

Serum deprivation enhances DNA synthesis of human hepatoma SMMC-7721 cells *

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Abstract

AIM To determine the relationship between serum deprivation or serum levels and cell proliferation of human hepatoma SMMC-7721 cells.

METHODS Human hepatoma SMMC-7721 cells were grown in RPMI 1640 supplemented with 10% fetal calf serum (FCS) in 5% CO₂ incubator at 37°C for 24h, and culture media were replaced to serum-free or different serum FCS levels (2.5%, 5%, 10%, 20% and 25%). Six h, 12h, 18h and 24h after the culture, the cells were incorporated [³H]-TdR for 4h. At last [³H]-TdR incorporation was detected with liquid scintillation counting.

RESULTS DNA synthesis of SMMC-7721 cells could be sharply stimulated by short-time (6h) serum deprivation (the cpm value of ³H-TdR incorporation of cells in serum-free was 39.32-fold higher than cells in 25% serum), and the incorporation of ³H-TdR was negatively related to the serum levels. Longer-time serum starvation (12h, 18h and 24h) also greatly stimulated DNA synthesis, although the cpm value of ³H-TdR incorporation was less than that in 6h serum deprivation. Morphology of cells cultured in different serum levels also showed significant difference.

CONCLUSIONS Compared with other cell lines such as BEL7404 and Swiss 3T3, human hepatoma SMMC-7721 cells had different response to the serum deprivation. Short-time serum deprivation could greatly stimulate DNA synthesis of human hepatoma SMMC-7721 cells. Precautions must be given to the changes of serum levels for the detection of growth factors and drugs using SMMC-7721 cells as a model.

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INTRODUCTION

Mammalian cells in culture required serum in order to proliferation^[1]. Serum was a mixture of protein and other substances, among which essential hormones and growth factors can support cell proliferation^[2]. In order to reduce the effects of growth factors in serum on cells, the procedure of serum deprivation was often involved to study growth regulation on cultured mammalian cells^[3-5]. Prolonged serum deprivation induced fibroblastic cells such as Swiss 3T3 to enter a quiescent state (G₀)^[6,7]. On the contrary, mouse embryo cells^[8] and HeLa S-3 cells^[9] showed reversal response to serum deprivation and lower serum levels. Human hepatoma SMMC-7721 cells were established in our country^[10] and were widely used for detecting the activity of growth factors and drugs. Therefore, it was very important to clarify the effects of serum deprivation and serum levels on the growth and metabolism of SMMC-7721 cells.

MATERIALS AND METHODS

Cell lines and culturing

Human hepatoma SMMC-7721 cells were obtained from the Shanghai Cell Bank of Chinese Academy of Sciences and maintained in our laboratory. The cells were grown as monolayers in RPMI 1640 medium supplemented with 10%-20% fetal calf serum (FCS) and incubated at 37°C in the humidified incubator with 5% CO₂ 95% air.

Serum deprivation and treatment of serum concentration

Exponent growing cells in flasks were harvested by trypsinization with 0.25% trypsin and suspended in RPMI 1640 medium plus 10% FCS. Cells were plated at 1×10⁵ cells/ml in 4 pieces of 24-well plates and incubated at 37°C in 5% CO₂ 95% air for 24 hours. After that, the medium was aspirated and the cells were washed with RPMI 1640 medium. The medium was replaced with RPMI 1640 plus different levels of serum (0%, 2.5%, 5%, 10%, 20% and 25%) in different treatment groups (each group having 4 wells of cells) respectively. The rates of DNA synthesis of cells cultured in different serum levels were detected at interval of 6 hours (6, 12, 18 and 24 hours).

Measurement of DNA synthesis

When cells were cultured in different serum levels for 6, 12, 18 and 24 hours respectively, rates of DNA synthesis were determined by pulse labeling for 4 hours in 74KBq ml^{-1} ^3H -TdR. The media were aspirated and the cells were gently rinsed with phosphate buffer saline (PBS), trypsinized properly with 0.25% trypsin, collected on 49 model filter membrane and rinsed with 5% TCA (trichloroacetic acid) and 100% ethanol (three times). The membranes were dried at 80°C for 30min. The incorporation of ^3H -TdR was determined by liquid scintillation counting.

