Metabolic interactions of parenchymal hepatocytes and dividing epithelial cells in co-culture

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When parenchymal hepatocytes isolated from adult liver are co-cultured with other epithelial cells, the production of various plasma proteins by the hepatocytes is preserved for much longer than in conventional culture. This study examines some of the metabolic interactions between parenchymal hepatocytes and epithelial cells maintained in co-culture. The leakage of lactate dehydrogenase by hepatocytes co-cultured with epithelial cells was lower than in conventional hepatocyte culture. The epithelial cells have a high glycolytic rate and provide the hepatocytes with a continual supply of lactate. The [lactate] was lower in co-cultures of hepatocytes and epithelial cells than in pure epithelial cultures of similar density, suggesting lactate clearance by the hepatocytes. Alanine uptake was higher in conventional hepatocyte cultures, which lack an exogenous supply of lactate, than in parenchymal hepatocytes in co-culture. Studies with pure parenchymal hepatocytes incubated with increasing [lactate] suggest that lactate is utilized in preference to alanine as a gluconeogenic substrate by hepatocytes co-cultured with epithelial cells. Ketogenesis and carnitine palmitoyltransferase activity declined more slowly in hepatocytes co-cultured with epithelial cells than in conventional culture. It is concluded that the co-culture model has potential for long-term studies of carbohydrate and lipid metabolism.

INTRODUCTION

Parenchymal hepatocytes isolated from adult liver and maintained in primary culture are the only system *in vitro* for long-term studies of differentiated liver functions [1]. However, a disadvantage of hepatocyte cultures, for experiments of several days duration, is that they show a progressive decline in expression of certain liver functions, e.g. the production of albumin and acute-phase proteins, and various metabolic functions [1]. Recently a technique has been developed by Guguen-Guillouzo and co-workers [2–4] which preserves some of the liver functions of hepatocytes in culture. This technique involves co-culturing parenchymal hepatocytes with liver-derived epithelial cells, which do not themselves express liver functions [5]. The production of albumin [2], various acute-phase proteins [3] and complement component C3 [4] remains stable in co-cultures with epithelial cells for several days, whereas they decline rapidly in conventional hepatocyte culture. Co-culture of human hepatocytes with epithelial cells derived from rat liver also preserves the activity of cytochrome *P*-450 [6]. The co-culture model is potentially attractive for long-term metabolic studies. However, for metabolic studies, the need to quantify the relative proportions of the two cell types in co-cultures is essential. The objectives of the present study were: (1) to examine whether co-culture of parenchymal hepatocytes with undifferentiated liver epithelial cells helps to preserve carbohydrate and lipid metabolism (gluconeogenesis and ketogenesis) in hepatocytes; (2) to examine the metabolic interactions between the hepatocytes and epithelial cells in co-culture.

MATERIALS AND METHODS

Isolation of rat parenchymal hepatocytes

Parenchymal hepatocytes were isolated from male Wistar rats (body wt. 190–200 g) by liver perfusion with collagenase [7]. The liver cells were dissociated in Earle's balanced salts solution (150 ml) and incubated for 20 min at 37 °C. The parenchymal hepatocytes were sedimented (50 g, 2 min), then washed twice in Earle's salts and once in Earle's salts with 1% (w/v) bovine serum albumin, and suspended in Minimum Essential Medium (MEM) with 5% (v/v) fetal bovine serum and antibiotics [8]. Part of the cell preparation was diluted to 4×10^5 cells/ml in the same medium and used for pure culture or co-culture immediately. Cell viability assessed by Trypan Blue exclusion was > 85%.

Isolation and culture of undifferentiated human epithelial cells

Human liver epithelial cells were isolated from a liver section from a kidney donor (female, 22 years old) [9]. The cells were maintained in Dulbecco's MEM supplemented with 5% fetal bovine serum and dexamethasone (10 nM), in 75 cm² flasks. They were passaged (1:5) every 4–5 days. They were characterized by cytokeratin staining with Labsystem PKK1 monoclonal antibody to cytokeratin polypeptides, by immunoperoxidase staining. These cultures did not secrete human albumin or α -fetoprotein. The epithelial cells were used for co-culture with the parenchymal hepatocytes after between 10 and 14 passages (between 46 and 61 days after isolation from the liver section). Throughout this time the cells retained

an epithelial-like shape [4,10]. However, after 95–100 days from isolation (about 23 passages) they showed gross morphological changes.

