of TBG synthesis. We have, therefore, utilized two cell lines in continuous culture which were established in 1966 by Dawe *et al.* (2) from a hepatocarcinoma chemically-induced in a Rhesus monkey. These cells were shown to have cytoplasmic alkaline phosphatase activity (2) and to synthesize and secrete alpha-fetoprotein (3). This report documents TBG production

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in one cell line as well as a direct effect of physiological concentrations of thyroxine (T_4) on TBG synthesis and/or secretion.

MATERIALS AND METHODS

Cell Cultures. Two hepatocarcinoma cell lines, NCLP-6-E and NCLP-6-F, were obtained through the generosity of Dr. Clyde J. Dawe, National Cancer Institute. These cells were maintained in continuous culture since 1966, through over 110 subcultures, and could be preserved frozen for over 2 years. The cells were cultured in 75 cm^2 plastic tissue culture flasks (Falcon Plastics, Los Angeles, CA) in a water-jacketed incubator at 37°C, in an atmosphere of 5% CO_2 and 95% air. The standard culture medium was Eagle's Minimal Essential Medium with Earle's balanced salt solution supplemented with 10% fetal calf serum (Microbiological Associates, Bethesda, MD), 2 mM glutamine, 1% non-essential amino acids (Gibco, Grand Island, NY) and 60 µg neomycin/ml.

Prior to all experiments, the cells were trypsinized (0.05%, 10 min, 37°C), counted in a hemocytometer, and transferred to 25 cm^2 tissue culture flasks (Falcon). Growth experiments were started by transferring 4 x 10^5 cells to each flask in standard media. After 4 hours, the media were discarded, the cells were washed 3 times, and 5 ml of one of the test media were added.

Synthesis and secretion experiments were performed after allowing the cells to grow past confluency. They were preincubated in serum-free media for 12-24 hours and then incubated in test media for 48 hours. De novo TBG synthesis was shown by incorporation of L-[U-^{14}C]leucine (308 Ci/mole; New England Nuclear Corporation, Boston, MA). The effect of T_4 on TBG production was shown by adding T_4, 10^{-14} to 10^{-7}M, to the serum-free incubation media.

TBG was identified in the media by immunochemical and T_4-binding techniques. The anti-TBG serum used in these studies was characterized previously (1). Newly synthesized TBG was identified by immunoelectrophoresis followed by autoradiography (1). T_4-binding by TBG in the media was shown after addition of trace amounts of [^{125}I]T_4 (Abbott Laboratories, Chicago, IL) followed by immunoprecipitation (1) and compared to binding by standard TBG. TBG was measured by a double antibody radioimmunoassay which was a modification of our assay for human serum (4). The standard curve, using the logit-log transformation, was linear between 0.1 and 10.0 ng TBG. Immunochemical identity between TBG measured in the media and standard TBG was shown by parallelism between the standard curve and dilutions of the incubation media. There was no cross-reaction with monkey albumin, human thyroxine-binding prealbumin, T_4, or triiodothyronine. TBG was purified from Rhesus monkey serum and used as a reference standard (1,5).

Total cell protein was measured by the method of Lowry et al. (6) after solubilization in 0.1% sodium dodecyl sulfate. One million cells contained 790±60 µg of protein (mean ± SE).

