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Effect of various factors and substrates on the growth of a human hepatoblastoma cell line, HuH-6 in a serum-free medium.

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Abstract

The effect of various factors and substrates on the growth of a human hepatoblastoma cell line, HuH-6, which was inoculated at low density in a serum-free medium was examined. Several supplements were required to enhance cell growth of HuH-6. These included cholera toxin (CT), glucagon (Glu) and selenium (Se). Type IV collagen (C-IV) provided the most conductive environment tested for cell growth. These results suggest that CT, Glu, Se, and C-IV are important stimulators for the continuous growth of HuH-6 in a serum-free medium at low density.

KEYWORDS: hepatoblastoma, cell line, serum-free medium, growth factor, substrate

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— Brief Note —

Effect of Various Factors and Substrates on the Growth of a Human Hepatoblastoma Cell Line, HuH-6 in a Serum-Free Medium

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The effect of various factors and substrates on the growth of a human hepatoblastoma cell line, HuH-6, which was inoculated at low density in a serum-free medium was examined. Several supplements were required to enhance cell growth of HuH-6. These included cholera toxin (CT), glucagon (Glu) and selenium (Se). Type IV collagen (C-IV) provided the most conductive environment tested for cell growth. These results suggest that CT, Glu, Se, and C-IV are important stimulators for the continuous growth of HuH-6 in a serum-free medium at low density.

Key words : **hepatoblastoma, cell line, serum-free medium, growth factor, substrate**

HuH-6 has been widely used as a basic cell model for the study of hepatocyte functions (1-8). Serum-free culture of hepatoma cells as well as other cells is a very useful means of characterizing the *in vitro* effects of hormones and growth factors on the proliferation or expression of hepatocyte functions. Our previous studies showed that HuH-6 grew much more poorly and could be less easily subcultured at a split ratio below 1 : 5 or 1 : 10 (low density) in a serum-free defined medium, IS, which was developed for the growth of a human hepatoma cell line, HuH-7, or in other serum-free media than at a split ratio over 1 : 2 (high density) (8, 9). In the present study, we examined the effect of various factors and substrates on the growth of HuH-6 at low density to investigate more improved conditions for serum-free culture of HuH-6.

HuH-6 produces α-fetoprotein and albumin at a high rate (1). In this study, the population doubling time of HuH-6 was about 83 h. HuH-6 was not transplantable into nude mice. Cultures were grown at 37° C in an atmosphere of 5 % CO_2 and 95 % air. Cells were routinely passaged in RPMI-1640 medium supplemented with 0.2 % lactalbumin hydrolysate (LA ; Sigma Chemical, Co., St. Louis, MO, USA) and 5 % dialyzed bovine serum (dBS) inactivated at 56° C for 30 min. Dialysis was performed through dialysis tubing (molecular weight cutoff, approximately 3500 ; Spectrum Medical Ind., Los Angeles, CA, USA). For serum-free growth assay, the dBS-containing medium was removed by aspiration, then monolayers were washed twice with Ca and Mg-free phosphate-buffered saline (PBS), and the cells were detached by incubation at 37° C with 0.1 % trypsin containing 0.02 % EDTA in PBS (trypsin-EDTA). The cell suspensions (0.5 ml/dish) thus obtained were inoculated into 24 multiwell cluster dishes (Falcon, Oxnard, CA,

