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EFFECTS OF EPIDERMAL GROWTH FACTOR ON APO B mRNA LEVELS AND APO B ACCUMULATION IN THE MEDIA OF PRIMATE HEPATOCYTES IN CULTURE

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SUMMARY: EGF has been shown to augment albumin and apolipoprotein A-I secretion by cynomolgus monkey hepatocytes in primary culture without stimulating cell division. This study was undertaken to determine what effect EGF had on apo B secretion by those hepatocytes. The results indicate that EGF (3 nM final concentration) severely inhibits the rate at which apo B accumulates in the culture medium of primate hepatocytes. That effect was evident within 48 hours of treatment, and by 72 hours the rate that apo B accumulated was less than half that of cells treated with a hormone-free medium. However, the apo B mRNA levels in the EGF-treated cells were more than double those of hepatocytes given the hormone-free medium. These data indicate that EGF has a potent effect on the rate at which apo B accumulates in the culture medium of primate hepatocytes and that the effect is independent of apo B gene expression.

With the availability of a procedure to cryopreserve primate hepatocytes (1), those cells have become more attractive as a model with which to study primate apolipoprotein metabolism. One of the problems originally encountered with those cells was that albumin accumulation in the culture medium began to decrease within a day or two after the cells were established in culture and reached virtually undetectable levels after 7-10 days, even in the presence of FBS (1). When a serum-free medium supplemented with human EGF was substituted for the original FBS-containing medium, albumin secretion was not only maintained, but actually increased during that same 7-10 day interval (2). Apo A-I accumulation also

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ABBREVIATIONS:
EGF, epidermal growth factor; apo B, apolipoprotein B; apo A-I, apolipoprotein A-I; FBS, fetal bovine serum; BSA, bovine serum albumin.

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increased during that interval (2). However, preliminary indications were that the rate at which apo B accumulated in the medium was suppressed by EGF. The purpose of the studies described here was to carefully delineate the effects of EGF supplementation on the rate at which apo B accumulated in the medium of cultured cynomolgus monkey hepatocytes.

METHODS

Methods : Cryopreserved hepatocytes, obtained from chow-fed cynomolgus monkeys, were thawed and established in culture as described previously (1). The cells ($3x10^6$ per 60 mm dish) were plated onto collagen coated dishes in L-15 medium supplemented with FBS (17%), glucose (6 mM), glutamine (1.5 mM), non-essential amino acids (0.08 mM), sodium bicarbonate (0.23%), Hepes (14 mM), BSA (0.15%), hydrocortisone (1.4 μM), and insulin (65 nM), hereafter referred to as sL-15 (supplemented L-15) medium. The cells were allowed 4 hr to attach, and then given fresh sL-15 medium. Sixteen hr later, the medium was replaced again, but this time with a serum-free, hormone-free sL-15 medium, identical in all other respects. All of the cells were allowed to adapt to that medium for an additional 24 hr after which the various treatments were started. The effect of a given treatment on apo B, apo A-I, and albumin accumulation was measured at 24 hr intervals for the next 72 hr. The accumulation rate, as used here, refers to the total amount of the designated protein in solution in the medium after a 24 hr interval. That value was normalized by cell protein for comparison purposes.

The concentrations of apo B and apo A-I in the conditioned media were determined by slot-blot immunoassay essentially as described previously (2). The albumin concentration was determined by electroimmunoassay, as described previously (2). The apo B mRNA levels were measured by the internal standard-RNAse protection assay also as described previously (3). The protein content of the cells was determined after the cells had been washed and then digested by incubation with a 1:1 mixture of 0.1 N NaOH and 1% deoxycholate. The Pierce BCA protein assay (Pierce; Rockford, IL) was used for protein measurements.

Statistics : The apo B, albumin, and apo A-I secretion data were analyzed using a 3-factor analysis of variance. The treatments and collection times were considered fixed effects and the monkeys (labeled 1,2,3, or 4) were considered a random effect (4). The SAS statistical procedure GLM (5) was used for all calculations. Hepatocytes from 4 monkeys were used, and each treatment at each time point was analyzed in at least triplicate, *i.e.*, 3 or more plates of a given monkey's cells were used for each treatment and each time point.

