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Collagen sandwich culture affects intracellular polyamine levels of human hepatocytes

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Abstract. Extracellular matrices, like collagen layers, play an important role in preventing dedifferentiation of hepatocytes in long-term culture experiments. It has also been shown that polyamines are crucial for cell growth and liver differentiation – regeneration. Primary cultured hepatocytes with their low mitotic activity might be a valuable tool in studying the role of polyamines in differentiation. Here, our goal was to investigate whether an extracellular cell culture matrix can influence intracellular polyamine levels in human hepatocytes during long-term culture. Primary human hepatocytes were isolated from surgical tissue resections and were maintained either in single collagen (SG) or double collagen gel (DG) layer (sandwich) culture systems. Cell viability and function were examined and intracellular polyamine levels were measured using a highly sensitive high performance liquid chromatography (HPLC) method. Hepatocytes showed high viability in both culture systems used, but albumin secretion was diminished in SG cultured hepatocytes after 14 days. In general, total intracellular polyamine levels of hepatocytes decreased markedly in both SG and DG within the first days of culture, but remained constant until day 21 with a SG/DG ratio of about 1.4. Individual polyamines levels were dependent on the culture time and system, where spermine decreased and putrescine increased in both SG and DG over time (day 14), but spermidine increased only in DG. Our results suggest that polyamine levels, in particular putrescine, might be important regulators of hepatocyte specific function *in vitro* and therefore serve as a marker of differentiation for cultivated human hepatocytes.

INTRODUCTION

In vitro cell culture techniques for long-term cultivation of primary hepatocytes are based on different strategies to maintain their differentiation and function. Several papers are published

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dealing with the influence of cell culture media conditions (Bader *et al.* 1999; Hino *et al.* 1999), cell–cell (Michalopoulos & DeFrances 1997; Bhatia *et al.* 1999) and cell–matrix interactions (Ezzell *et al.* 1993; Dunn *et al.* 1989; Chen *et al.* 1998) on the viability and function of primary cultured hepatocytes. Single- and double-(sandwich) gel culture techniques resemble *in vivo* conditions by embedding cells in collagen layers. Typically, one layer is set on the bottom of a culture dish, and an additional layer placed on top of the hepatocyte monolayer (Dunn *et al.* 1989; Ryan *et al.* 1993; Knop *et al.* 1995). Under these conditions, hepatocytes can be shown to maintain their function and differentiation for up to 4–6 weeks, depending on the system used. Changes in cultured hepatocyte function were monitored by determination of specific mRNA expression (Chen *et al.* 1998; Dunn *et al*. 1992) or protein secretion into the culture media (Ryan *et al.* 1993; Pahernik *et al.* 1996) or protein induction with drugs (Kern *et al.* 1997; Silva *et al.* 1998; Hosagrahara *et al.* 2000). Because regulation of transcriptional and translational levels is highly regulated and not fully understood, markers of hepatocyte differentiation involved in these processes are lacking.

The polyamines putrescine (Put), spermidine (Spd), spermine (Spm), and their acetylated derivatives, are a prerequisite for cellular metabolism (Pegg 1988). A role of polyamines for inducing a proliferative response, or to maintain cell differentiation, has been demonstrated for a variety of cell types (Verma & Sunkara 1982; Frostesjo *et al.* 1997; Duranton *et al.* 1998). It has also been shown that polyamines are essential for hepatic regeneration in that inhibition of their synthesis by chemical agents significantly impairs regenerative activity after partial hepatectomy (Diehl *et al.* 1990; Luk 1986). One of the early genes expressed during liver regeneration is *odc*, which encodes for ornithine decarboxylase (ODC), a key enzyme in polyamine metabolism (Fig. 1). Furthermore, polyamines improved the survival rate of rats with an orthotopic liver transplant (Terakura *et al.* 1995) and play an important role in growth-factor-induced DNA synthesis in cultured rat hepatocytes (Nagoshi & Fujiwara 1994; Higaki *et al.* 1999).

