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Letter to the Editor

EPIDERMAL GROWTH FACTOR, DNA SYNTHESIS AND HUMAN HEPATOCYTES

Dear Editor:

Because of the controlled environment and the use of chemically-defined culture medium, primary cultures of hepatocytes, mainly from rats, have been used extensively as an experimental system to study factors affecting cell proliferation. It has been well-established that the DNA synthesis activity of primary cultures of rodent hepatocytes can be stimulated by the addition of exogenous epidermal growth factor (EGF) (Richman et al., 1976; McGowan et al., 1981; Francavilla et al., 1986; Chan et al., 1989). However, while isolation and culturing of human hepatocytes have been reported to be successful in several laboratories (Ballet et al., 1984; Gomez-Lechon et al., 1990; Gugen-Guillouzo et al., 1982; Le Bot et al., 1988), including ours (Loretz et al. 1988, 1989), very little information is yet available on the effects of EGF on human hepatocytes in culture. In the only two reports that we could find, one (Chan et al., 1989) stated that human hepatocytes in primary culture do not respond to EGF: in more than 20 separate cases of individual human donors that were studied, none were shown to have DNA synthesis stimulated by EGF. The stimulatory effects of EGF was demonstrated only if the primary human hepatocytes were pretreated for 7 days in culture with DMSO and EGF and then changed to a DMSO-free medium. On the other hand, Parzefall et al. (1991) showed that such a pretreatment was unnecessary for the induction of DNA synthesis in cultured human hepatocytes by EGF.

We report here our findings on the effect of EGF on DNA synthesis in primary cultures of human hepatocytes. Human hepatocytes were cultured from normal liver fragments obtained primarily from patients undergoing abdominal operations. The specimens were obtained with explicit informed consent of the donor and approval of the St. Louis University Medical School Institutional Review Board. Information on the donors are as follows: Donor 1: 73 year old male; Donor 2: 76 year old male; Donor 3: 34 year old female; Donor 4: 57 year old male; Donor 5: 41 year old female; Donor 6: 49 year old male.

The two-step collagenase perfusion procedures of Reese and Byard (1981) adopted in our laboratory (Loretz et al., 1988; 1989) were used in the preparation of human hepatocytes. The viability of the hepatocytes were consistently above 80% as determined using trypan blue dye exclusion (0.5% final concentration). The cells were then cultured on rat tail collagen coated tissue culture plastic plates (Costar, Cambridge, MA) in a 1:1 mixture of Ham's F12 and Dulbecco's Modified Eagle's medium (Media Tech, Washington, D. C.) supplemented with 2 g/L fatty acid-poor bovine serum albumin, 5 mg/L oleic acid, 5 mg/mL linoleic acid, 0.5 mg/L d,l-tocopherol, 0.288 mg/L testosterone, 0.272 mg/L estradiol, 0.393 mg/L dexamethasone, 7.9 mg/L d-thyroxine, 0.03 mg/L glucagon, and 20 U/L insulin. Synthesis of DNA in cultured human hepatocytes in the presence and absence of growth factors was determined by the incorporation of [^{3}H]thymidine (New England Nuclear; 19.4 Ci/mmol) into trichloroacetic acid insoluble materials based on a procedure reported by Marceau et al., 1982. Isolated hepatocytes were plated at ca. 0.2×10^6 cells in a 35 mm diameter collagen coated dish in 2 mL medium. After a 2 h attachment period, medium was changed to that containing the desired concentration of EGF (Gibco, Long Island, N.Y.). [^{3}H]Thymidine at 0.5 μCi/mL was added 24 h before each designated harvest time except for donor 1 where it was added two h before harvest. The percent of hepatocytes undergoing DNA synthesis was also quantified by autoradiography using a procedure modified from that previously described by McGowan et al., 1981. Approximately 500 nuclei were counted for the determination of labelling index which was calculated as the ratio of darkly-labelled nuclei to the total number of nuclei counted.

The initial 2 experiments were performed to evaluate the time course of DNA synthesis for human hepatocytes cultured in the absence and presence of EGF. The EGF concentration used of 10 ng/ml was then commonly used by other investigators to induce DNA synthesis in cultured rat hepatocytes. For hepatocytes from Donor 1, ^{3}H-thymidine was added at 24, 68, 112, and 161 h after plating (EGF was added 2 h after plating) and harvested 2 h later. No induction was observed at 24 h. Induction was observed at all subsequent time points, with induction levels of approximately 4 fold, 2.5 fold, and 2 fold of the untreated cultures for the 68, 112, and 161 culture periods, respectively (Fig. 1 *A*). For hepatocytes from Donor 2, the labelling period with ^{3}H-thymidine was increased to 24 h. In this experiment, the induction levels were approximately 2, 7, and 2 fold for hepatocytes cultured for 48, 72, and 96 h. At 120 h after culturing, no apparent induction was observed (Fig. 1 *B*).

The next experiments were performed to evaluate the effects of different concentrations of EGF in the induction of DNA synthesis. In these two experiments, ^{3}H-thymidine was added at different times after initial plating and the cells were harvested for the quantitation of incorporation 24 h later. Hepatocytes cultured from Donor 3 (Fig. 1 *C*) showed dose-dependent EGF induction of ^{3}H-thymidine incorporation into DNA. As observed with Donors 1 and 2, highest levels of incorporation was observed for hepatocytes cultured for 73 and 97 h. Induction of DNA synthesis appeared to plateau at 2 ng/ml and higher levels of EGF, reaching an approximately 4 fold induction over the corresponding medium control samples. No apparent induction was observed at the 166 h culture time. For Donor 4 (Fig. 1 *D*), EGF appeared to be ineffective in the induction of DNA synthesis at all culture times and EGF concentrations tested.

