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Abstract

To select a suitable medium for serum-free primary culture of adult rat hepatocytes, ten commercially-available synthetic media were compared for their ability to maintain the cells under serum-free and serum-supplemented conditions with special reference to attachment, survival and albumin secretion. It was found that Williams' medium E and DM-160 medium were the best among the ten media for maintaining hepatocytes under serum-free conditions in primary culture.

KEYWORDS: adult rat hepatocytes, serum-free primary culture, maintenance, Williams' medium E, DM-160 medium

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Selection of Medium for Serum-Free Primary Culture of Adult Rat Hepatocytes

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To select a suitable medium for serum-free primary culture of adult rat hepatocytes, ten commercially-available synthetic media were compared for their ability to maintain the cells under serum-free and serum-supplemented conditions with special reference to attachment, survival and albumin secretion. It was found that Williams' medium E and DM-160 medium were the best among the ten media for maintaining hepatocytes under serum-free conditions in primary culture.

Key words : **adult rat hepatocytes, serum-free primary culture, maintenance, Williams' medium E, DM-160 medium**

Following the introduction of the collagenase-liver-perfusion method for the preparation of intact hepatocytes from rat liver (1), the primary culture of hepatocytes has been widely used as an experimental system for studies on hepatic metabolism, regeneration and carcinogenesis (2-5). Most of these studies, however, employed serum-containing media. Serum contains hormones, growth factors and many other undefined substances. The complex and undefined nature of serum complicates the design and interpretation of experiments aimed at understanding the interaction of hormones, growth factors and so forth. Thus serum-free primary culture of hepatocytes would be desirable for such studies.

Unfortunately, no medium for serum-free hepatocyte primary culture is commercially available at present. So in developing serum-free primary culture of hepatocytes, a suitable medium needs to be selected from among commercially-available synthetic media. In the present study, ten commercially-available synthetic media were compared with special reference to attachment, survival and albumin secretion of adult rat hepatocytes under serum-free and serum-supplemented conditions in primary culture.

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Materials and Methods

Materials. Male Donryu rats (3 months old) inbred in this laboratory were used in the present study. Bovine serum (BS) was prepared as reported previously (6). The followings were purchased: DM-160 medium (7) from Kyokuto Pharmaceutical Industrial Co., Ltd., Tokyo, Japan; Eagle's minimal essential medium (MEM) and RPMI 1640 medium from Nissui Pharmaceutical Co., Ltd., Tokyo, Japan; Williams' medium E (WE), Waymouth's medium MB 752/1 (MB 752/1), Medium 199 (M199), basal medium Eagle (BME), McCoy 5a medium, Ham's medium F12 (F12) and Leibovitz's medium 15 (L15) from Flow Laboratories, Inc., McLean, VA, USA; dexamethasone sodium phosphate (dexameth-

asone) from Banyu Pharmaceutical Co., Ltd., Tokyo, Japan; EDTA, HEPES, insulin and collagenase type I from Sigma Chemical Co., St. Louis, MO, USA and trypsin (1:250) from Difco, Detroit, MI, USA. Other chemicals were from various sources and of reagent grade.

Isolation and primary cuture of hepatocytes. As reported previously, hepatocytes, having an initial viability of 85 to 90 % as measured by trypan blue exclusion, were isolated from male adult rats by a collagenase-liver-perfusion method (8). The isolated hepatocytes were inoculated at a cell number of 1.4×10^6 cells onto Falcon plastic dishes (60 mm in diameter) containing 4 ml of various serum-free or 5 % BS-containing media supplemented with dexamethasone (10 μM), insulin (10 μg/ml) and HEPES (5 mM, pH 7.2), and cultured in a humidified atmosphere of 5 % CO_2 / 95 % air at 37 °C. After a one-day attachment period, the culture media were replaced by the respective hormone-free media.