Very few experiments have been undertaken to investigate intracellular polyamine levels of cultured hepatocytes. It has been shown that polyamine levels change during the first days of

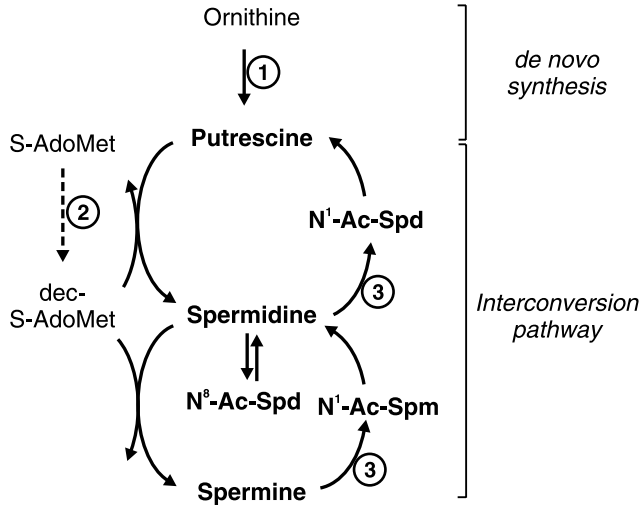

Figure 1. Intracellular metabolism of polyamines: synthesis and metabolism of polyamines is catalysed by a variety of enzymes of which the rate-limiting enzymes are indicated in the diagram. 1, ornithine decarboxylase (ODC); 2, S-adenosylmethionin decarboxylase (AdoMetDC); 3, spermidine/spermine acetyltransferase (SAT).

culture with different short-term rat hepatocyte culture systems (Wallace 1989; Colombatto & Grillo 1991), but data using primary human hepatocytes in long-term culture experiments are missing. Primary hepatocytes keep their function over a certain time of culture, depending on the use of a single collagen (SG) or double collagen (DG) culture system, but there is no evidence for proliferation in either systems (Bhatia *et al.* 1994; Thomson & Arthur 1999). Therefore, human hepatocytes could be a valuable tool in studying the role of polyamines in hepatic cell function and differentiation. It may be important to understand the changes in hepatocyte differentiation *in vitro* if culture models are used to study pharmacological metabolism and liver regeneration.

In this article we present data that demonstrate the influence of different cell culture systems (SG or DG) and the culture time on the intracellular polyamine content of human hepatocytes. The changes in polyamine concentrations in cultured human hepatocytes correlate with cell-specific function and may therefore be a marker for regulation of hepatic cell function and differentiation.

MATERIALS AND METHODS

Reagents

Polyamines, boric acid and ethylene glycol-bis(beta-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA) were obtained from Sigma (Munich, Germany). Acetic acid was purchased from Fluka Chemical (Neu-Ulm, Germany). Methanol and acetonitrile were of HPLC-grade (Baker, Groβ-Gerau, Germany), and water was purified by a Milli-Q System (Millipore, Eschborn, Germany). Triethylamine and all other chemicals were of analytical or HPLC-grade and were obtained from Merck (Darmstadt, Germany) apart from N-hydroxysuccinimidyl 6-quinolinyl carbamate (HSQC), which was purchased as part of the AccQ amino acid analysing reagent kit from Waters (Eschborn, Germany). Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/l glucose, collagenase (type IV) and all other media additives were purchased from Serva (Heidelberg, Germany).

Hepatocyte preparation and culture

Tissue samples from human liver resection were obtained after pathological evaluation from four male patients (age 43–64 years) undergoing partial hepatectomy for metastatic liver tumours of colorectal cancer. Experimental procedures were performed according to the guidelines of the local Ethics Commitee, University Hospital of Regensburg, with informed patient's consent.

Cells were isolated using a modified two-step EGTA/collagenase perfusion procedure as described previously (Ryan *et al.* 1993; Pahernik *et al.* 1996). Viability of the isolated hepatocytes was determined by trypan blue exclusion, and cells with a viability greater than 85% were used for cell culture. For SG culture, cells were plated on a collagen gel layer (1.1 mg/ml type I collagen, Serva) at a density of 0.15×10^6 cells/cm^2 in culture media (DMEM with 5% FCS and supplements as follows: 125 mU/ml insulin, 60 ng/ml hydrocortisone, 10 ng/ml glucagon, 100 μg/ml streptomycin, 100 mU/ml penicillin). After 24 h, cell adhesion media were replaced by supplemented DMEM without FCS, and cells were incubated at 37 °C in a humidified incubator with 5% CO_2. For DG culture, cells were covered by a second collagen layer after 24 h of plating and were maintained under the conditions described for single-gel cultures. In both cases, media were changed on a daily basis. At the specified times, cells with collagen matrix were digested with collagenase (0.02% v/v) for 10 min at 37 °C, followed by resuspension in

phosphate buffered saline (PBS). The hepatocytes were washed twice with PBS and cell pellets were stored at −80 °C until analysis.

