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INDUCTION OF DNA SYNTHESIS IN ADULT RAT HEPATOCYTES CULTURED IN A SERUM-FREE MEDIUM

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SUMMARY

Isolated hepatocytes from adult rats were cultured for 3 days in a serum-free synthetic medium. Supplementation with fibrinogen digests, glucagon and insulin remarkably increased DNA synthesis in hepatocytes. DNA synthesis began to increase at 35 h and reached a maximum at 41 to 54 h after plating. At this time, cells were morphologically identifiable as hepatocytes. Glucagon could be replaced by dibutyryl cyclic AMP or isobutyl-methyl-xanthine. Addition of amiloride (a Na^+ influx inhibitor) during the initial 22 h completely inhibited DNA synthesis. These results suggest that influx of Na^+ during early prereplicative period and increase in cellular cyclic AMP levels during late prereplicative period are necessary for the induction of DNA synthesis in hepatocytes.

INTRODUCTION

Although regenerating liver after partial hepatectomy has long been used as a useful model for studies of the regulatory mechanisms of cell proliferation, the stimuli that lead quiescent hepatocytes to ploliferation are not well known (1,2). Proliferation of hepatocytes has also been induced in intact rats by various means (3-7). However, definitive examinations have been difficult to perform because of the presence of complex neuro-humoral interactions in vivo. To overcome this in vivo complexity, attempts to induce hepatocyte DNA synthesis and cell division in primary cultures have been tried in our (8) and other laboratories (9-11).

In this paper, we report conditions necessary for the induction of hepatocyte DNA synthesis in a serum-free synthetic medium. Evidences that suggest the importance of Na^+ influx and increased intracellular cyclic AMP levels for DNA synthesis are also presented.

Abbreviations used: FD= fibrinogen digests, Bt_2cAMP= dibutyryl cyclic AMP, IMX= 3-isobutyl-1-methyl-xanthine

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MATERIALS AND METHODS

Chemicals. Glucagon was purchased from Calbiochem, San Diego, Calif., USA. Insulin and 3-isobutyl-1-methyl-xanthine were obtained from Sigma Chemical Co., St. Louis, Mo., USA. Dibutyryl-cyclic AMP was from Boehringer Mannheim GmbH., Germany. [Methyl-^{3}H]thymidine (sp. act., 2 Ci/mmol) was from New England Nuclear, Boston, Mass., USA. Amiloride®-HCl (a Na^+ influx inhibitor) was kindly supplied by Dr. C. Stone of Merck Sharp & Dohme, West Point, Penn., USA. Leupeptin was a gift from Dr. T. Aoyagi of Institute of Microbial Chemistry, Tokyo, Japan.

Hepatocyte Culture. Breeding conditions of rats (170-200 g), method of hepatocyte isolation and composition of synthetic basal medium were essentially the same as the previous report (8) except that increased amount of Cys (80 mg/l), Gln (700 mg/l) and ascorbic acid (60 mg/l) were used in the present medium. About 1.5 x 10^6 viable hepatocytes (as determined by trypan blue exclusion) in 6 ml of basal medium were plated in a TD-40 (50 cm^2) glass culture flask and were incubated at 37°C under air in a closed system. Culture period was divided into P1 (0 to 4 h), P2 (4 to 22 h) and P3 (22 h to labeling). Medium was changed at 4 h and 22 h. For hepatocyte attachment, degradation products of fibrinogen (Sigma, Type I) by urokinase (EC 3.4.4.a) were supplemented (0.12 A_{280} unit) to 6 ml of basal medium during P1. At the indicated periods, appropriate hormones and other chemicals (0.1 ml) were added to the basal medium. Leupeptin (1 μg/ml) and Trasylol®(Bayer, 2 U/ml) were added in P2 and P3 to prevent protein degradation. Incorporation of [^{3}H]thymidine was not inhibited by these drugs.

Assay of DNA Synthesis. DNA synthesis in hepatocytes was estimated by the incorporation of [^{3}H]thymidine (sp. act., 0.5 Ci/mmol, 5 μCi/flask) into hepatocyte nuclei as reported previously (8).

RESULTS

For the initial attachment of hepatocytes, FD was used during the initial 4 h (P1). FD stimulated spreading of hepatocytes as well as attachment. The efficiency of attachment by FD was 70 to 80% of the plated cells. This value was nearly equal to that observed with 5 to 10% fetal bovine serum.