RESULTS AND DISCUSSION

Figure 1 illustrates growth curves of NCLP-6-E in the standard media,

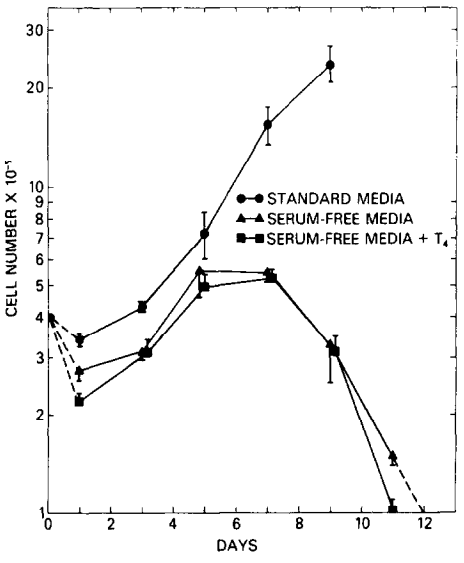

FIGURE 1. GROWTH CURVES FOR NCLP-6-E CELLS IN STANDARD, SERUM-FREE, AND SERUM-FREE MEDIA WITH T_4 (10^{-11}M). Each of 32 flasks (25 cm^2) was inoculated with 4 x 10^5 cells and incubated with standard media for 4 hours. The media was then replaced with 5 ml of one of the 3 test media and the cultures incubated at 37°C. The cells were harvested by trypsinization every other day and counted in a hemocytometer. The number of cells plotted represent the mean ± S.D.

serum-free media, and serum-free media containing T_4 (10^{-11}M). An initial decrease in cell number occurred in all 3 media. In the standard media, the subsequent rise in cell number continued for the duration of the experiment; the mean doubling time was 58 hours. In the serum-free media, with or without T_4, there was an increase in cell number for 5 days with an identical mean doubling time of 58 hours, and then a steady decrease until the 14th day at which time no attached cells remained. There was no effect of T_4 on the growth rate or survival of the cells. The pH of the media was between 7.5 and 7.3 after the first day in all flasks. Although the cell lines were maintained in serum-containing media, their ability to survive and grow at an identical rate in the absence of serum for up to 5 days permitted study of TBG production in chemically defined media. Furthermore, in the absence of serum there were no T_4-binding

proteins present initially and, therefore the T_4 added was all in the unbound state, which correlates best with biological activity (7). As TBG, albumin and other T_4-binding proteins were secreted into the media, a fraction of the T_4 would have become bound leaving less T_4 unbound as the incubation continued.

Newly synthesized TBG was identified by incorporation of [^{14}C]leucine. After a 24 hour incubation in leucine-free media with dialyzed fetal calf serum (10%) and 0.1 mCi [^{14}C]leucine, the cells were washed and incubated in fresh standard media for 24 hours. Immunoelectrophoresis and autoradiography of the media from NCLP-6-E, concentrated 50-fold by vacuum dialysis, showed a radioactive arc which was identical to non-radioactive standard TBG. No radioactive TBG was detected in the media from NCLP-6-F. These results demonstrate that the NCLP-6-E cells synthesize and secrete a protein with physical and immunochemical properties of TBG.

TABLE I

Immunoprecipitation of [^{125}I]T_4 bound by TBG in the media and by standard TBG

Standard TBG (ng/ml)	[^{125}I]T_4 bound (cpm)
50	1656
100	2073
200	3540
500	5119
1000	5634
1500	6641
Media*	3373

*Media concentrated 50-fold by vacuum dialysis; it contained 150 ng TBG/ml as measured by radioimmunoassay

TBG secreted by NCLP-6-E was also demonstrated by its ability to bind T_4. The amount of $[^{125}I]T_4$ precipitated by anti-TBG serum from the concentrated media was compared with that bound by increasing amounts of standard monkey TBG (Table I). The TBG level estimated from its $[^{125}I]T_4$ binding was 175 ng/ml of concentrated media. TBG was also measured by radioimmunoassay which gave a value of 150 ng/ml. The close similarity in the concentration of TBG obtained by these different methods is further proof that the TBG measured in the media is identical to standard TBG.