Polyamine determination

For the determination of polyamines and their acetylated derivatives, we used a highly sensitive reversed-phase high-performance liquid chromatography method (RP-HPLC), as we have described recently (Weiss *et al.* 1997). Briefly, cell pellets were resuspended in 50 mM borate buffer (pH 7.4), homogenized by sonication and centrifuged (13 000 ***g*** for 10 min). 50 µl of the supernatant were mixed with 10 µl of internal standard (1,7 diaminoheptane, 60 µM) and 30 µl of 0.6 M trichloroacetic acid was added for deproteinization. After a second centrifugation step (13 000 ***g*** for 10 min), 10 µl of the resulting supernatant were mixed with 30 µl of 0.2 M borate buffer (pH 9.3). Derivatization was accomplished by adding 20 µl of a 10-mM HSQC solution; after gently shaking, the samples were loaded onto the HPLC column (RP-C18).

Other methods

For DNA determination, Hoechst dye H33259 was used with calf thymus DNA (Sigma) as a reference (Labarca & Paigen 1980). Albumin secretion into the supernatant medium was assayed immunologically as described by the manufacturer (Dako Diagnostika, Hamburg, Germany). Lactic dehydrogenase (LDH) was measured colourimetrically within the framework of routine clinical diagnosis. Results are expressed as the mean ± standard error of the mean (± SEM) and the statistical significance was calculated according to the student's *t*-test.

RESULTS

Freshly isolated primary human hepatocytes were cultured on collagen-coated dishes, either with a second collagen layer on top, DG, or without, SG. Morphology of the human hepatocytes was monitored by light microscopy over the culturing period (Fig. 2). Results show that, within a culture time of 14–21 days, human hepatocytes kept in SG (Fig. 2a and b) start to spread and develop a flattened morphology, compared with cells in DG (Fig. 2c and d) that maintain their cubic-polygonal structure. Additionally, albumin and LDH levels were measured to assess cell function and viability, respectively (Fig. 3). Albumin secretion of hepatocytes in SG decreased after 14 days and was almost undetectable at day 21, whereas hepatocytes in DG continued to show a good function at 14 days, but the function decreases by day 21 (Fig. 3a). In contrast, in both the SG and DG culture systems, low levels of LDH were observed during the first 7 days, with an increase in LDH activity at day 14 that persisted until day 21 (Fig. 3b). These LDH activities in the medium of cultured cells at day 14 and 21 are low compared to day 28 (approximately 175 mU/ml, data not shown) when cells dramatically lose viability. These results indicate that primary human hepatocytes cultured either in the SG or DG culture system maintain their viability over 21 days, whereas hepatic-specific function persists longer in DG than SG cultures.

The content of intracellular acetylated and non-acetylated polyamines in freshly isolated and cultured human hepatocytes was determined using a highly specific HPLC method. Acetylated polyamines like N^1-acetyl-put, N^1-acetyl-spd and N^1-acetyl-spm were determined, but showed no statistically significant difference over culture time (data not shown). The initial highly elevated content of putrescine (Put), spermidine (Spd), spermine (Spm), N^8-acetylspermidine (N^8-Ac-spd) and total polyamines in freshly isolated primary human hepatocytes at day 0 (Table 1)

Table 1. Levels of polyamines and their acetylated derivatives determined in primary human hepatocytes cultivated in either single collagen gel (SG) or double (sandwiched) collagen (DG) gel culture systems (± SEM, one experiment out of four was done in triplicate)