Fibroblasts were isolated and grown from the above cultures from the cells that remained attached to the flasks during trypsin treatment of the epithelial cells in the early passages. They were grown in Dulbecco's MEM containing 5% fetal bovine serum and passaged every 8 days.

Hep-G2 cells (human hepatoma [11]) were grown as in [9].

Co-culture of parenchymal hepatocytes with epithelial cells or fibroblasts

The co-cultures were set up in parallel with pure cultures of these cell types seeded at the same cell density per flask as the co-cultures. Immediately after preparation of the diluted parenchymal hepatocyte suspension, 3 ml (1.2×10^6 cells) was added to 25 cm² flasks (Nunc; Gibco). Some of the flasks were kept as pure parenchymal hepatocyte cultures; the rest were used for co-culture. The epithelial cells and fibroblasts were removed from the 75 cm² flasks with trypsin and suspended (at 10^5 cells/ml) in MEM containing 5% fetal bovine serum. The epithelial cells were added (2 ml per flask) either to flasks containing 3 ml of parenchymal hepatocyte suspension immediately after addition of the parenchymal cells or to flasks that had been incubated with parenchymal cells for 4 h to allow the cells to attach and spread. In the latter case, the medium was decanted before addition of the epithelial cells. Pure cultures of epithelial cells (2 ml per 25 cm² flask) were set up in parallel. Fibroblast co-cultures were set up as for the epithelial cells, but with half the cell density. All cultures were equilibrated with 5% CO₂ in air and incubated at 37 °C. At 4 h after inoculation of epithelial cells of fibroblasts, the medium was replaced by 5 ml of Williams E medium containing 1% fetal bovine serum. After 24 h the medium was replaced with 4 ml of serum-free Williams E medium containing 10 nM-dexamethasone and 10 nM-insulin, and thereafter daily with the same medium. The spent medium was collected for determination of lactate dehydrogenase activity and albumin. A 1 ml sample of the medium was deproteinized with 0.2 M-HClO₄ for determination of metabolites.

The data presented are from two experiments out of four. The numbers of replicate flasks set up are shown in Table 2. In the course of the culture, some of the flasks were used for determination of ketogenesis and enzyme activities. In preliminary experiments it was found that lactate dehydrogenase activity and metabolite concentrations varied by less than 8% between replicate flasks of each cell type. Thereafter, assays were performed on pooled medium. When activities or concentrations were constant over several consecutive days, the values are presented as means $\pm$ S.E.M. for the number of days indicated.

Assays

Metabolites. Glucose [12], lactate, pyruvate, 3-hydroxybutyrate, acetoacetate [13] and alanine [14] were assayed enzymically. Urea and ammonia were determined enzymically with a kit from Roche Diagnostics (Nutley, NJ, U.S.A.). The final assay contained 59 mM-Tris, 0.29 mM-NADH, 1.5 mM-ADP, 15 mM-2-oxoglutarate, 40 units of urease/ml and 2.5 units of glutamate

dehydrogenase/ml. The reaction was monitored from the decrease in NADH absorption at 340 nm and is expressed as nmol of urea or ammonia nitrogen per ml.

Ketogenesis. This was determined by incubation of the monolayers with 0.5 mM-palmitate and 0.5 mM-L-carnitine for 2 h as in [15].

Enzymes. Cells were harvested as in [13]. Enzymes were assayed at 30 °C. Pyruvate kinase (EC 2.7.1.40) was assayed with 0.5 mM-phosphoenolpyruvate without or with 100 μ M-fructose 1,6-bisphosphate as in [13]; fructose-1,6-bisphosphatase (EC 3.1.3.11) was assayed as in [16], and other enzymes were measured as in [17].