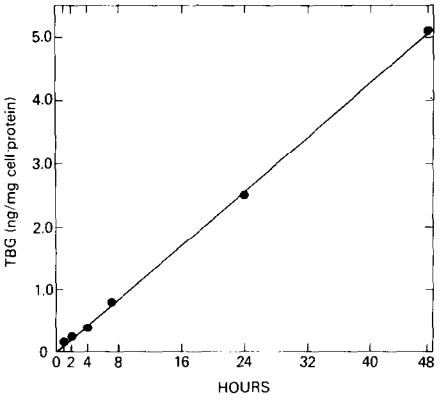

FIGURE 2. ACCUMULATION OF TBG IN THE MEDIA OF NCLP-6-E CELLS. Each of 12 flasks (25 cm^2) was inoculated with 2 x 10^5 cells and incubated in standard media at 37°C until the cells had grown past confluency. The media was then replaced with serum-free media and the cultures preincubated for 24 hours. This media was then replaced with 3.0 ml of fresh serum-free media. At the times indicated the media and cells were separated. TBG was measured in the media and the cells were solubilized in 0.1% sodium dodecyl sulfate and quantitated by determination of total cell protein. Each point represents the average of a pair of flasks which agreed within 10%.

Figure 2 illustrates the rate of TBG accumulation during a 48 hour incubation in serum-free media of NCLP-6-E cells. Newly secreted TBG was identified as early as 1 hour after the beginning of the incubation. There was a linear increase in TBG over the 48 hour period. At the end of 48 hours, there was 4.9 $\pm$ 0.2 ng TBG/mg cell protein (mean $\pm$ SD in 4 experiments). There was no TBG measureable in the media from NCLP-6-F cells.

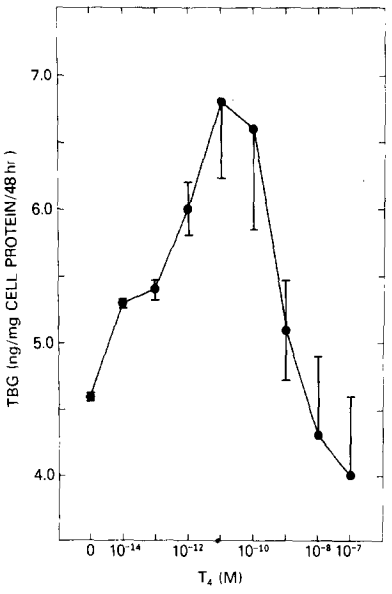

FIGURE 3. DOSE-RESPONSE RELATIONSHIP OF TBG ACCUMULATION IN THE MEDIA FROM NCLP-6-E CELLS TO T_4. The cultures were prepared as described in the legend to Figure 2. The final serum-free media contained the indicated concentration of T_4. The cells were incubated in this media for 48 hours at 37°C. The results at each T_4 concentration represent the mean $\pm$ S.D. of a pair of cultures in 3 separate experiments.

Figure 3 illustrates the effect of T_4 on TBG accumulation in the media of NCLP-6-E cells after 48 hours of incubation. The T_4 stock solution was prepared immediately prior to each experiment and sterilized by Millipore filtration. T_4 concentration was determined by its molar extinction coefficient - E_{325} = 6180 (8) - and then serially diluted to 10^{-7} to 10^{-14}M. The TBG response to T_4 was biphasic. There was a significant increase in TBG secretion at a T_4 concentration as low as 10^{-14}M and a progressive increase in TBG accumulation from 10^{-14}M to 10^{-11}M T_4. The latter level of T_4 corresponds to the physiological concentration of unbound T_4 ($6x10^{-12}$M) in the Rhesus monkey (9). TBG accumulation decreased from the maximum at T_4 greater than 10^{-10}M and was depressed below control at T_4 greater than 10^{-8}M. These results suggest that T_4 had at least 2 effects on TBG synthesis and/or secretion. The first, associated with T_4

concentrations up to 10^{-11}M, was a stimulation of TBG accumulation, while the second, with T_4 greater than 10^{-10}M, was an inhibition.

These observations indicate that the NCLP-6-E hepatocarcinoma cell culture system, which responds to T_4 concentrations as low as 10^{-14}M, could serve as a very sensitive model to define the role of T_4 in the regulation of protein synthesis. Although the T_4 effect which we observed may be a general one on hepatic protein synthesis, this is the first demonstration that T_4 regulates the synthesis and secretion of its major transport protein.

Acknowledgments

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