Vol. 104, No. 1, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 15, 1982 Pages 259-265

INDUCTION OF MITOSIS IN PRIMARY CULTURES OF ADULT RAT HEPATOCYTES UNDER SERUM-FREE CONDITIONS

Kaoru Hasegawa, Kazuto Watanabe and Mutuyosi Koga

Department of Physiology, Dokkyo University School of Medicine, Mibu, Tochigi 321-02, Japan

Received November 30, 1981

When adult rat hepatocytes were cultured in a serum-free, hormone-supplemented medium containing 1 mM of Ca^{2+}, DNA synthesis was induced in about 50% of hepatocytes. However, mitosis could not be observed since hepatocytes became aggregated and detached from culture flasks after 3 days. On the other hand, when Ca^{2+} concentration was reduced to below 0.4 mM, not only survival time of hepatocytes was prolonged but cell divisions were observed in morphologically identified hepatocytes. About 50% of hepatocytes synthesized DNA and divided under the latter conditions. These results indicate that low concentration of Ca^{2+} in the culture medium permit post DNA synthetic hepatocytes to complete cell division.

INTRODUCTION

Adult rat hepatocytes in primary culture exhibit specific characteristics and functions at levels similar to the hepatocytes in vivo during the first few days, and therefore, have been used for the study of regulatory mechanisms in hepatocyte metabolism (1-3). Such cultured cells, however, show little cell division. As an initial study to induce cell division, we have previously reported that marked DNA synthesis was induced in adult rat hepatocytes cultured in the presence of serum (4). In recent years, Sato and co-workers have developed methods for replacement of serum with hormones and chemically defined agents in culture medium (5). Recently, we have also tried to cultivate adult rat hepatocytes under serum-free conditions and succeeded in inducing DNA synthesis by combination of hormones and other chemicals (6,7). A lag period of initial rise in DNA synthesis in these cultured hepatocytes was close to that observed after partial hepatectomy (6). However, mitosis of hepatocytes have not been observed in culture owing to the progressive degeneration of hepatocytes after 3 days.

Abbreviations used: HEPES= N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid, FD= fibrinogen digests, EDTA= ethylenediamine-tetraacetic acid.

0006-291X/82/010259-07$01.00/0

During the course of the study to improve culture conditions, we found that adult rat hepatocytes could undergo mitosis when Ca^{2+} concentration was reduced to below 0.4 mM.

In this communication, we report the culture conditions necessary for the induction of mitosis in adult rat hepatocytes in primary culture, and also report microscopical observations of the process of hepatocyte divisions.

MATERIALS AND METHODS

Isolation of Hepatocytes and Primary Cultures. Male Wistar rats weighing 200 to 300 g were used for experiments. Hepatocytes (parenchymal liver cells) were isolated aseptically using a modification of the previously reported methods (4,8) which was firstly described by Haward et al. (9). Liver was perfused in situ via the portal vein with O_2-saturated warm (37°C) perfusion buffer [in mg/l; NaCl (7,000), KCl (400), $NaHCO_3$ (420), Na-pyruvate (550), HEPES (40 mM, pH 7.4)] at a flow rate of 20 ml/ min for 5 to 6 min. The perfusate was then changed to 30 ml of 0.05% of collagenase buffer (perfusion buffer plus 5 mM of Ca^{2+}). After perfusion, the liver was removed and minced finely with scissors. Minced liver was suspended in 30 ml of collagenase solution and incubated at 37°C for 10 to 15 min with shaking. The digests were filtered through double layers of stainless mesh (167 and 67 μm pore size). The cells in the filtrate were sedimented at 10 g for 3 min. The cell pellet was washed thrice by suspending in glucose-free, pyruvate (5 mM)-supplemented Hanks' solution followed by centrifugation. The cells were finally resuspended in an appropriate volume of the culture medium. At the expence of cell yield (1 to 2 x 10^8 cells from 5 to 7 g of liver), the resulting cell suspension consist almost of hepatocytes (98% or more). For hepatocytes to anchorage to culture flask, 0.12 A_{280} unit of FD (4,6) was supplemented to 5 ml of culture medium. FD was prepared as follows. To 0.5% bovine fibrinogen (Sigma, Type I) in perfusion buffer, Urokinase® (E.C.3.4.21.31, Green Cross Corp., Japan) was added (100 U/ml) and incubated at 37°C for 60 min. At the end of incubation, Trasylol® (Bayer, 200 I.U./ml) was added to stop the reaction. Other procedures for primary culture of hepatocytes were essentially the same as reported previously (4,6) and briefly described in the legend of Fig. 2.