Proteins. Rat albumin was measured immunoturbidimetrically [18] with goat anti-(rat albumin) IgG (GARa/Alb), obtained from Nordic Immunological Laboratories, Tilburg, The Netherlands. Protein was assayed by the Lowry method [19], with bovine albumin standards.

RESULTS AND DISCUSSION

Enzyme activities in dividing epithelial cells and parenchymal hepatocytes

The dividing epithelial cells had a different morphology from the parenchymal hepatocytes and were similar to dividing epithelial cells from rat liver [4,5,10]. Table 1 shows some of the enzyme activities that differed in the dividing epithelial cells and hepatocytes. The activities of human hepatoma cells are included for comparison. In the dividing epithelial cells: pyruvate kinase was not activated by fructose 1,6-bisphosphate, suggesting the absence of the L isoenzyme; fructose-bisphosphatase was not detectable (< 0.2 munit/mg of protein), indicating lack of gluconeogenic function; and activities of various mitochondrial enzymes, e.g. carnitine palmitoyl-transferase, citrate synthase, hydroxyacyl-CoA dehydrogenase and glutamate dehydrogenase, were much lower than in hepatocytes or hepatoma cells (Table 1). Although there are differences in enzyme activities between human and rat liver [15], enzyme activities in human parenchymal hepatocytes (results not shown) were more similar to those in rat hepatocytes than in dividing epithelial cells from human liver.

Lactate dehydrogenase release in the medium

The leakage of lactate dehydrogenase during the first 9 days of culture was lower in co-cultures of hepatocytes with epithelial cells or fibroblasts than in pure hepatocyte cultures (Table 2), suggesting better parenchymal-cell integrity in co-cultures.

Pyruvate kinase activity

Since pyruvate kinase is activated by fructose 1,6-bisphosphate in parenchymal hepatocytes, but not in dividing epithelial cells, pyruvate kinase activity assayed without the bisphosphate was used to assess epithelial-cell density in co-culture. On day 3 of culture, pyruvate kinase activity (assayed without fructose 1,6-bisphosphate) was approximately equal in co-cultures (104 munits/flask) to the sum of activities in pure parenchymal and pure epithelial cultures (16 and 84 munits/flask respectively), suggesting that epithelial cell number was similar in pure and co-cultures. On days 5 and 8, the activity was 30 and 50% higher in co-cultures than in

Table 1. Enzyme activities in parenchymal hepatocytes, dividing liver epithelial cells and hepatoma cells

Parenchymal hepatocytes and dividing epithelial cells were isolated as described in the Materials and methods section. Enzyme activities are expressed as munits/mg of protein and are means $\pm$ S.E.M., for eight hepatocyte preparations (cultured for 1–3 days) and for five different passages for the epithelial and hepatoma cells. N.D., not detectable.

Cell type ... Species ... n ...	Enzyme activity (munits/mg of protein)		
	Parenchymal hepatocytes	Dividing epithelial cells	Hep-G2 hepatoma
	Rat 8	Human 5	Human 5
Pyruvate kinase	20 $\pm$ 3	46 $\pm$ 6	285 $\pm$ 46
+ 100 μ M-fructose bisphosphate	124 $\pm$ 9	42 $\pm$ 4	281 $\pm$ 46
Fructose-bisphosphatase	38 $\pm$ 6	N.D.	N.D.
Carnitine palmitoyltransferase	31 $\pm$ 1.5	2.1 $\pm$ 0.7	5.0 $\pm$ 1.1
Citrate synthase	126 $\pm$ 9	42 $\pm$ 7	157 $\pm$ 17
Hydroxyacyl-CoA dehydrogenase	102 $\pm$ 6	6.6 $\pm$ 1.5	32 $\pm$ 4
Glutamate dehydrogenase	1660 $\pm$ 41	33 $\pm$ 12	381 $\pm$ 70
Aspartate aminotransferase	2353 $\pm$ 162	165 $\pm$ 30	695 $\pm$ 67

Table 2. Lactate dehydrogenase release in the medium by pure hepatocyte cultures and co-cultures with epithelial cells and fibroblasts

