Maintenance of bile acid synthesis and cholesterol 7 α -hydroxylase activity in cultured rat hepatocytes

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Addition of foetal-bovine serum to rat hepatocytes cultured in Williams E medium resulted in improved maintenance of bile-acid-synthetic capacity and cholesterol 7 α -hydroxylase activity as compared with cultures supplemented with rat or newborn-bovine serum or cultures in a hormonally defined serum-free medium. Minimally, 5% (v/v) foetal-bovine serum was necessary to maintain these liver-specific functions. Serum factor(s) responsible for these effects were not dialysable or associated with lipoproteins, but were removed by charcoal extraction.

INTRODUCTION

One of the specific functions of the liver is the synthesis of bile acids, which is the major pathway for removal of cholesterol from the body. In recent years, various groups have shown that primary monolayer cultures of hepatocytes from different species (rat [1–4], rabbit [5] and pig [6]) express this liver-specific function during culture. This ‘*in vitro*’ system offers the opportunity to investigate the effects of mediators thought to be involved in cholesterol and bile acid metabolism (e.g. hormones, bile acids, drugs and cholesterol supply by lipoproteins) directly on hepatocytes in a controlled and chemically defined environment, provided that bile acid synthesis is maintained during culture. We have shown that the activity of cholesterol 7 α -hydroxylase, the first and rate-limiting enzyme in the bile-acid-synthetic pathway, decreases with culture age [6–8]. However, enzyme activity can be restored to the initial level found in freshly isolated hepatocytes of rat and pig by addition of dexamethasone and insulin [6,7]. This recovery was only found in the presence of foetal-bovine serum (FBS) [6]. Furthermore, bile acid synthesis in pig hepatocytes was low in serum-free medium as compared with culture medium containing FBS [6]. Addition of albumin to serum-free medium lowered the formation of bile acids by rat hepatocytes even further [2]. These observations led us to the idea that serum may be necessary to maintain this liver-specific function.

Cholesterol 7 α -hydroxylase and several other enzymes involved in the synthesis of bile acids are specific cytochrome *P*-450 isoenzymes [9]. Loss of total cytochrome *P*-450 is a well-known phenomenon in cultured rat hepatocytes [10–12]. However, contradictory observations were made with respect to the influence of serum on this decrease. The decrease was found to be independent of the supplementation with serum [10,11], but addition of rat serum (RS) partially prevented the decline in cytochrome *P*-450 [13]. Furthermore, FBS was shown to be beneficial for maintenance of other differentiated liver functions [14]. On the other hand, deleterious effects of FBS on the maintenance of liver-specific functions have been reported [15,16]. Groups studying bile acid synthesis *in vitro* either employ serum-free conditions [1,2,5] or use medium supplemented with rat serum [3].

Since little is known about the culture conditions that may preserve bile acid synthesis and cholesterol 7 α -hydroxylase activity, we have evaluated the effect of different widely used sera on the levels of these parameters in cultured rat hepatocytes. In parallel incubations we investigated the effects of a hormonally

defined serum-free medium (HDM), which prolonged several liver-specific functions during culture [15,16]. Here we report that addition of FBS to cultures markedly improved culture conditions for the study of the regulation of bile acid synthesis and cholesterol 7 α -hydroxylase activity.

MATERIALS AND METHODS

Materials used for isolation and culturing of rat hepatocytes, determination of mass production of bile acids and assaying cholesterol 7 α -hydroxylase activity were obtained from sources described previously [4,7,8].

Williams E (WE) and RPMI 1640 culture medium, and foetal- (FBS) and newborn- (NBBS) bovine serum were from Flow Laboratories (Irvine, Ayrshire, Scotland, U.K.). All ingredients (freshly prepared) for the HDM according to Reid and co-workers [15,16] were purchased from Sigma (St. Louis, MO, U.S.A.). RS was prepared from male Wistar rats weighing 300–350 g. All sera were heat-inactivated at 56 °C for 30 min. Activated charcoal (250–350 mesh) was from Sigma, and Dextran T-70 was from Pharmacia Fine Chemicals (Uppsala, Sweden).