Table 1 shows the effect of glucagon and/or insulin supplemented in P2 and/or P3 on DNA synthesis. When both glucagon and insulin were supplemented in P3, a marked increase in DNA synthesis was observed as compared with basal medium alone throughout the culture period. Only a slight increase in DNA synthesis was observed when P3 received basal medium alone regardless of the presence of glucagon and insulin in P2 (Exp.1 and Exp. 2). As shown in Exp. 3, addition of either glucagon or insulin alone to P3 increased DNA synthesis. However, the levels of DNA synthesis were much lower than those observed with a combination of glucagon and insulin. Although basal levels of DNA synthesis were not the same, similar results were obtained from more than 10 repeated experiments.

Table 1. Effect of glucagon and/or insulin on DNA synthesis in rat hepatocytes in primary culture.

Addition			[^{3}H]Thymidine Incorporation (46-48 h)		
P1 (0 - 4 h)	P2 (4 - 22 h)	P3 (22 - 48 h)	cpm / 10^5 nuclei		
			Exp. 1	Exp. 2	Exp.3
FD	–*	–	1,670 (1.0)	750 (1.0)	90 (1.0)
FD	G & I	–	2,340 (1.4)	2,130 (2.8)	–
FD	–	G & I	11,430 (6.8)	8,830 (11.8)	2,860 (31.8)
FD	G & I	G & I	14,930 (8.9)	–	1,710 (19.0)
FD	–	G	–	–	550 (6.1)
FD	–	I	–	–	800 (8.9)

FD was added to synthetic basal medium during P1, and glucagon (G, 50 ng/ml) and/or insulin (I, 50 ng/ml) were supplemented to P2 and/or P3. The values shown are average cpm per 10^5 nuclei from duplicate experiments, and ratios relative to each control in the parentheses. *, no addition

As shown in Fig. 1, the rise in DNA synthesis was shifted to earlier time when glucagon and insulin supplied to both P2 and P3. DNA synthesis began to increase at 35 h and reached a peak at 41 to 47 h when both P2 and P3 received glucagon and insulin. On the contrary, the begining and the peak of DNA synthesis were observed at 41 and 54 h, respectively, when glucagon and insulin were supplemented only in P3. Slight increase in [^{3}H]thymidine incorporation was observed after 50 h when hepatocytes were cultured in synthetic basal

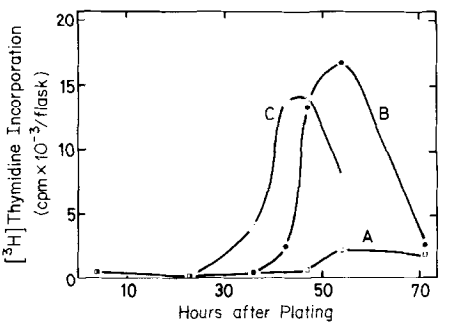

Fig. 1. Time course of DNA synthesis in adult rat hepatocytes in primary culture.
About 1.5 x 10^6 viable cells per flask were plated at zero time. Medium was changed at 4 h and 22 h. At the times indicated, [^{3}H]thymidine (5 μCi/flask) was added and pulse labeled for 2 hours. A; P1=basal medium (BM) + FD, P2, P3 = BM alone. B; P1= BM + FD, P2= BM alone, P3= BM + glucagon (50 ng/ml) + insulin (50 ng/ml). C; P1= BM + FD, P2, P3= BM + glucagon (50 ng/ml) + insulin (50 ng/ml)

Table 2. Replacement of glucagon-stimulated DNA synthesis in hepatocytes by dibutyryl cyclic AMP or isobutyl-methyl-xanthine.

Addition to P3	[^{3}H]Thymidine Incorporation (46-48 h) cpm / 10^5 nuclei	
	Exp. 1	Exp. 2
Glucagon + Insulin	4,860	4,550
Bt_2cAMP + Insulin	5,090	5,640
IMX + Insulin	3,580	6,120
Bt_2cAMP + IMX + Insulin	4,470	9,590

FD was added to basal medium during P1. P2 received basal medium alone. Each compound was supplemented to P3 (22 to 48 h). The values shown are average cpm per 10^5 nuclei from duplicate experiments. Glucagon (50 ng/ml), insulin (50 ng/ml), Bt_2cAMP (10^{-6} M) and IMX (10^{-4} M) were used.

medium alone. Although DNA synthesis was observed in this culture system, most of the hepatocytes became aggregated and detached from the flasks after 72 h. Mitotic fugures were not seen in hepatocytes throughout the culture.