Pure cultures of parenchymal hepatocytes (Par), epithelial cells (Ep) and fibroblasts (Fib) and co-cultures of parenchymal hepatocytes and epithelial cells (Par + Ep) or fibroblasts (Par + Fib) were set up as described in the Materials and methods section. In the co-cultures in Expt. I, the parenchymal hepatocytes and epithelial cells were inoculated simultaneously, and in Expt. II the parenchymal hepatocytes were allowed to attach and spread for 4 h, before inoculation of the epithelial cells or fibroblasts. The medium was changed daily from days 3–9 after start of the culture. The numbers of replicate flasks on days 3 and 9 are shown in parentheses. Values for lactate dehydrogenase activity in the medium (munits/ml) are means $\pm$ S.E.M. for the 7 days. The values for co-cultures that are significantly lower (*t* test) than for pure parenchymal hepatocyte cultures are indicated.

Expt. no.	Cells	No. of flasks		Lactate dehydrogenase activity (munits/ml)
		Day 3	Day 9	
I	Par	3	1	76 $\pm$ 11
	Ep	3	1	19 $\pm$ 3
	Par + Ep	3	1	26 $\pm$ 3 (<i>P</i> < 0.001)
II	Par	10	5	72 $\pm$ 10
	Ep	5	2	22 $\pm$ 4
	Par + Ep	10	5	31 $\pm$ 5 (<i>P</i> < 0.003)
	Fib	4	1	10 $\pm$ 3
	Par + Fib	6	2	46 $\pm$ 5 (<i>P</i> < 0.05)

pure epithelial cultures, suggesting higher epithelial-cell proliferation in co-culture. This may be due to the production of transferrin or insulin-like growth factors by the hepatocytes.

The incremental increase in pyruvate kinase activity after addition of fructose 1,6-bisphosphate was similar in pure parenchymal cultures and in co-cultures on day 3, but it then declined more rapidly in pure parenchymal cultures, and on days 5 and 8 the bisphosphate-dependent activity was 76 and 31 %, respectively, of that in co-cultures. On day 8 the activity in co-cultures and pure cultures was 50 and 18 %, respectively, of the original, indicating that the L isoenzyme of pyruvate kinase decreases more rapidly in conventional culture than in co-culture.

Albumin secretion

Albumin production by pure parenchymal cultures declined after 10 days to less than 5 % of that at 2–6 days

(36 $\pm$ 8 μ g/ml), whereas in the co-cultures with epithelial cells it was sustained up to day 20 (38 $\pm$ 4 μ g/ml). Similar results were reported by Guguen-Guillouzo *et al.* [2].

Ketogenesis and carnitine palmitoyltransferase activity

In the absence of exogenous fatty acids, acetoacetate and 3-hydroxybutyrate were present at higher concentrations in the medium of co-cultures than in parenchymal pure cultures (parenchymal, 49 $\pm$ 4 μ M; + epithelial cells, 136 $\pm$ 8 μ M; + fibroblasts, 70 $\pm$ 5 μ M; means $\pm$ S.E.M., days 4–7), and were not detectable in the medium of pure epithelial or fibroblast cultures. The 3-hydroxybutyrate/acetoacetate ratio was also higher in co-cultures (parenchymal, 0.81 $\pm$ 0.08; + epithelial cells, 1.48 $\pm$ 0.09; + fibroblasts, 0.98 $\pm$ 0.05).

The rate of ketogenesis determined with 0.5 mM-palmitate (and 0.5 mM-carnitine) as substrate was constant on days 1, 2 and 3 of culture, but slightly lower in parenchymal/epithelial (by 28 %) and parenchymal/

Table 3. Lactate, pyruvate, alanine and glucose concentrations in the medium in pure cultures and co-cultures

For experimental details, key to abbreviations and numbers of replicate flasks see Table 2. The medium was changed daily between days 3 and 9. Metabolite concentrations were determined in a deproteinized extract of the spent medium. Values are means $\pm$ S.E.M. for the 7 days. Values in parentheses for alanine and glucose indicate the difference between the final concentration in the 24 h (spent) medium and the initial in the fresh medium (727 μ M-alanine; 7.04 mM-glucose). Minus sign denotes net uptake; plus sign denotes net output. Statistics (*t* test): **P* < 0.01 relative to Par; †*P* < 0.05 relative to Ep cultures.