Morphological observation of cells cultured in different serum levels

Before collected for liquid scintillation counting, the cells in different treatment groups were observed and photographed under inverted phase contrast microscope.

Materials RPMI 1640 medium was from Gibco, USA; trypsin was from Sigma; ^3H -thymidine (^3H -TdR) was from the Institute of Atomic Energy of China; 24-well plates were from NUNC; and Triton X100, POPOP, PPO were from Serva.

RESULTS

Effects of serum deprivation and concentration on ^3H -TdR incorporation

Serum contained significant amounts of thymidine, which would affect the incorporation of their appropriate exogenous labeled forms. This was because the formulated media such as RPMI 1640 for cell culture did not usually contain unlabeled nucleotides, therefore serum provided the only source other than the labeled form. SMMC-7721 cells were pulse labeled with ^3H -TdR for 4 hours at 74KBq ml^{-1} after serum deprivation and treatment of different serum concentrations for different times. The results showed decreased specific activity of the incorporation of ^3H -TdR with increased serum concentrations, that was, the incorporation of ^3H -TdR had negative relation with serum levels in medium. Cells cultured in serum-free medium for 6 hours after media replacement incorporated ^3H -TdR 39.32-fold higher than cells in 25% serum medium (Figure 1A). On the other hand, within 18 hours as the time of serum deprivation going down, the incorporation of ^3H -TdR decreased (Figure 1B, C, D). The ratio of ^3H -TdR incorporation between cells in serum free and in 25% serum decreased to 3.53-fold (Table 1).

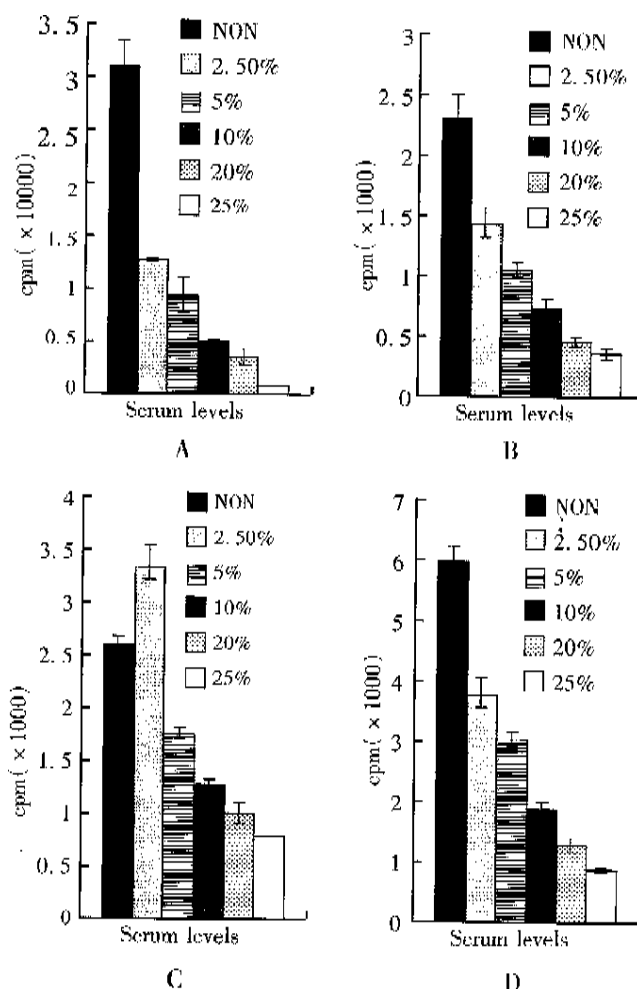


Figure 1 Effects of serum deprivation and serum levels on the incorporation of ^3H -TdR.