Culture Medium. Koga's Medium L (KL) was used for primary culture of adult rat hepatocytes. Composition of KL was as follows (in mg/l). NaCl (7,400), KCl (250), $CaCl_2 \cdot 2H_2O$ (20), $MgSO_4 \cdot 7H_2O$ (150), $NaH_2PO_4 \cdot 2H_2O$ (150), $MnCl_2 \cdot 4H_2O$ (0.05), $ZnSO_4 \cdot 7H_2O$ (0.75), $FeSO_4 \cdot 7H_2O$ (0.5), H_2SeO_3 (0.002), $CuSO_4 \cdot 5H_2O$ (0.2) $NaHCO_3$ (420), L-alanine (200), L-asparagine·H_2O (40), L-aspartic acid (10), L-cysteine HCl H_2O (80), L-cystine HCl (40), L-glutamine (700), L-glutamic acid (60), L-histidine·HCl·H_2O (40), L-isoleucine (50), L-leucine (90), L-lysine·HCl (150), L-methionine (30), L-ornithine·HCl (100), L-phenylalanine (40), L-proline (120), L-serine (60), L-threonine (90), L-tryptophan (45), L-tyrosine (60), L-valine (75), glutathione (reduced, 20), ascorbic acid (60), biotin (0.01), Ca-pantothenate (0.5), choline chloride (10), cyanocobalamine (0.1), folic acid (1.0), inositol (10), menadione (0.01), nicotinamide (0.5), pyridoxine·HCl (0.5), riboflavin (0.1), thiamine·HCl (1.0), orotic acid (0.1), lipoic acid (0.04), Na-linoleate (0.5), DL-carnitine·HCl (3), DL-mevalonic acid lactone (2), adenine·HCl (12), thymidine (0.15), galactose (200), Na-pyruvate (4,400), phenol red (5), kanamycin (30), bovine serum albumin (Fraction V, Reheis Chemical Co., 500), transferrin (human, Sigma, 5) Trasylol® (2,000 U), and HEPES (20 mM, pH 7.4). In the present experiments, Ca^{2+} concentration was adjusted to levels between 0 and 2 mM by addition of $CaCl_2 \cdot 2H_2O$ to Ca^{2+}-free KL. The special features of KL are: 1) Glucose was omitted. Instead, a high concentration of Na-pyruvate was supplemented. An

unusually high concentration of Na-pyruvate is required to replace serum and to enhance DNA synthesis (7). 2) Considerable amounts of all non-essential amino acids were supplemented, while arginine was omitted. 3) A protease inhibitor Trasylol was supplemented. This is also required for serum-free cultivation (6). Although albumin (Fraction V) and transferrin are not necessary for the induction of DNA synthesis and mitosis, these components prolonged survival of hepatocytes under serum-free conditions.

RESULTS AND DISCUSSION

About 80% of plated hepatocytes attached to the culture flask within 4 h and formed a monolayer with pavement-like epithelial morphology one day after plating. Photographs in Fig. 1 show that the monolayer consists almost exclusively of hepatocytes without contaminating non-hepatocytes. Fig. 1A shows aggregates of hepatocytes cultured for 75 h in the medium containing 1 mM of Ca^{2+}. Several hours after the photograph was taken, hepatocytes were detached from the flask and none of the cells except cell debris remained. Fig. 1B shows hepatocytes cultured at 0.1 mM of Ca^{2+} for 75 h. Several dividing hepatocytes (encircled) are seen. Fig. 1C shows magnified figures of dividing hepatocytes cultured for 50 h in 0.1 mM Ca^{2+} medium, three of them are in metaphase and one is in telophase. In metaphase figures, granules are distributed homogenously in the cytoplasm and non-granular rhombic area is situated at the center of the cytoplasm. This rhombic area is composed of a spindle body and an equatorial plate made by chromosomes. Although marked morphological difference was observed between Fig. 1A and Fig. 1B, C, degree of DNA synthesis was almost the same. As shown in the autoradiograph (Fig. 1D), about 50% of hepatocytes (mononucleated and binucleated) were labeled with [^{3}H]thymidine between 26 and 50 h after plating.