Expt. no.	Cells	Lactate + pyruvate (μ M)	Lactate (μ M)	Pyruvate (μ M)	Lactate pyruvate	Alanine		Glucose	
						Final (μ M)	(final – 727 μ M)	Final (mM)	(final – 7.04 mM)
I	Par	522 $\pm$ 22	447 $\pm$ 17	90 $\pm$ 9	5.2 $\pm$ 0.7	482 $\pm$ 31	(–245)	7.18 $\pm$ 0.08	(+0.14)
	Ep	2467 $\pm$ 189*	2447 $\pm$ 195*	26 $\pm$ 7*	98 $\pm$ 32*	780 $\pm$ 19*	(+53)	5.15 $\pm$ 0.17*	(–1.89)
	Par + Ep	1507 $\pm$ 109*†	1381 $\pm$ 130*†	151 $\pm$ 13*†	8.7 $\pm$ 1.1*†	685 $\pm$ 17*	(–42)	5.42 $\pm$ 0.17*	(–1.62)
II	Par	514 $\pm$ 23	432 $\pm$ 24	82 $\pm$ 7	5.6 $\pm$ 0.7	497 $\pm$ 31	(–230)	7.13 $\pm$ 0.11	(+0.09)
	Ep	2427 $\pm$ 169*	2396 $\pm$ 173*	31 $\pm$ 5*	116 $\pm$ 36*	766 $\pm$ 15*	(+39)	5.02 $\pm$ 0.15*	(–2.02)
	Par + Ep	1775 $\pm$ 205*†	1610 $\pm$ 195*†	165 $\pm$ 15*†	10 $\pm$ 1.1*†	712 $\pm$ 12*	(–15)	5.21 $\pm$ 0.17*	(–1.83)
	Fib	1039 $\pm$ 74*	1006 $\pm$ 77*	32 $\pm$ 6*	43 $\pm$ 12*	721 $\pm$ 35*	(–6)	6.52 $\pm$ 0.07	(–0.52)
	Par + Fib	997 $\pm$ 85*	905 $\pm$ 90*	92 $\pm$ 14	8.1 $\pm$ 0.8	536 $\pm$ 16	(–191)	5.96 $\pm$ 0.015*	(–1.08)

fibroblast (by 21 %) co-cultures than in pure parenchymal cultures (350 $\pm$ 7 nmol/h per flask). Whether the lower apparent rate of ketogenesis was due to ketone-body utilization by epithelial cells and fibroblasts is not clear. By day 6, ketogenesis declined to 18 %, 46 % and 30 % in pure parenchymal, parenchymal/epithelial and parenchymal/fibroblast co-cultures respectively (62, 112 and 83 nmol/h per flask respectively; means of duplicate flasks). In two other experiments, rates of ketogenesis on day 8 were 80 % higher in parenchymal/epithelial co-cultures than in pure parenchymal cultures (247 $\pm$ 8 and 137 $\pm$ 12 nmol/h per flask respectively; means $\pm$ S.E.M., duplicate flasks from two expts.).

Carnitine palmitoyltransferase activity in pure parenchymal cultures and co-cultures showed similar trends to the rate of ketogenesis. It declined to 16 % and 43 % respectively in pure parenchymal and parenchymal/epithelial co-cultures (5 $\pm$ 1 and 14 $\pm$ 3 munits/flask respectively; means $\pm$ S.E.M., *n* = 4).

Glucose, lactate, pyruvate and alanine concentrations in the medium

In pure parenchymal cultures, there was net output of glucose, lactate and pyruvate, but net uptake of alanine (Table 3) and presumably other amino acids, including glutamine. In pure epithelial cultures, there was net uptake of glucose, but not of alanine, and the output of lactate and pyruvate accounted for 65–66 % of the glucose uptake. In fibroblast cultures, the changes in glucose, lactate and pyruvate were similar to but lower than for the epithelial cultures (Table 3).