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USA) at densities of 8.5×10^4 (1 : 5 split ratio) (Fig. 1) or 4.5×10^4 (1 : 10 split ratio) (Fig. 2) cells per well coated with type IV collagen (C-IV ; Nitta Gelatin Co., Osaka, Japan) as described later. For the study of various factors, RPMI-1640 supplemented with 0.2 % LA, 5 μg/ml insulin (Ins), 20 ng/ml EGF and 5×10^{-7} M hydrocortisone (HC) was used as a control medium. For the study of substrates, the control medium was supplemented with 0.2 μg/ml choleratoxin (CT), 2 μg/ml glucag n (Glu) and 10^{-10} M selenium (Se). Triplicate dishes were taken after several days, and the cells were dissociated with trypsin-EDTA in order to count the cells with a Coulter counter (Coulter Electronics, Hialeah, FD, USA). CT, Glu, Se, transferrin (Tf), fetuin (Fet), triiodothyronine (T3) and phosphoethanolamine (PE) were purchased from Sigma and tested. Fibronectin (FN ; Sigma) and laminin (LAM ; Collaborative Research, Bedford, MA, USA) were dissolved in PBS. Type I collagen (C- I ; Nitta Gelatin) and C-IV were diluted 1 : 1000 with acetic acid. Multiwell cluster dishes were coated with 20 μg/ml of each solution, allowed to incubate at 37 °C for 1 h and then air-dried. Before use, the coated dishes were washed twice with PBS.

In Fig. 1, the effect of various chemical factors on the growth of HuH-6 is shown. In the control medium (cont), HuH-6 rarely grew more than 14 days after inoculation. The growth of HuH-6 was significantly stimulated by the addition of 0.2 μg/ml CT, 2 μg/ml Glu or 10^{-10} M Se and insignificantly by the addition of T3 to the control medium. Gatmaitan *et al.* (10) described the colony-forming ability of some growth factors,

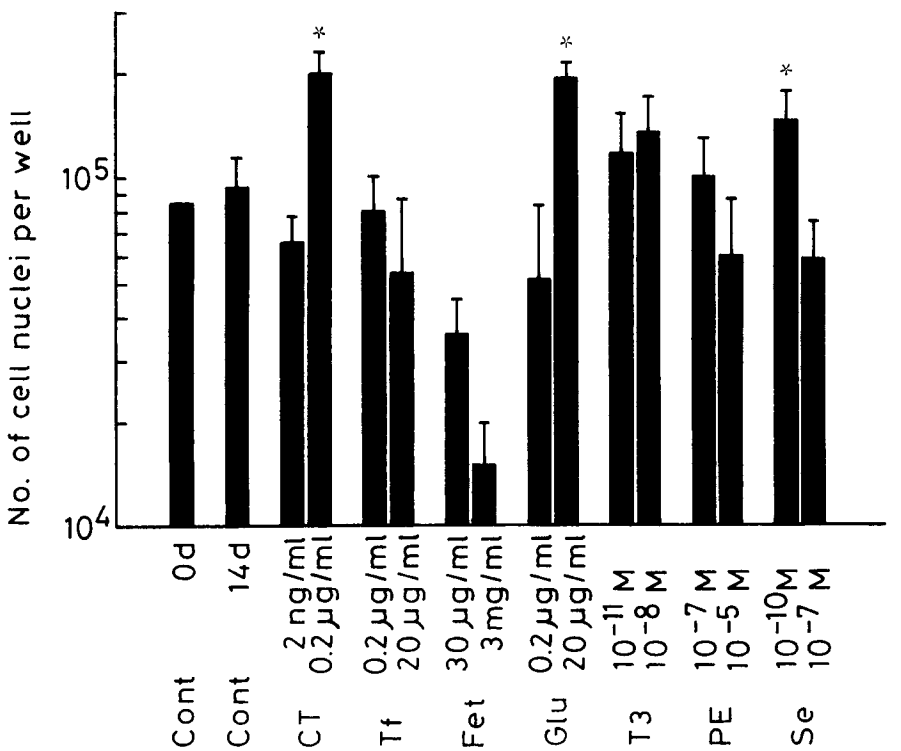

Fig. 1 Effect of various chemical factors on the growth of HuH-6, a human hepatoblastoma cell line. HuH-6 was plated in a serum-free medium supplemented with or without a factor and harvested at 14 days. Abbreviations : Cont Od, untreated control (0 day) ; Cont 14 d, untreated control (14 day) ; CT, choleratoxin ; Tf, transferrin ; Fet, fetuin ; Glu, glucagon ; T3, triiodothyronine ; PE, phosphoethanolamine ; Se, selenium. *, Significantly different from Cont 14 d ($p < 0.05$).