FBS was treated with active charcoal (50 mg/ml) and Dextran T-70 (5 mg/ml) for 30 min at 56 °C as described by Sato [17]. Lipoprotein-depleted FBS (LPD-FBS) was obtained by ultracentrifugation of FBS at a density of 1.21 g/ml, followed by extensive dialysis of the infranant against WE as described previously [18].

Male Wistar rats (250–350 g) were used throughout and maintained on standard chow (Hope Farms, Woerden, The Netherlands) and water *ad libitum* as described previously [8]. For the preparation of hepatocytes, animals were killed between 09:00 and 10:00. Institutional guidelines for animal care were observed in all experiments.

Rat hepatocyte preparation and culture

Rat liver cells were isolated by perfusion with 0.05% collagenase and 0.005% trypsin inhibitor as described previously [4,7,8]. Viability, as determined by Trypan Blue exclusion, was higher than 90%. The cells were seeded on 60 mm-diameter plastic tissue-culture dishes (Costar, Cambridge, MA, U.S.A.) at a density of 1×10^5 cells/cm² in WE medium supplemented with 10% heat-inactivated FBS, 2 mM-L-glutamine, insulin (20 munits/ml), 50 nM-dexamethasone, penicillin (100 i.u./ml) and streptomycin (100 μ g/ml), and maintained at 37 °C under

Abbreviations used: HDM, hormonally defined serum-free medium; WE, Williams E; FBS, foetal-bovine serum; NBBS, newborn-bovine serum; RS, rat serum; LPD-FBS, lipoprotein-depleted serum prepared from foetal-bovine serum.

CO₂/air (1:19) [4,7]. After a 4 h attachment period and 24 h thereafter, medium was refreshed with 2.5 ml of the appropriate culture medium without hormones.

Quantification of mass production of bile acids in cultured rat hepatocytes

Mass production of bile acids by rat hepatocytes was measured during the 24 h culture period from 28 to 52 h as described previously [8].

Cholesterol 7 α -hydroxylase

Cholesterol 7 α -hydroxylase activity in homogenates of hepatocytes cultured for 52 h was measured as reported previously [7].

Protein and cholesterol determinations

Protein and cholesterol were assayed as described by Lowry *et al.* [19] and Gamble *et al.* [20] respectively.

Statistical analysis

Statistical significance of differences was calculated using Student's *t* test for paired data, with the level of significance selected to be $P < 0.05$. Values are expressed as means $\pm$ S.D.

RESULTS

Effect of sera and culture media

Mass production of bile acids by rat hepatocytes cultured in WE medium containing 10% FBS varied markedly between different cell isolations. A similar phenomenon was found with cholesterol 7 α -hydroxylase activity ([7]; Table 1) and was caused by inter-individual variation between different animals [7]. The synthetic rate was 1.35 ± 0.68 μ g/24 h per mg of cell protein ($n = 8$) during the second day of culture. Hepatocytes synthesized predominantly cholic acid ($22 \pm 7\%$ of total bile acid synthesis) and β -muricholic acid, together with a small amount of its precursor, chenodeoxycholic acid (sum of the latter two is $78 \pm 7\%$). Replacement of FBS by 10% NBBS or RS lowered the synthetic rate markedly (Table 1). Since promising effects of HDM on expression of tissue-specific functions in cultured hepatocytes were reported [15,16], cells were maintained in this

Table 1. Effect of sera and culture media on bile acid synthesis and cholesterol 7 α -hydroxylase activity

Hepatocytes were cultured as described in the Materials and methods section. Bile acid synthesis was measured in the period from 28 to 52 h, and cholesterol 7 α -hydroxylase activity was determined in cells cultured for 52 h. Data are expressed as a percentage of the values found in cultures containing WE/10% FBS and are the means ($\pm$ S.D., or range where $n = 2$) for duplicate incubations with hepatocytes from n (in parentheses) rats. The absolute values for bile acid synthesis were 1.35 ± 0.68 μ g/24 h per mg of cell protein and for cholesterol 7 α -hydroxylase activity 231 ± 223 pmol/h per mg of cell protein. N.D., not detectable.