Determination of viable hepatocyte number. The suspensions of cultured hepatocytes were prepared as reported previously (6). Briefly, all the cultures were washed once with 4 ml of Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS). Then the cultures were incubated with 2 ml of 0.1 % trypsin in PBS containing 0.02 % EDTA for about 5 min at 37 °C. Immediately after the enzyme treatment, 2 ml of cold media supplemented with 20 % BS were added to the cultures to inhibit further proteolytic activity of the enzyme. Then the suspensions were gently pipetted to disperse the hepatocytes. The final suspensions of hepatocytes were kept in an ice-water bath until determination of viable cell number. The numbers of viable hepatocytes were determined by trypan blue exclusion in a hemacytometer and expressed as the mean from three dishes.

Determination of secreted albumin contents in the spent culture media. As reported previously, quantitative determinations of albumin secreted into the culture media were carried out by a double antibody method using anti-rat albumin rabbit serum and anti-rabbit IgG sheep serum (9). These antisera and purified rat albumin were kindly supplied by Professor Dr. H. Watabe, Department of Biochemistry, Faculty of Pharmaceutical Science, Higashi-Nippon-Gakuen University, Hokkaido, Japan.

Results

Under serum-free conditions, the efficiency of hepatocyte attachment to plastic substratum was reduced when four (MEM, RPMI 1640, F12 and L15) of the ten commercially-available synthetic media were used, but it was unaffected by the other six media (DM-160, WE, MB 752/1, M199, BME and McCoy 5a) (Table 1). The

Table 1 Attachment efficiency and survival rate of adult rat hepatocytes in various media under serum-free and serum-supplemented conditions in primary culture[a]

Medium[b]	Attachment efficiency (%)		Survival rate of hepatocytes (%)	
	Serum-free	5 % BS-supplemented	Serum-free	5 % BS-supplemented
DM-160	82.9 ± 5.0	83.6 ± 6.5	91.3 ± 11.2	86.8 ± 11.7
WE	81.1 ± 4.4	80.8 ± 7.2	92.8 ± 4.8	89.2 ± 3.7
MB 752/1	78.4 ± 8.0	80.2 ± 7.9	80.7 ± 4.2	92.3 ± 2.9(**)
M199	77.5 ± 2.8	79.2 ± 7.1	80.1 ± 2.5	81.6 ± 9.5
BME	76.6 ± 5.1	79.5 ± 5.8	90.9 ± 8.5	80.9 ± 15.8
McCoy 5a	76.4 ± 4.0	78.6 ± 5.6	70.2 ± 1.8*	82.8 ± 6.4(*)
MEM	72.9 ± 1.7*	73.0 ± 2.6*	89.6 ± 4.7	85.1 ± 12.7
RPMI 1640	72.2 ± 2.2*	80.0 ± 1.4(***)	81.9 ± 9.8	71.4 ± 6.0
F12	35.2 ± 5.9****	70.6 ± 3.4*,(****)	53.7 ± 3.1***	85.7 ± 4.7(****)
L15	30.2 ± 1.0****	77.8 ± 4.2(****)	0 ± 0****	51.5 ± 3.9***,(****)

a: Attachment efficiency is expressed as the percentage of viable cells attached 24 h after seeding. Survival rate of hepatocytes is expressed as the percentage of the attached viable cells 24 h after the inoculation, which survived on Day 2 of primary culture. Results are expressed as the mean ± SD of three dishes. Asterisks denote statistical difference against the respective DM-160 by Student's *t*-test as follows: *, $p < 0.05$; **, $p < 0.025$; ***, $p < 0.01$; ****, $p < 0.005$. Asterisks in parentheses denote the statistical difference between serum-free and 5% BS-supplemented conditions.

b: DM-160, DM-160 medium; WE, Williams' medium E; MB 752/1, Waymouth's medium MB 752/1; M199, Medium 199; BME, basal medium Eagle; MEM, Eagle's minimal essential medium; F12, Ham's medium F12; L15, Leibovitz's medium 15.

latter group of six media did not require serum to yield maximal attachment efficiency of hepatocytes. However, three of the other four media (RPMI 1640, F12 and L15) required serum.