(A) The cpm value after cells were treated in different serum concentrations for 6 hours; (B) for 12 hours; (C) for 18 hours; (D) for 24 hours.

Table 1 Ratios of the ^3H -TdR incorporation among cells cultured in serum-free and different serum levels

Treatments	6 h	12 h	18 h	24 h
SF/2.5% FCS	2.47	1.63	0.778	1.67
SF/5% FCS	3.53	2.26	1.51	1.98
SF/10% FCS	7.34	3.35	2.19	3.27
SF/20% FCS	11.61	5.30	2.72	4.88
SF/25% FCS	39.32	7.21	3.53	7.35

Effects of serum deprivation and serum concentration on cellular morphology

Cells cultured in different serum levels showed significant difference in morphology under inverted phase contrast microscope. Cells cultured in serum-free or lower serum level (2.5%) (Figure 2A) were less well spread and smaller than that in higher serum levels. Cells cultured in 5% serum and more (Figure 2B, C) were epithelial-like and well spread.

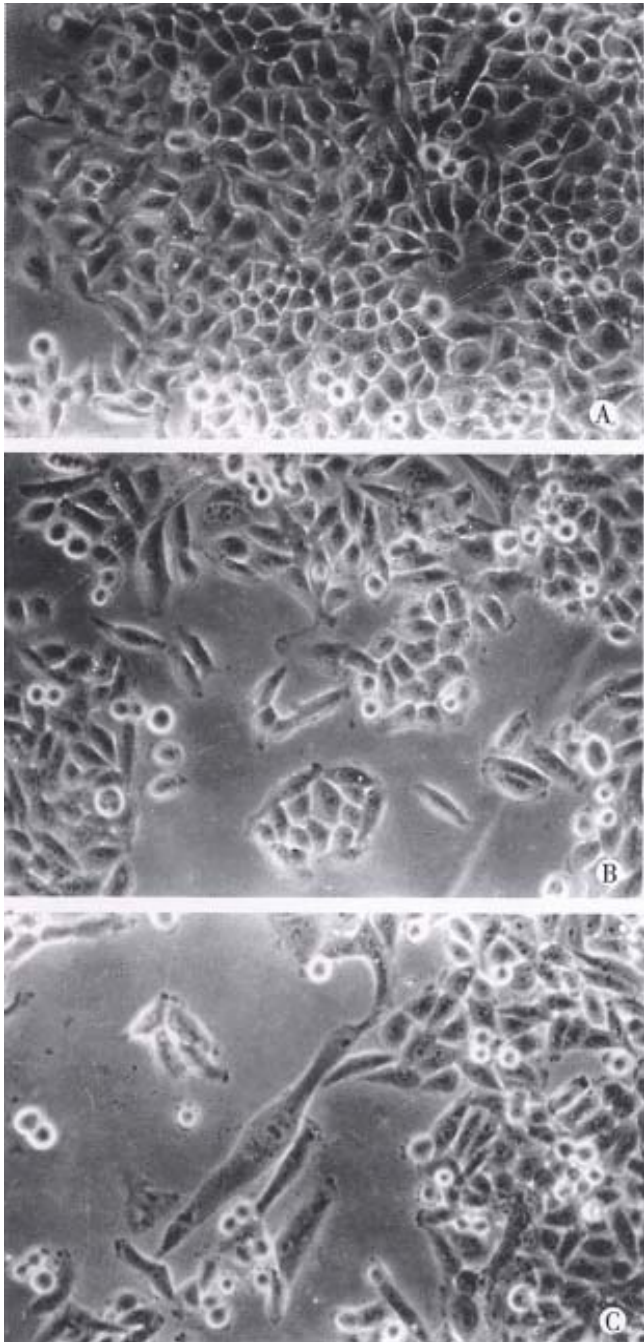


Figure 2A Morphology of human hepatoma SMMC-7721 cells cultured in serum-free medium for 6 hours photographed under phase contrast microscope (200×)