For the last two experiments, DNA synthesis was monitored by autoradiography. Nuclei undergoing DNA synthesis appeared as darkly labelled nuclei which could be easily discerned from the background radioactivity. Significant induction of DNA synthesis by

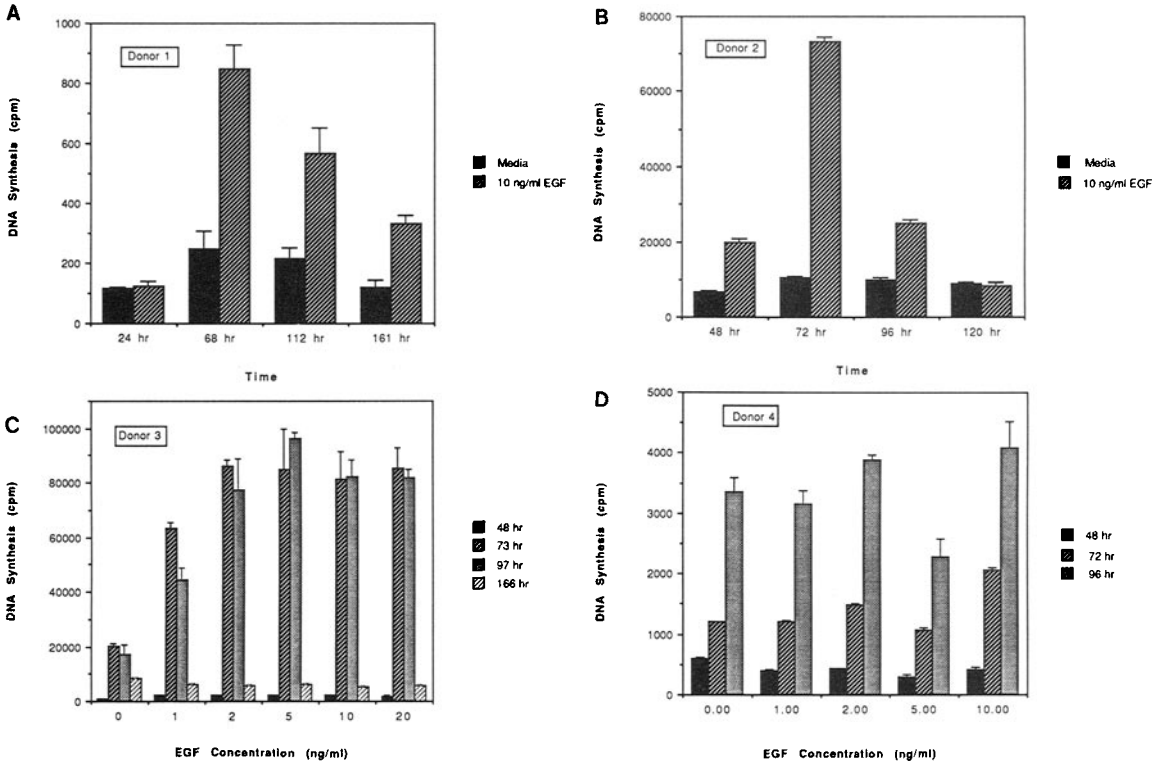

FIG. 1. Effect of EGF on DNA synthesis of human hepatocytes in culture determined biochemically as incorporation of ^{3}H-thymidine into TCA-insoluble materials. *A*: Donor 1 *B*: Donor 2 *C*: Donor 3 *D*: Donor 4.

EGF (10 ng/ml) was observed for hepatocytes cultured from both donors, as indicated by statistically significant increases in labelling indexes (Table 1). With hepatocytes cultured from Donor 5, induction was approximately 7 and 15 fold for the 48 and 72 h culture times. With Donor 6, induction was approximately 9 and 4.5 fold for the two culture times. The labelling indexes reached approximately 9% for hepatocytes cultured for 72 h in the presence of EGF.

TABLE 1

EFFECT OF EGF (10 NG/ML) ON INDUCTION OF DNA SYNTHESIS IN HUMAN HEPATOCYTES AS DETERMINED BY AUTORADIOGRAPHY

Culture Time	EGF	Labelled Nuclei/ Total Nuclei	Labelling Index (%)	Fold Induction[a]
A. Donor 5				
48 h	−	3/506	0.59	
	+	22/501	4.39**	7.4
72 h	−	3/513	0.58	
	+	45/504	8.93**	15.4
B. Donor 6				
48 h	−	2/500	0.40	
	+	19/509	3.73**	9.3
72 h	−	10/512	1.95	
	+	47/520	9.04**	4.6

[a] Fold induction = Labelling Index (EGF)/Labelling Index (medium control).

** Statistically significant ($p < 0.001$) to be higher than concurrent control values as determined by chi-square analysis.

Our results therefore appear to confirm that of Parzefall et al. (1991). Of the six isolations of human hepatocytes from individual donors studied, five of the isolations demonstrated significant induction of DNA synthesis by EGF. The one nonresponding culture was from an excessively fatty liver which may or may not be the reason for the difference. For the five responding cultures, the EGF effect was dependent both on dose and culture time. Also as observed by Parzefall et al., the range of labelling index for human hepatocytes stimulated by EGF was substantially lower than that reported for rat hepatocytes (Houck et al., 1988; Houck and Michalopoulos, 1989).

Our results suggest that, under our experimental conditions, human hepatocytes respond to the growth stimulatory effects of EGF similar to that reported for rat hepatocytes. While the discrepancy between our results and that of Chan et al. (1989) is unclear, the condition of the liver specimen as well as the isolated hepatocytes may be one of the critical factors. Our results suggest that primary cultures of human hepatocytes can be a useful experimental system to study the role of EGF in growth and regeneration of human liver.

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