Culture medium	Bile acid production		Cholesterol 7 α -hydroxylase	
	(%)	(n)	(%)	(n)
WE/10% FBS	100	(8)	100	(11)
WE/10% NBBS	$29 \pm 23^*$	(8)	$26 \pm 16^*$	(10)
WE/10% RS	$68 \pm 16^*$	(4)	$48 \pm 12^*$	(4)
HDM	$12 \pm 12^*$	(6)	N.D.*	(6)
RPMI/10% FBS	87 ± 8	(2)	77 ± 14	(2)

* Significant difference ($P < 0.05$) compared with values found in WE/10% FBS.

Table 2. Effect of concentration and treatment of FBS on bile acid synthesis and cholesterol 7 α -hydroxylase activity

Experiments were performed, and data are presented, as described in the legend to Table 1.

Culture medium	Bile acid production		Cholesterol 7 α -hydroxylase	
	(%)	(n)	(%)	(n)
WE/10% FBS	100	(8)	100	(11)
WE/5% FBS	109 ± 20	(6)	100 ± 25	(5)
WE/2% FBS	$80 \pm 13^*$	(7)	100 ± 24	(6)
WE/1% FBS	$65 \pm 14^*$	(6)	$54 \pm 20^*$	(5)
WE	$48 \pm 10^*$	(7)	$25 \pm 10^*$	(9)
WE/10% FBS, charcoal-treated	$49 \pm 17^*$	(5)	$20 \pm 10^*$	(6)

culture medium. However, a dramatic decline in bile acid synthesis was observed, which could, for the greater part, be counteracted by the addition of 10% FBS to the culture medium (RPMI 1640) and by the omission of hormones and growth factors. Under the various culture conditions, no significant change in the ratio of the individual bile acids was found, except for cultures in WE/10% NBBS, in which medium the contribution of cholic acid to total bile acid formation decreased to $7 \pm 7\%$.

The enzyme activity of cholesterol 7 α -hydroxylase followed a pattern similar to that of bile acid synthesis (Table 1).

Effect of serum concentration and treatment of FBS

As Table 2 shows, minimally 5% and 2% FBS respectively were necessary to maintain bile-acid-synthetic capacity and 7 α -hydroxylase activity. At lower FBS concentrations a gradual decrease of these functions was observed. Subjecting FBS to charcoal extraction diminished the beneficial effect of FBS noticeably, to levels found in serum-free WE medium. No changes in bile acid production and enzyme activity were detected in hepatocytes cultured in FBS that had been dialysed extensively against WE medium (molecular-mass cut-off 10 kDa) or depleted of lipoproteins.

Effect of dexamethasone on cholesterol 7 α -hydroxylase

Recently we have shown that glucocorticoids stimulate bile acid production in rat hepatocytes by inducing cholesterol 7 α -hydroxylase [8]. Since a large decrease in enzyme activity occurred on culturing of cells in sera other than FBS, or on omitting serum, we investigated whether hepatocytes cultured under these conditions were still responsive to dexamethasone. Addition of 1 μ M-dexamethasone stimulated 7 α -hydroxylase activity 7-fold (S.D. = 3, $n = 9$) in cultures containing 10% FBS. Enzyme activity was enhanced 10-fold (S.D. = 4, $n = 4$) in WE medium containing 10% NBBS, 6-fold (S.D. = 3, $n = 4$) in serum-free WE medium and 4-fold (S.D. = 1, $n = 4$) in culture medium supplemented with charcoal-treated FBS. No cholesterol 7 α -hydroxylase activity could be detected in HDM in the presence of 1 μ M-dexamethasone ($n = 6$).