Reduced Ca^{2+} concentration also induced morphological changes. When Ca^{2+} concentration was higher than 0.4 mM, hepatocytes contacted closely to one another. On the other hand, when Ca^{2+} concentration was below 0.4 mM contact among hepatocytes was very loose and each hepatocyte showed simple outlined shape (Fig. 1B,C,D). It would be easier for post-DNA synthetic hepatocytes to complete cell division under loose cell-to-cell contact caused by low concentration of Ca^{2+}. Observations by the time lapse cinemicrography support this idea. Several hepatocytes actually entered mitotic phase even

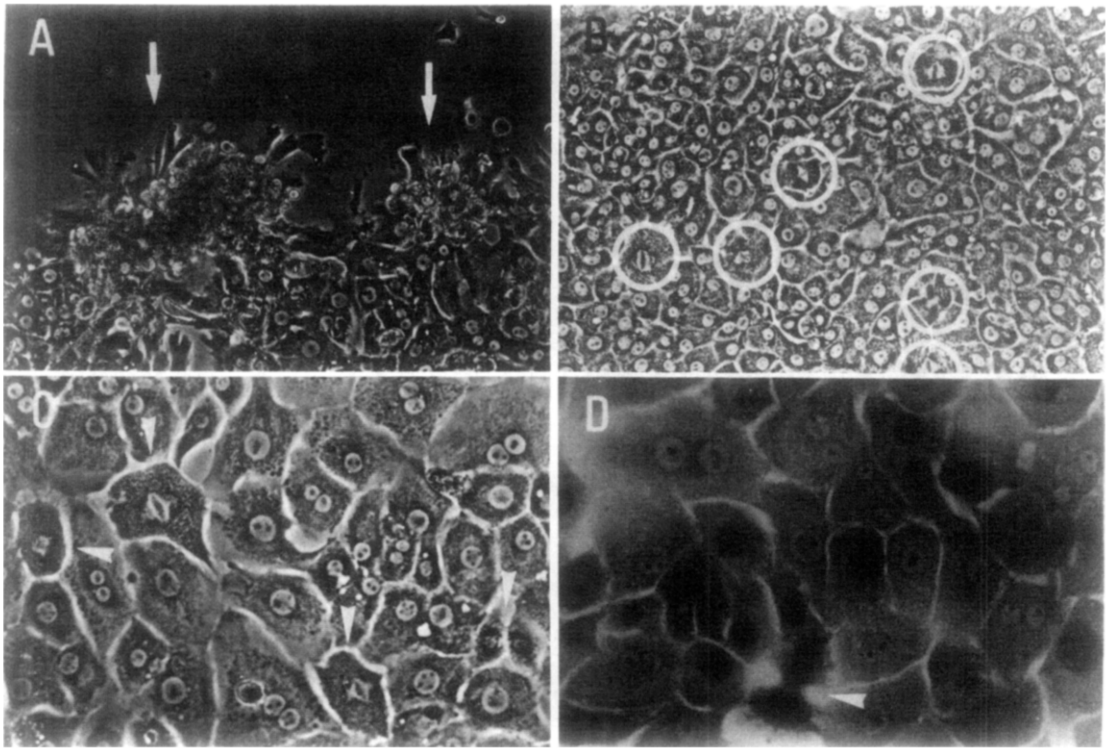

Fig. 1. Photomicrographs of adult rat hepatocytes cultured in a serum-free medium containing 1 mM (A) and 0.1 mM (B,C,D) of Ca^{2+}. (A) Phase contrast photomicrograph of hepatocytes which formed aggregates at 75 h in high Ca^{2+} medium. Arrows indicate aggregates of hepatocytes that exhibits blebing and balooning of cell membrane. (B) Phase contrast photomicrograph of hepatocytes cultured for 75 h in low Ca^{2+} medium. Dividing hepatocytes are indicated by circles. (C) Phase contrast photomicrograph showing 4 dividing hepatocytes. Arrows indicate three metaphase and one telophase hepatocytes. Fifty hours after plating. (D) Autoradiograph of Giemsa stained hepatocytes labeled with [^{3}H]thymidine between 26 and 50 h. The arrow indicate a telophase hepatocyte.

in high Ca^{2+} (1mM) medium when observed by cinemicrography. However, such mitotic figures were almost unnoticed when observed under phase contrast microscope, since ruffling of cell membrane was active and hepatocytes exhibited extensive blebing and balooning of the cell membrane (Fig. 1A) which resulted in detachment of cells from the flask. On the other hand, under loose cell-to-cell contact in low Ca^{2+} medium (0.1 mM), ruffling of cell membrane was relatively quiet and not only 50% of hepatocytes underwent cell division but survival time was prolonged twice of that of cultures with 1 mM of Ca^{2+}.