Day of culture	Putrescine (pmol/μg DNA)		Spermidine (pmol/μg DNA)		Spermine (pmol/μg DNA)		N^8-acetylspermidine (pmol/μg DNA)		Total polyamines (pmol/μg DNA)	
	SG	DG	SG	DG	SG	DG	SG	DG	SG	DG
0*	21.3		67.2		266.2		49.1		413.6	
7	6.3 ± 0.7	4.0 ± 0.7	57.2 ± 6.7	27.0 ± 1.9	105.8 ± 11.4	55.8 ± 4.5	30.2 ± 3.4	34.9 ± 3.4	209.3 ± 18.9	137.8 ± 6.2
14	77.0 ± 2.5†	55.9 ± 6.6†	63.4 ± 6.8	44.0 ± 4.1†	51.3 ± 2.3†	39.2 ± 1.4†	30.5 ± 4.2	17.4 ± 1.5†	226.0 ± 11.0	176.8 ± 12.9
21	135.6 ± 7.6‡	73.5 ± 13.7	53.5 ± 7.2	41.0 ± 3.3	55.9 ± 4.6	45.8 ± 4.0	29.8 ± 0.2	10.6 ± 2.3	274.2 ± 15.7	192.8 ± 4.5†

*Freshly isolated primary hepatocytes, †differs significantly from day 7 ($P < 0.05$) and ‡differs significantly from day 14 ($P < 0.05$).

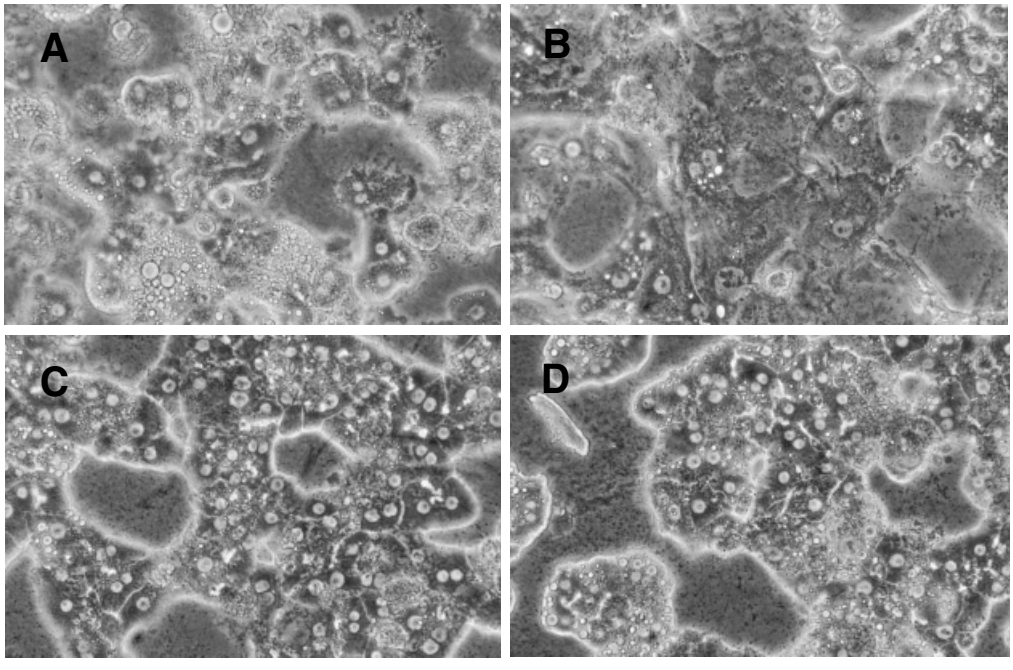

Figure 2. Morphology of primary human hepatocytes grown either in SG (a, b) or DG (c, d) culture systems under standard conditions. Pictures were taken at day 14 (a, c) and 21 (b, d), using phase-contrast microscopy (magnification × 320). Human hepatocytes cultured in DG maintain their polygonal morphology (c, d) whereas hepatocytes in SG spread out and flatten (b).

might be due to stress of isolation, as, within the subsequent 7 days, levels were markedly decreased accompanied by high function in both SG and DG systems (see also Fig. 3a). The observed decrease was most dramatic in the case of DG cultured hepatocytes for Put, Spd, Spm and total polyamines. During the consecutive cultivation period in the DG system, total polyamine content increased from day 7 to day 21. Furthermore, between day 7 and day 14 in DG systems, concentrations of Put and Spd were increased, whereas Spm and N^8-Ac-spd levels were decreased. In contrast, using the SG technique we determined constant total polyamine levels from day 7–21, but an increase of Put from day 7 through day 21 and a decrease of Spm between day 7 and 14. During cultivation of hepatocytes, polyamine levels changed in SG and DG systems over time and alterations of polyamine content was dependent on the system used. This suggests that over culture time the extracellular matrix induces enzymes of polyamine metabolism, regulating their homeostasis in the cell.