Table 2 shows that glucagon could be replaced by low doses of Bt_2cAMP (10^{-6} M) or IMX (10^{-4} M). Effect of combination of Bt_2cAMP with IMX was additive in Exp. 2, although no additive effect was observed in Exp. 1.

Inhibitory effect of amiloride on DNA synthesis was shown in Table 3. Effect of amiloride was more prominent when added in P1 than in P2 or P3. Amiloride addition in both P1 and P2 inhibited DNA synthesis completely. In-

Table 3. Effect of Amiloride on DNA synthesis in adult rat hepatocytes.

Addition			[^{3}H]Thymidine Incorporation (46-48 h)	
P1 (0 - 4 h)	P2 (4 - 22 h)	P3 (22 - 48 h)	cpm / flask	%
-*	-	-	26,850	100
Amil.	-	-	4,270	16
-	Amil.	-	9,020	34
-	-	Amil.	8,330	31
Amil.	Amil.	-	710	3
-	Amil.	Amil.	780	3

FD was supplemented in all flasks in P1, and glucagon (50 ng/ml) and insulin (50 ng/ml) were supplemented in P2 and P3. The values shown are average cpm per flask from duplicate experiments, and per cent of control. Amil. : Amiloride®-HCl (10 μg/ml)
*; no addition

hibition was dose dependent (data not shown). Complete inhibition of DNA synthesis was observed with 5 to 10 μg of amiloride per ml of medium when supplemented in P1 and P2. Hepatocytes were degraded by addition of 20 μg of amiloride per ml.

DISCUSSION

Since serum contains many unidentified factors including growth factors, we preferred to use a serum-free culture medium with known chemicals and hormones for the analysis of control mechanisms of cell proliferation.

We used FD for stimulation of hepatocyte attachment in a serum-free medium. SDS-polyacrylamide gel electrophoresis showed that FD is composed of a considerable number of proteins and polypeptides including fibronectin and its digests. We are currently investigating whether fibronectin in FD is the only agent which stimulates hepatocyte attachment.

Combination of glucagon with insulin had a remarkable stimulatory effect on the induction of DNA synthesis (Table 1). Synergistic action of glucagon and insulin in promoting DNA synthesis has been reported *in vivo* (12) and *in vitro* (8,9,11,13). In our experimental conditions, addition of glucagon and insulin was necessary in P3. Glucagon and insulin supplemented to P2 shifted the time course of DNA synthesis to earlier time only when P3 received both glucagon and insulin.

Previously, we observed progressive degradation of hepatocytes already at 48 h of culture. One of the reasons for hepatocyte degradation seemed to be protein degradation in hepatocytes. Therefore, we used leupeptin and Trasylol® to prevent hepatocyte degradation. The doses used were not inhibitory for DNA synthesis. However, despite supplementation of leupeptin and Trasylol®, most of the hepatocytes were detached from flasks after 72 h under the present experimental conditions. Similar decrease in the number of attached hepatocytes at day 3 of culture has been reported (14). Since mitoses in hepatocytes have not been observed as yet, culture conditions should be further improved.

In vivo experiments showed that glucagon could be replaced by cyclic AMP (15). Armato et al. (13) reported that cyclic AMP may be a mediator of glucagon action in neonatal rat hepatocytes in primary culture. The present study confirms these observations using adult rat hepatocytes in primary culture. The rise in cellular cyclic AMP content late in prereplicative phases of rat hepatocytes in vivo (16) and T51B rat liver epithelioid cells in vitro (17) seems to be necessary for initiation of DNA synthesis.

Amiloride is one of the diuretics and known as an inhibitor of Na^+ influx into cells (11,18). Inhibition of DNA synthesis by amiloride in the present results suggest that Na^+ influx is necessary in inducing DNA synthesis in hepatocytes. Intracellular Na^+ concentration increased in early prereplicative period in the liver of intact rat stimulated to proliferate by partial hepatectomy or by chemical manipulations such as administrations of isoproterenol in combination with glucagon (19). Recently, Koch and Leffert (11) have also reported the necessity of Na^+ influx for initiation of proliferation in 10- to 15-day-old culture of rat hepatocytes.

We propose from the present results that Na^+ influx in the early prereplicative period and synergistic action of glucagon and insulin which results in an increase in cellular cyclic AMP content in the late prereplicative period are involved in the induction of DNA synthesis in rat hepatocytes.

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