Concerning the hepatocyte degeneration in high Ca^{2+} medium, it has been reported (10) that cell death by membrane-active toxins in primary cultures

of adult rat hepatocytes was prominent when Ca^{2+} concentration in the medium was 3.6 mM, while little cell death was observed in the absence of Ca^{2+} in the medium. Therefore, low concentration of Ca^{2+} protects hepatocytes from the observed cell degeneration (damage) which may be caused by accumulation of intracellular Ca^{2+} in high Ca^{2+} medium (11).

In the time lapse cinemicrography, we have observed three different types of hepatocyte division during primary culture. First, one mononucleated hepatocyte divided into two mononucleated daughter hepatocytes. Second, one binucleated hepatocyte divided into two mononucleated daughter hepatocytes; both nuclei synthesized DNA prior to cell division as observed by autoradiogram. Third, soon after one mononucleated hepatocyte divided, two daughter hepatocytes fused, forming one binucleated hepatocyte. The former two types of division accompanied cell number increase, while the latter one did not. The second and third types of division are noteworthy, since many cells are binucleated or polyploid in the liver of intact rats (12).

Fig. 2A shows that cell number began to increase over the 1-day level at 2-day, and reached a peak at 4-day (usually 3- to 4-day), since then decreased gradually (not shown). DNA and protein content (Fig. 2B,C) changed in patterns similar to that of the cell number. Maximal increase in cell number was 35% in this experiment. Up to 50% of increase in cell number was observed in other experiments. These values suggest that almost all hepatocytes which synthesized DNA (50%, see Fig. 1D) might have divided.

It has been reported that low Ca^{2+} concentration stimulated growth of human epidermal keratinocytes (13,14) and urinary bladder epithelium (15). Our results which show proliferation of hepatocytes by low Ca^{2+} concentration are consistent with these reports. Contrary to our results, Whitfield *et al.* (16) have reported that Ca^{2+} deprivation inhibited DNA synthesis in the liver after partial hepatectomy and in cultured T51B rat liver cells. However, restoration of Ca^{2+} concentration to the normal level enabled the cells to initiate DNA synthesis and enter mitosis in both cases. It is unclear why Ca^{2+} requirement of hepatocytes in primary culture differs from that *in vivo* and in the established cell line.

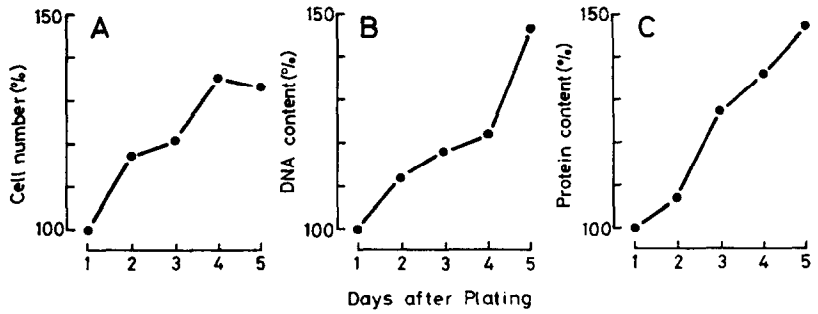

Fig. 2. Time courses of cell number (A), DNA content (B) and protein content (C) during primary culture of adult rat hepatocytes. About 1.5 x 10^6 viable hepatocytes in 5 ml of culture medium (KL) were plated in a glass culture flask (40 cm^2) and incubated at 37°C under air in a closed system. Culture period was divided into P1 (plating to 4 h), P2 (4 to 22 h) and P3 (22 h -). Medium was changed at 4, 22 h and at 24-h interval thereafter. Only during the initial 4 h, FD was added. Glucagon (50 ng/ml) and insulin (50 ng/ml) were supplemented after 4 h. Hepatocytes were harvested by EDTA -trypsin and cell number was counted with a hemocytometer. For determination of DNA and protein content, hepatocytes were lysed with 1 N NaOH and the lysate was assayed by the method of Burton (21) and Lowry et al. (22), respectively. Each point shows the average of duplicate determinations in one typical experiment of five similar experiments, and expressed as per cent of values obtained at 1-day.