B Morphology of human hepatoma SMMC-7721 cells cultured in 5% serum medium for 6 hours photographed under phase contrast microscope (200×)

C Morphology of human hepatoma SMMC-7721 cells cultured in 25% serum medium for 6 hours photographed under phase contrast microscope (200×)

DISCUSSION

Most mammalian cells were serum dependent and usually passaged in medium containing serum, and they would die if they were cultured in serum-free medium for long time. Therefore, short-time serum-free (serum deprivation) cell culture was one

of the main protocols for the study of cell growth and regulation. How cell responses to the serum deprivation has called extensive attention. The results varied greatly even reversal because of the different cells used. Brooks, Larsson^[3,4] and Zetterberg^[7] indicated that the proliferation of non-transformed fibroblastic cells usually depend on serum or purified growth factors in the tissue culture medium. If the serum concentration was drastically reduced, the cells ceased proliferation and entered a reversible state of quiescence (G_0). Larsson *et al*^[4] reported serum-dependent proliferating 3T3 cells prolonged their intermitotic time by 9-10 hours after exposure to serum-free medium for only 1 hour and a short exposure to serum-free medium was sufficient for cells to leave the cell cycle. Zetterberg^[7] showed that in 3T3 cells, in all stages of cell cycle, serum deprivation resulted in inhibition of protein synthesis, but only in postmitotic cells in the first 3-4 hours of G_1 did it produce cell-cycle arrest, a 1-hour exposure to serum-free medium was sufficient to force most G_1 cells into a state of quiescence (G_0). Loo *et al*^[8] demonstrated that mouse embryo cells established and maintained in the absence of serum depend on epidermal growth factor for survival and their proliferation was reversibly inhibited by serum or platelet-free plasma. Yin *et al*^[9] showed that Hela cells cultured in 10% serum medium incorporated ^3H -TdR to 25% of cells cultured in 0.2% serum medium. Xu *et al*^[11,12] had demonstrated that human hepatoma BEL-7404 could grow in serum-free medium and there were only less than 2%-3% apoptotic cells after serum starvation for 24 hours. Human hepatoma SMMC-7721 cell were serum-dependent proliferating cell line and widely used in the detection of growth factors and anti-cancer drugs, therefore it was very important to ascertain the effects of short-time serum deprivation and serum levels on growth and metabolism of SMMC-7721 cells, which would affect the correct assessment of the activity of growth factors and drugs. Our results suggested that short-time serum deprivation (6 hours) could stimulate the synthesis of SMMC-7721 cells and the incorporation of ^3H -TdR was negatively related to the serum levels in medium. For longer (12, 18, and 24 hours) exposure to serum^{a2}free medium, the incorporation of ^3H -TdR was also negatively related to the serum level, although the ratio of ^3H -TdR incorporation of cells in serum^{a2}free medium and in 25% serum medium decreased from 39.32-fold (6 hours) to 3.53-fold (18 hours). These results were reversal to that with 3T3 cells, which could easily be overlooked, or even mistakenly attributed to the activity of growth factors and drugs. The results might be due to two main reasons, one was that DNA synthesis of SMMC-7721 cells cultured in serum-free medium was

inhibited by the lack of thymidine in RPMI 1640 medium and the cells were accumulated to the G₁/S. Once ³H-TdR was added to the medium, the cells began to enter S phase quasisynchronously and start DNA synthesis, the other reason was that most of malignant cells appeared less dependent on serum and could secrete some growth factors and stimulate themselves by feedback mechanism. The differences of cell morphology in serum-free and different serum levels were mainly induced by fibronectin and fetuin in serum^[13], which promoted cell attachment and spread on the surfaces of culture plates. So necessary precautions must be given to the serum level changes in the medium in detecting growth factors and drugs with human hepatoma SMMC-7721 cells, otherwise false conclusion might be implied.

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