In co-cultures of parenchymal and epithelial cells, [glucose] was higher (*P* < 0.05, paired *t* test) and [lactate + pyruvate] lower (*P* < 0.05) than in pure epithelial cultures (Table 3). Since the pyruvate kinase data suggest similar epithelial-cell density on day 3 in pure culture and co-culture, but higher (30–50 %) epithelial-cell pyruvate kinase in co-culture on days 5 and 8, the likely explanation is that the lower [lactate + pyruvate] in co-cultures reflects lactate uptake by the hepatocytes and the higher [glucose] reflects gluconeogenesis. Alanine uptake was very low (< 20 %) in hepatocyte/epithelial

co-cultures, but not in hepatocyte/fibroblast co-cultures, compared with hepatocyte pure cultures (Table 3). In co-cultures with fibroblasts there was no difference in [lactate + pyruvate] relative to pure fibroblast cultures. The low utilization of alanine in hepatocyte/epithelial co-cultures, despite net glucose output, suggests preferential utilization of lactate as gluconeogenic, glycogenic or oxidative substrate. The [lactate]/[pyruvate] in the medium was higher in epithelial and fibroblast cultures than in hepatocyte cultures (Table 3). If this reflects the cytoplasmic [lactate]/[pyruvate] ratio, it suggests a more reduced NADH/NAD⁺ redox couple in the glycolytic epithelial cells and fibroblasts than in the hepatocytes, which were in net gluconeogenic flux. In co-cultures of parenchymal and epithelial cells the [lactate]/[pyruvate] ratio was higher than in pure parenchymal cultures (Table 3).

Effects of lactate on alanine uptake by pure parenchymal cultures

In order to test the hypothesis that the lower depletion of alanine by parenchymal/epithelial co-cultures compared with pure parenchymal cultures is due to lactate (produced by the epithelial cells) replacing alanine as a substrate for the parenchymal cells, experiments were performed with the pure parenchymal cultures incubated with different lactate concentrations. The uptake of alanine by parenchymal hepatocytes precultured for either 24 h or 48 h was decreased by lactate added to the medium by 35–45 % (Table 4). Glucagon (100 nM) increased the metabolism of alanine (by 50–100 %; *P* < 0.01). Lactate also decreased alanine metabolism (26–28 %) in the presence of glucagon (Table 4). The production of urea and ammonia was suppressed in the incubations with lactate (Table 4). This suppression was greater than the decrease in alanine uptake, suggesting that the metabolism of other amino acids may also be suppressed by lactate. These findings support the hypothesis that in co-cultures the availability of lactate (produced by the epithelial cells) substitutes for alanine (and probably also other amino acids) as a substrate for the parenchymal hepatocytes. The [lactate]/[pyruvate]

Table 4. Effects of lactate on the uptake of alanine in pure parenchymal cultures

Parenchymal hepatocytes were cultured in MEM containing 10 nM-dexamethasone and 10 nM-insulin for either 24 h or 48 h. The medium was then replaced with 3 ml of MEM containing 1 mM-alanine, either without or with 100 nM-glucagon and containing the concentrations of lactate shown. Incubations were terminated after either 5 h or 7.5 h. Alanine uptake was determined by subtraction of the [alanine] at the end of the incubation from the initial concentration and is expressed as nmol/ml. The production of urea and ammonia is expressed as nmol of amino groups/ml present in the medium at the end of the incubations. The sum of the concentrations of lactate, pyruvate and alanine on termination of the incubations is expressed as nmol/ml. Values are means $\pm$ S.E.M.; $n = 4$ hepatocyte preparations. Total cell protein was 1.06 ± 0.06 mg/flask. Values that are significantly different (Student t test, unpaired) from the respective controls without added lactate are indicated by: * $P < 0.05$; ** $P < 0.005$.