DISCUSSION

We have shown that FBS has a stabilizing effect on the ability of cultured rat hepatocytes to synthesize bile acids and on the activity of cholesterol 7 α -hydroxylase during culture. In this respect, the cholesterol 7 α -hydroxylase, and possibly also other

cytochrome *P*-450-dependent enzymes involved in bile acid formation, clearly differ from other classes of cytochrome *P*-450 isoenzymes, since culturing in FBS did not prevent the decrease of total or specific *P*-450 enzymes as compared with other sera or serum-free cultures [10,12,21]. In agreement with the finding by Stenberg & Gustafsson [13] on maintenance of total cytochrome *P*-450, we also found a significant improvement by addition of RS to WE medium, but the stabilizing effect was less than with FBS. Hepatocytes cultured in HDM virtually lost bile-acid-synthetic capacity, and cholesterol 7 α -hydroxylase activity could not be detected and was not inducible by dexamethasone. The disappearance of these functions was in strong contrast with the beneficial effects of this culture medium on the maintenance of other liver-specific capacities, e.g. prolonged synthesis of albumin and α_1 -antitrypsin [15,16]. Lowering of the high insulin concentration, which is one of the constituents of HDM and which decreased cholesterol 7 α -hydroxylase activity and bile acid synthesis in rat hepatocytes (H. M. G. Princen, P. Myer & E. M. Lehmann, unpublished work), did not alter the latter two. The reason for the adverse effect of HDM remains unknown, but addition of 10% FBS to RPMI 1640 medium largely restored these functions, indicating that it cannot be attributed to the medium *per se*.

At least 2% FBS was necessary to maintain cholesterol 7 α -hydroxylase activity and 5% for the bile-acid-synthetic capacity. It is unlikely that this difference is the result of shortage of exogenous (lipoprotein) cholesterol as substrate for bile acid synthesis, since production of bile acids was not diminished in medium supplemented with LPD-FBS. It was shown that lipoprotein-depleted serum stimulated synthesis and secretion of cholesterol in cultured rat hepatocytes [22], which was obviously sufficient to maintain the bile-acid-synthetic rate. This is supported by the finding by Sutton & Botham [23] that there is a significant correlation between bile acid synthesis and the secretion of non-esterified and esterified cholesterol, which may be utilized as substrate for bile acid production. We suggest that FBS at higher concentrations may also stabilize other enzymes in the bile-acid-synthetic pathway apart from cholesterol 7 α -hydroxylase.

Differences in cholesterol 7 α -hydroxylase activity cannot be explained by changes in substrate availability, since the cellular free cholesterol content was not significantly altered under the various culture conditions (16.7 ± 1.7 μ g/mg of protein in hepatocytes cultured in WE/10% FBS) and since enzyme activities were assayed with saturating cholesterol concentrations [7]. These findings suggest that, under the conditions given, cholesterol 7 α -hydroxylase activity may be regulated by modulation of synthesis or degradation of the enzyme, although changes in the catalytic activity of the enzyme cannot be excluded.

Dialysis and depletion of lipoproteins from FBS did not affect the stabilizing effect of this serum, indicating that no dialysable low-molecular-mass compounds or lipoproteins are involved. Charcoal extraction led to a strong decline in maintenance of bile acid synthesis and enzyme activity. It is known that this treatment effectively removes some classes of steroid hormones and, less effectively, protein factors and probably other components of possible biological importance [17]. It is not known which factors were responsible for the preservation of these functions. It is unlikely, however, that removal of steroid hormones provoked the fall, since most of these hormones were unable to stimulate bile acid synthesis [8]. Glucocorticoids are active inducers, but FBS in our experiments was used at a concentration of 10% or

less, reducing the concentration of cortisol and corticosterone in the culture medium to levels (giving 50–200 nM; [24]) below that which was stimulatory [8]. In addition, no (or a less) stabilizing effect was found with NBBS or RS also containing these glucocorticoids.

The beneficial effect of FBS on bile acid synthesis and cholesterol 7 α -hydroxylase activity was also observed in pig hepatocyte cultures [6], indicating that this is not a species-specific effect. We conclude that FBS is an *in vitro* requirement of cultured parenchymal cells necessary for better maintenance of these specific functional activities, making them more suitable for regulation studies.

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