RESULTS AND DISCUSSION

Figure 1 shows the effect that increasing concentrations of EGF had on the rate that apo B accumulated in the culture media of cynomolgus monkey hepatocytes. Those cells were from a single cynomolgus monkey, and had been exposed to EGF-containing media for 72 hr. The accumulation rates shown are those measured

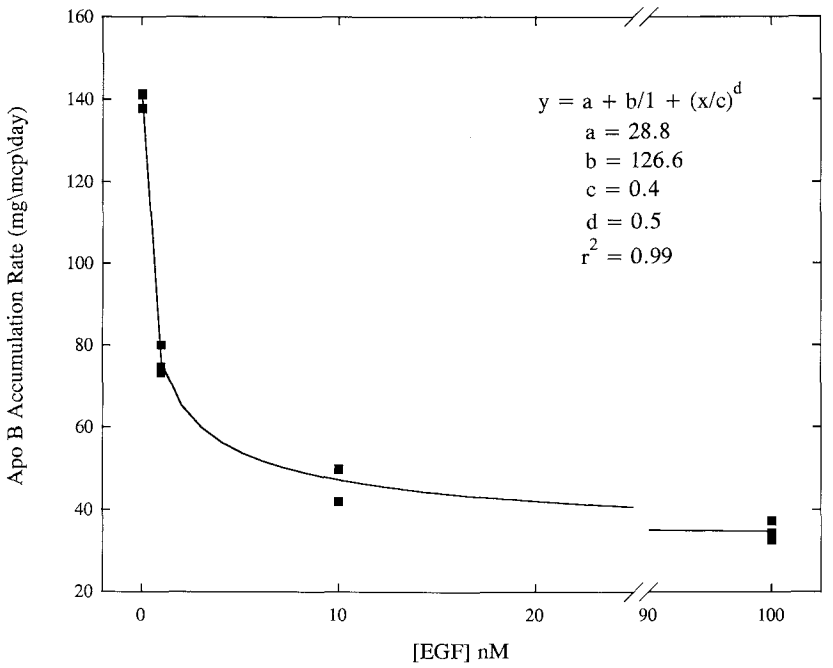

Figure 1. Effect of EGF concentration on the rate at which apo B accumulates in the culture medium of primate hepatocytes. Cryopreserved hepatocytes from a single cynomolgus monkey were thawed and established in culture. The cells were allowed to attach to the plate, and then maintained for an additional 16 hr in a supplemented L-15 (sL-15) medium containing FBS (17%). At that point, all of the cells were switched to a serum-free, hormone-free sL-15 medium for 24 h. Finally, the cells were switched to sL-15 medium containing the indicated concentration of EGF and maintained for 72 hrs (the culture medium was changed daily). The apo B accumulation rate shown is that between 48 and 72 h. Three plates of cells were used for each dose, and the data fitted to the equation shown using a nonlinear least squares method.

between hours 48 and 72. Those data indicated that EGF had a profound inhibitory effect on the rate that apo B accumulated in the media of primate hepatocytes. To confirm that observation, hepatocytes from 3 different monkeys were established in culture and the effect of EGF on the rate at which apo B, apo A-I, and albumin accumulated was measured daily over a 72 hr period. Those data are shown in Table 1. Note that the rates at which apo B and apo A-I accumulated in the media tended to increase slightly with time in those dishes in which the cells were maintained in the hormone-free media (control), whereas the rate of accumulation of albumin in those same dishes didn't change substantially. That contrasts sharply with what occurred in the cells given EGF-containing media. In the latter dishes, the rate of accumulation of apo B (measured over the entire 72 hr interval) was approximately half that of the control dishes, and the rate of accumulation of albumin

Table 1. Effect of EGF on the quantity of apo B, apo A-I, and Albumin recovered in the conditioned media of cynomolgus monkey hepatocytes

Time (hr)	Apo B (ng/mcp/day)		Apo A-I (μg/mcp/day)		Albumin (μg/mcp/day)	
	Control	EGF	Control	EGF	Control	EGF
24	40 ± 12	30 ± 13	2.3 ± 1.4	1.7 ± 1.0	23 ± 6	20 ± 4
48	59 ± 14	28 ± 12	2.6 ± 1.4	2.1 ± 1.2	27 ± 8	32 ± 6
72	98 ± 41	45 ± 18	3.0 ± 1.4	2.9 ± 1.3	21 ± 6	42 ± 8
Total	197 ± 48	102 ± 39	7.8 ± 4.0	6.7 ± 3.4	70 ± 20	95 ± 16