To further compare the influence of extracellular matrix on primary hepatocytes *in vitro*, we calculated the ratios of polyamine levels of hepatocytes cultivated in SG or DG systems (Fig. 4). Interestingly, levels of total polyamines were approximately 1.4-fold higher in the SG systems during the entire cultivation period (Fig. 4). Significantly elevated levels (~2-fold ratio) in SG over DG systems were found for Spd and Spm at day 7, which decreased from day 14–21 to about 1.3-fold. In contrast, SG/DG ratios of N^8-Ac-spd and Put increased over culture time after 21 days to a ratio of 2.7 and 2, respectively (Fig. 4). Enhanced Spd levels in SG over DG might be responsible for the high N^8-Ac-spd content, as its generation is regulated by Spd concentration (Fig. 1). The use of a sandwich configuration (DG) for culturing primary human hepatocytes lowers intracellular polyamine content compared with mono-layer systems (SG). Activation of

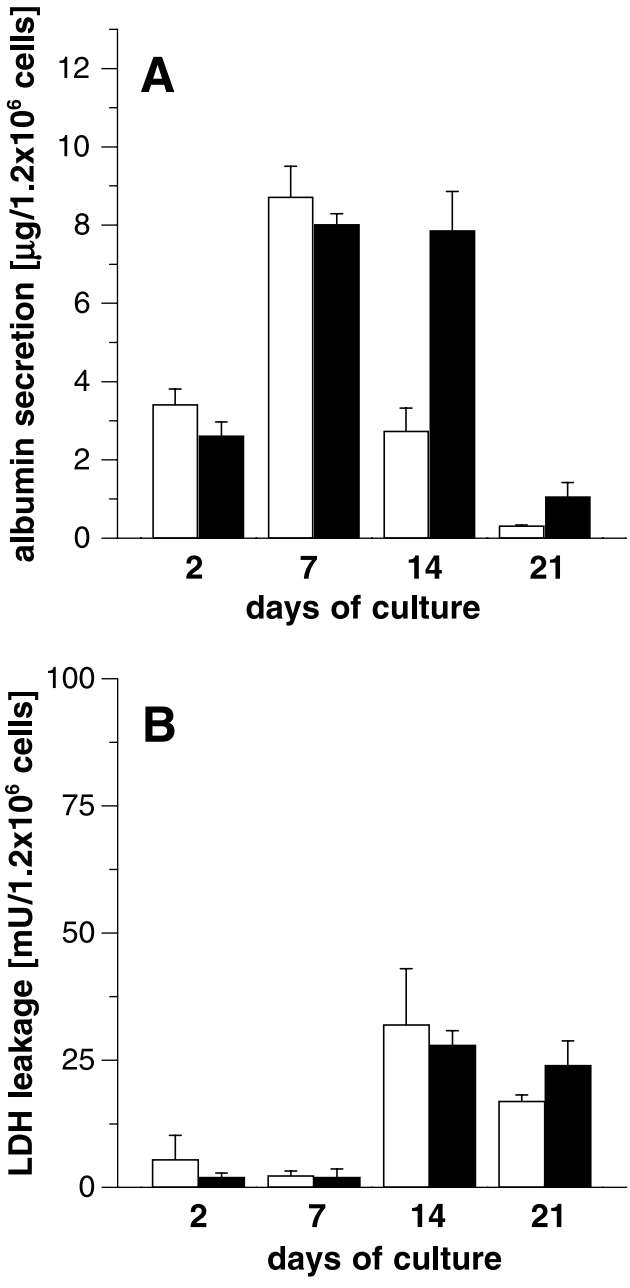

Figure 3. Function and viability of cultured primary human hepatocytes. (a) 24-h albumin secretion into culture media was determined as a parameter for hepatocyte cell function. Primary human hepatocytes grown in DG (black bar) compared to SG (open bar) systems demonstrate function through 21 days of culture. (b) Measurement of LDH activity in medium of cultured cells was used as a marker for cell integrity. After 14 days in culture, an increase of LDH activity was observed in both SG (open bar) and DG (black bar) systems (± SEM, one experiment out of four was done in triplicate).