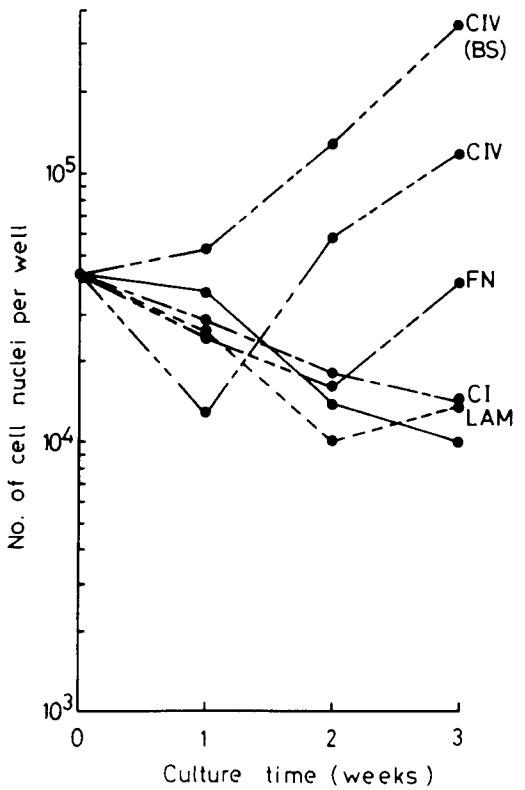

Fig. 2 Effect of various substrates on the growth of HuH-6, a human hepatoblastoma cell line, in a serurm-free medium. HuH-6 was plated in a serum-free medium and harvested at the indicated times. Dishes were coated with an extracellular matrix material at a concentration of 20 μg/ml. Abbreviations : CIV (BS), type Ⅳ collagen in a serum-supplemented medium ; CIV, type Ⅳ collagen ; FN, fibronectin ; CI, type Ⅰ collagen ; LAM, laminin. No label : No coating.

including Glu and Se, in rat hepatoma cell culture. Of the factors tested in the present study, Glu, Se and CT were active in promoting growth of HuH-6. IS medium contains Se as an important factor for the serum-free growth of HuH-7 (8). Se may be an essential factor for promoting growth of hepatoma cells in culture. The effect of various substrates on the growth of HuH-6 was examined (Fig. 2). In the serum-free medium used in this study, a decrease in cell number after inoculation (lag time) was more prominent than in the serumsupplemented medium. The results show that C-IV substrates most effectively promote the growth of HuH-6 among the substrates tested. HuH-6 failed to grow on C-I, LAM and uncoated substrates. Extracellular matrix materials are well known to play an important role in the expression of differentiated functions and growth of mammalian cells in culture (9–11). HuH-6 exhibited active cell growth when it was cultured on C-IV-coated substrates in a serum-free medium. Epithelial cells attach via LAM to a C-IV substrate (11). HuH-6 is capable of synthesizing LAM (Tokiwa T, unpublished data). It can be assumed that HuH-6 also utilizes the mechanism of LAM-mediated cell binding to C-IV for adhesion and growth in a serum-free medium. Our previous studies showed that HuH-6 grew poorly and could not be easily subcultured at low density in serum free-media (9). We have shown that the growth of HuH-6 at low density is stimulated by the presence of CT, Glu, Se and C-IV in a serum-free medium. It is well known that hepatoma cells in culture as well as other tissue cultured cells will secrete attachment factors and/or growth-promoting factors into the medium, especially when they are cultured at high density (8). The present finding suggests that the poor growth capacity of HuH-6 at low density in a serum-free medium may be due to the insufficient secretion of such factors as mentioned above. Whether or not HuH-6 can be serially subcultured in a serum free-medium containing CT, Glu, Se and C-IV at low density is under investigation.

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