Data are expressed as mass of the indicated protein in the media per mg of cell protein (mcp) per 24 h. Cryopreserved hepatocytes from 4 different monkeys were used. All studies were done in triplicate, *i.e.*, three dishes of each monkey's cells were used for each time point. The mean for each of the three dish sets was taken as the best estimate of the protein secretion rate for a given monkey's cells during a given time interval. The Mean ± standard deviation of those means are shown in the table. The bottom row shows the total amount of the specified protein recovered during the entire 3 days. EGF treatment significantly slowed the rate of apo B accumulation ($p<0.05$) and significantly increased the rate of albumin accumulation ($p<0.02$).

was increased by more than 1/3. That effect of EGF on the rates of apo B and albumin accumulation was clearly evident after 48 hr of treatment, and most striking at 72 hrs (Table 1). Apo A-I accumulation in the cells maintained in the EGF supplemented media was not substantially different from that of the cells given the hormone-free medium.

Statistical analysis of the apo B data showed that there was a significant difference between the two treatments ($p=0.045$) and between the collection times ($p=0.033$). Although the treatment by time interaction term was not significant ($p=0.10$), when the components of the treatment by time interaction were tested in a pairwise fashion (control *vs* EGF at 24, 48, and 72 hr) the p-values obtained were: 24 hr, 0.35; 48 hr, 0.037; and 72 hr, 0.007. Thus, by 48 hours, a statistically significant inhibitory effect of EGF on apo B accumulation was evident which was even more pronounced at 72 hr.

Statistical analysis of the albumin data showed a significant treatment by time interaction ($p<0.001$). At 24 hours, the control had a slightly larger value than the EGF group ($p=0.06$), but by 48 hours albumin accumulation in the EGF treated cells was significantly higher ($p=0.004$) than that in the controls and that difference remained significant through 72 hrs ($p<0.001$). Thus, by 48 hrs, a statistically significant stimulatory effect of EGF on albumin accumulation was evident which, like apo B, was even more pronounced by 72 hours.

Table 2. Effect of EGF treatment on apo B mRNA levels in cynomolgus monkey hepatocytes

	Total RNA (pg/culture dish)	apo B mRNA (pg/μg total RNA)	apo B mRNA (ng/culture dish)
Control	26 ± 8	149 ± 26	4.0 ± 1.8
EGF	44 ± 9**	196 ± 19*	8.6 ± 2.7**

These are the mean (± s.d.) RNA levels of the same cells used for Table 1. The RNA content of the cells was measured at 72 h. EGF treatment caused a statistically significant increase in the Total RNA and apo B mRNA whether the latter was expressed as pg/μg RNA or ng/culture dish. * $P<0.05$; ** $P<0.01$

To gain some insight into the mechanism by which EGF reduced the apo B accumulation rate, we compared the apo B mRNA levels in cells after 72 hrs exposure to either the control or EGF supplemented medium. Those data are contained in Table 2, and indicate that the apo B mRNA levels were significantly increased in the EGF treated cells whether expressed as pg apo B mRNA/μg total RNA, or as ng apo B mRNA/culture dish. Thus, it would not appear that the EGF-mediated reduction in apo B accumulation in the media is the result of an inhibited gene expression. In fact, given the observation that the total cellular apo B mRNA levels more than doubled in response to EGF treatment, it is tempting to speculate that apo B synthesis was actually increased in the EGF treated cells. If that is correct, it would indicate that the marked reduction in the rate at which apo B accumulated in the media of the EGF treated cells was due either to an inhibited secretion rate (*i.e.*, that newly synthesized apo B was degraded to a greater extent in EGF-treated cells and thus never secreted) or a stimulated re-uptake of apo B-containing particles from the culture media. A key difference between hepatocytes in culture and those in the liver is that nascent lipoproteins secreted by the former probably remain in close proximity to the hepatocytes for extended periods, since there is no mechanism (other than diffusion) for their removal. Thus, it is conceivable that apo B synthesis and secretion are both stimulated by EGF, but that re-ingestion of the newly secreted particles is also stimulated by EGF, and the net result is a decreased accumulation of that apoprotein.

Finally, the decreased levels of apo B in the culture media of EGF-treated cells may be due to some extent to increased secretion of extracellular matrix material by those cells in response to the hormone. It is well known that glycosaminoglycans efficiently bind apo B containing particles and the decreased levels of apo B in the

culture media may simply be the result of increased production of particles with an affinity for apo B.

In summary, these data suggest that although EGF has a positive effect on albumin synthesis and secretion by these cells, it appears to have a potent inhibitory effect on apo B accumulation. Therefore, studies of apo B synthesis and secretion by hepatocytes in culture, in which EGF is a component of the culture medium, should be interpreted with some caution.

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