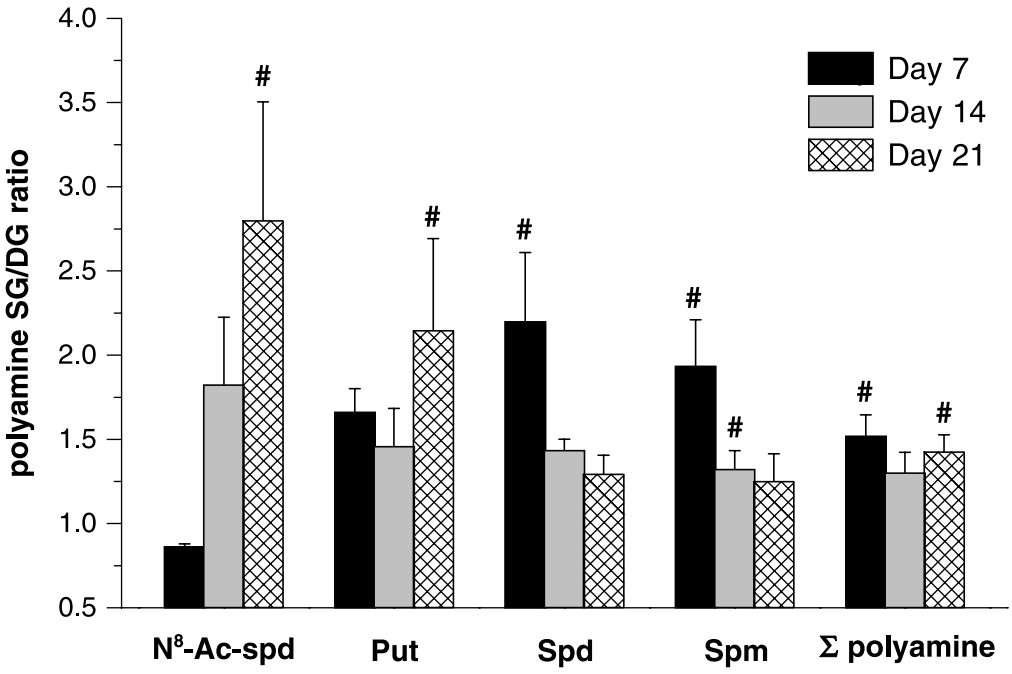

Figure 4. Ratios of polyamine levels of human hepatocytes cultured either in SG or DG systems. Ratios were calculated using data from Table 1. Significantly elevated levels of polyamines in SG over DG cultured cells were observed within 21 days of culture (#$P > 0.05$, SG vs. DG) (± SEM, experiment was done in triplicate). N^8-Ac-spd, N^8-Acetylspermidine; Put, putrescine; Spd, spermidine; Spm, spermine.

polyamine metabolism through the presence of a second collagen layer, leads to a higher decrease of Spm until day 14 and to a reduction of the Put increase until day 21 compared to SG.

DISCUSSION

To maintain primary hepatocytes *in vitro* requires specific cell-culture systems to prevent cells from retrodifferentiation over culture time. For both rat (Dunn *et al.* 1989; Dunn *et al.* 1992; Ezzell *et al.* 1993; Knop *et al.* 1995) and human hepatocytes (Ryan *et al.* 1993) collagen DG (sandwich gel) culture systems have been shown to be suitable in maintaining specific morphology and functionality. It is crucial to monitor cell-specific products of cultured hepatocytes as a marker for their function, but additional parameters might be necessary to indicate retrodifferentiation events at an early stage.