Five media (DM-160, WE, MB 752/1, M199 and BME) were efficient for supporting not only attachment but also survival of hepatocytes under serum-free conditions (Table 1). Both MEM and RPMI 1640 also efficiently supported hepatocyte survival under serum-free conditions. However, the hepatocyte survival rate was reduced when the remaining three media (McCoy 5a, F12 and L15) were used. Especially, in L15, hepatocytes could not survive even for two days under serum-free conditions. However, under serum-supplemented conditions, hepatocyte survival rates in the nine media excepting L15 did not differ from each other (Table 1).

The most striking difference among the media was the ability to stimulate albumin secretion of hepatocytes. Under serum-free conditions, hepatocytes secreted the largest amount of albumin in WE followed by DM-160 among the ten media (Table 2). The effects of serum addition on albumin secretion of hepatocytes varied from enhancement to suppression in the ten media, although these effects were not remarkable.

Table 2 Amounts of albumin secreted by adult rat hepatocytes into various media under serum-free and bovine serum (BS)-supplemented conditions in primary culture[a]

Medium[b]	Secreted albumin ($\mu g/10^5$ cells/24 h)	
	Serum-free	5 % BS-supplemented
WE	11.8 ± 0.3	10.1 ± 0.2(****)
DM-160	10.0 ± 0.8**	11.1 ± 0.4**
MB 752/1	8.2 ± 0.3****	9.0 ± 0.3***,(*)
M199	7.4 ± 0.4****	5.6 ± 0.1****,(****)
McCoy 5a	5.3 ± 0.4****	6.4 ± 1.3***
RPMI 1640	4.9 ± 1.0****	6.1 ± 0.6****
BME	3.6 ± 0.1****	3.4 ± 0.3****
MEM	3.1 ± 0.2****	5.0 ± 0.2****,(****)
F12	2.8 ± 0.4****	4.3 ± 0.3****,(***)
L15	ND	1.2 ± 0.1****

a: Results show the amounts of albumin secreted by hepatocytes into various media for 24 h, from Day 1 to Day 2 of primary culture, and are expressed as the mean ± SD of three dishes. Asterisks denote statistical difference against the respective WE by Student's *t*-test as follows: *, $p < 0.05$; **, $p < 0.025$; ***, $p < 0.01$; ****, $p < 0.005$. Asterisks in parentheses denote the statistical difference between serum-free and 5 % BS-supplemented conditions. ND: not determined.

b: See the legend to Table 1.

Discussion

Recent reports indicate that primary cultured hepatocytes produce matrix components, such as fibronectin and collagen, which are known to enhance attachment and survival of the cells (10–12). Synthesis of these matrix components in hepatocytes occurs a few hours after inoculation (10). In the present study, WE and DM-160 were found to support attachment, survival and especially albumin secretion of adult rat hepatocytes with relatively high efficiency under serum-free conditions in primary culture. This result may be related to the relatively active synthesis of matrix components in hepatocytes due to an abundance in both sort and concentration of amino acids in these two media, as compared with the other eight media. Hepatocytes inoculated into WE and DM-160 secreted remarkably large amounts of albumin, which were 2.8 to 3.8-fold higher than those secreted into the amino acid-poor media, such as BME and MEM. The present results are consistent with the previous report that synthesis of extracellular proteins, *e.g.*, albumin, by hepatocytes decreased when the cells were cultured in medium deficient in amino acids (13).

Murad *et al.* (14) have reported that collagen synthesis in cultured human skin fibroblasts was stimulated approximately 8-fold by L-ascorbic acid with no significant change in synthesis of noncollagen protein. Therefore, the lack of L-ascorbic acid may be one of the causes for the reduction of efficiency of hepatocyte attachment with the use of MEM, RPMI 1640, F12 and L15 under serum-free conditions.

Addition of serum remarkably improved hepatocyte attachment and survival in the media

which did not support the attachment and survival of the cells as efficiently as did WE and DM-160. This may be due to the supply from serum of an attachment factor, *i.e.*, fibronectin (15, 16), and other factors which are required for the survival of hepatocytes in primary culture (16).

In conclusion, WE and DM-160 are the best among the ten media tested for serum-free primary culture of adult rat hepatocytes.

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