Low concentration of Ca^{2+} as well as other factors in the culture medium [FD (4,6), non-essential amino acids, especially proline (manuscript in preparation), hormones (insulin and glucagon)(4,6,7), and high concentration of Na-pyruvate (7,17) is essential for the induction of hepatocyte proliferation in primary monolayer culture under serum-free conditions. In our serum-free culture system, the use of commercially available media which have been adopted by other groups in original or modified forms [Eagle's minimum essential medium (18), Dulbecco and Vogt's modification of Eagle's medium (19), L-15 (Leibovitz) medium (3), Ham's F-12 medium (2), Wiiliams' mediumE (20), and Waymouth's MB 752/1 (17)] was not successful for the induction of mitosis. However, mitosis was observed when each of the above media was supplemented with these factors.

ACKNOWLEDGEMENT: We thank Professors H. Terayama and M. Yamada of University of Tokyo and Drs. J. Enami and H. Ohtake in our laboratory for reading the manuscript. Supported in part by grants from the Ministry of Education, Science and Culture of Japan.

REFERENCES

1. Bissell, D. M., Hammaker, L. E., and Meyer, U. A. (1973) J. Cell Biol. 59, 722-734.
2. Bonney, R. J., Becker, J. E., Walker, P. R., and Potter, V. R. (1974) In Vitro 9, 399-413.

3. Sirica, A. E., Richards, W., Tsukada, Y., Sattler, C. A., and Pitot, H. C. (1979) Proc. Natl. Acad. Sci. USA 76, 283-287.
4. Koga, M., Namai, K., and Hasegawa, K. (1979) Dokkyo J. Med. Sci. 6, 69-75.
5. Barnes, D., and Sato, G. (1980) Anal. Biochem. 102, 255-270.
6. Hasegawa, K., Namai, K., and Koga, M. (1980) Biochem. Biophys. Res. Commun. 95, 243-249.
7. Hasegawa, K., and Koga, M. (1981) Biomed. Res. 2, 217-221.
8. Umeda, T., and Koga, M. (1977) Biochim. Biophys. Acta 478, 115-127.
9. Howard, R. B., Lee, J. C., and Pesch, L. A. (1973) J. Cell Biol. 57, 642-658.
10. Schanne, F. A. X., Kane, A. B., Young, E. E., and Farber, J. L. (1979) Science 206, 700-702.
11. Farber, J. L. (1981) Life Sci. 29, 1289-1295.
12. Bucher, N. L. R., and Malt, R. A. (1971) Regeneration of Liver and Kidney, pp 42-45, Little, Brown and Co., Boston.
13. Peehl, D. M., and Ham, R. G. (1980) In Vitro 16, 526-538.
14. Hawley-Nelson, P., Sullivan, J. E., Kung, M., Hennings, H., and Yuspa, S. H. (1980) J. Invest. Dermatol. 75, 176-182.
15. Reese, D. H., and Friedman, R. D. (1978) Cancer Res. 38, 586-592.
16. Whitfield, J. F., Boynton, A. L., MacManus, J. P., Rixon, R. H., Sikorska, M., Tsang, B., Walker, P. R., and Swierenga, S. H. H. (1980) Ann. N. Y. Acad. Sci. 399, 216-240.
17. McGawan, J. A., and Bucher, N. L. R. (1981) In Vitro 17, 256.
18. Richman, R. A., Claus, T. H., Pilkis, S., and Friedman, D. L. (1976) Proc. Natl. Acad. Sci. USA 73, 3589-3593.
19. Koch, K. S., and Leffert, H. L. (1979) Cell 18, 153-163.
20. Williams, G. M., Bermudez, E., and Scaramuzzino, D. (1977) In Vitro 13 809-817.
21. Burton, K. (1968) Methods in Enzymol. XIIB 163-166.
22. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275.