Primary cultured hepatocytes with their low mitotic activity might be a valuable tool in studying the role of polyamines in differentiation. Interestingly, after an initial decrease of all polyamine levels in both SG and DG cultured human hepatocytes, Put content increased, while Spm decreased over culture time. These results could be explained by an activated degradation of Spm through Spd to Put, while synthesis of Put is inactive (see Fig. 1). Therefore, these results indicate an active interconversion pathway versus inactive biosynthesis of polyamines in cultured human hepatocytes. Our findings are consistent with a low activity of ornithine decarboxylase (ODC), the rate limiting enzyme in polyamine biosynthesis as reported in cultured rat

hepatocytes (Higaki *et al.* 1993; Higaki *et al.* 1999). The major activity of polyamine catabolic enzymes, e.g. diamine oxidase (Seiler 1987) and secretion of polyamines (i.e. acetylated polyamines) may have a minor impact as total polyamine levels did not decrease over time. Further uptake of exogenous polyamines could be excluded by usage of defined serum-free medium without polyamine addition.

The impact of an extracellular matrix on intracellular polyamine metabolism is shown by lower polyamine levels of human hepatocytes cultured in DG versus SG. Similar results were obtained with rat hepatocytes (Colombatto & Grillo 1991) where decreasing polyamine concentrations were evident after a 5-day delay after addition of a second collagen layer to SG cultures. Furthermore, distribution of the individual polyamines of human hepatocytes, as seen by the calculated ratio of spermidine to spermine changes from day 7–21, but nevertheless remains low (Table 1). This ratio was suggested as an index for growth in non-transformed cells with ratios of 1.5–2.0 being indicative of rapid rates of cell growth (Wallace 1989). In general, a low Spd/Spm ratio in hepatocytes is indicative of their quiescent state, as we have observed for human hepatocytes in SG, as well as in DG collagen culture systems.

Only a few reports have been published dealing with polyamine levels of cultured hepatocytes. In these studies primary rat hepatocytes were maintained for 2–10 days (Wallace 1989; Colombatto & Grillo 1991) in culture either on SG (Schulz *et al.* 1989; Colombatto & Grillo 1991) or DG collagen systems (Colombatto & Grillo 1991), or on plastic (Wallace 1989). In the present study, we show that primary cultured hepatocytes from human tissues have a marked loss of all polyamine levels between the time of isolation and day 7 of culture. To date this has only been observed by others for Spm (Schulz *et al.* 1989; Wallace 1989) and Spd (Wallace 1989) using primary rat hepatocytes within 2 days of SG culture. Furthermore, they found increased levels of Put (Schulz *et al.* 1989; Wallace 1989; Colombatto & Grillo 1991) and Spd (Schulz *et al.* 1989; Colombatto & Grillo 1991) during the first days of culture. This is in contrast to our findings with human hepatocytes, where Put levels started to rise between day 7 and 14 of culture, and Spd levels in SG remained almost unchanged. Obviously we did not use the same time frame of culture for our investigations of polyamine levels, but these differences might also be partly explained by the different species. Several reports have been published indicating that hepatocytes from rat and human (Silva *et al.* 1998; Jo *et al.* 2000) have different metabolic activities (Hogemann *et al.* 2000) which may be related to the differences in polyamine levels observed.

It has also been shown previously that rat (Gupta 2000) and human (Kern *et al.* 1997) hepatocytes in sandwich configuration had higher activities of cytochrome P450 isoenzymes compared to cells cultured on SG. Additionally it was found that CYP450 3A4 activities of cultured human hepatocytes decline within the first 5 days on SG (Silva *et al.* 1998), and within 9 days on DG systems (Kern *et al.* 1997). Based on these studies and our findings regarding polyamine levels of hepatocytes, one could speculate that increasing Put and decreasing Spm levels might be responsible for CYP450 3A4 function *in vitro*. This hypothesis is supported by a recently published paper reporting Put decreases of cytochrome P450 3A4 levels in rat hepatocytes during liver regeneration (Favre *et al.* 1998).

Spm levels may be significant for cell differentiation, as it has been shown that for rat hepatocytes (Colombatto & Grillo 1991) Spm content could be inversely correlated with hepatocyte differentiation during a 10-day culture experiment. Our present study partly confirms this for human hepatocytes, but during a 21-day cultivation, only Put content was correlated to albumin secretion. Our data suggest that besides Spm, Put levels might play an important role in hepatocyte differentiation, as high levels were shown to affect metabolic function. Therefore, polyamines might be a valuable marker for early events that reflect hepatocellular function. Further studies needs to be done to elucidate the role of polyamines in hepatocyte metabolism.

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