	Alanine uptake (nmol/ml)		Production of urea and NH_3 (nmol/ml)		[Lactate + pyruvate + alanine] (nmol/ml)		[Lactate]/[pyruvate]	
	0-7.5 h		0-7.5 h		After 5 h		After 7.5 h	
	0-5 h	0-7.5 h	0-5 h	0-7.5 h	After 5 h	After 7.5 h	After 5 h	After 7.5 h
24 h cultures								
Controls	404 $\pm$ 17	445 $\pm$ 16	676 $\pm$ 20	680 $\pm$ 14	1057 $\pm$ 39	1002 $\pm$ 30	4.5 $\pm$ 0.5	4.2 $\pm$ 0.1
2 mM-lactate	359 $\pm$ 19	405 $\pm$ 16	554 $\pm$ 60	632 $\pm$ 58	1838 $\pm$ 93	1589 $\pm$ 64	7.6 $\pm$ 0.2**	7.2 $\pm$ 0.3**
5 mM-lactate	260 $\pm$ 49*	292 $\pm$ 63	492 $\pm$ 70*	552 $\pm$ 32*	4291 $\pm$ 60	3997 $\pm$ 35	8.8 $\pm$ 0.2**	8.7 $\pm$ 0.3**
10 mM-lactate	287 $\pm$ 6**	285 $\pm$ 21**	400 $\pm$ 14**	500 $\pm$ 30**	6693 $\pm$ 63	6664 $\pm$ 64	14.0 $\pm$ 0.3**	14.1 $\pm$ 0.7**
100 nM-glucagon								
Controls	622 $\pm$ 5	893 $\pm$ 8	1318 $\pm$ 18	1882 $\pm$ 38	774 $\pm$ 37	559 $\pm$ 17	3.8 $\pm$ 1.2	5.5 $\pm$ 0.4
2 mM-lactate	586 $\pm$ 17	807 $\pm$ 19*	1226 $\pm$ 36	1744 $\pm$ 30*	2036 $\pm$ 250	1472 $\pm$ 266	10.8 $\pm$ 0.6**	10.7 $\pm$ 0.8**
5 mM-lactate	495 $\pm$ 13**	696 $\pm$ 11**	908 $\pm$ 38**	1448 $\pm$ 54**	3850 $\pm$ 92	2961 $\pm$ 70	13.6 $\pm$ 0.3**	13.9 $\pm$ 0.4**
10 mM-lactate	465 $\pm$ 17**	645 $\pm$ 21**	934 $\pm$ 86**	1462 $\pm$ 96*	6106 $\pm$ 72	5349 $\pm$ 70	13.6 $\pm$ 0.5**	14.6 $\pm$ 0.7**
48 h cultures								
Controls	249 $\pm$ 31	362 $\pm$ 19	402 $\pm$ 52	546 $\pm$ 126	650 $\pm$ 29	523 $\pm$ 36	3.7 $\pm$ 1.1	6.3 $\pm$ 1.6
2 mM-lactate	196 $\pm$ 23	300 $\pm$ 11	390 $\pm$ 48	462 $\pm$ 94	2604 $\pm$ 107	2266 $\pm$ 75	6.2 $\pm$ 0.8	8.4 $\pm$ 1.0
5 mM-lactate	152 $\pm$ 27	234 $\pm$ 12**	380 $\pm$ 102	314 $\pm$ 106	4585 $\pm$ 110	4277 $\pm$ 139	9.6 $\pm$ 0.4**	12.7 $\pm$ 0.3*
10 mM-lactate	136 $\pm$ 4*	209 $\pm$ 21**	270 $\pm$ 42	250 $\pm$ 104	6858 $\pm$ 52	6474 $\pm$ 45	12.6 $\pm$ 0.6**	13.7 $\pm$ 0.4**
100 nM-glucagon								
Controls	355 $\pm$ 16	558 $\pm$ 13	620 $\pm$ 72	866 $\pm$ 66	572 $\pm$ 31	401 $\pm$ 25	4.5 $\pm$ 0.6	4.5 $\pm$ 0.5
2 mM-lactate	328 $\pm$ 21	498 $\pm$ 17	550 $\pm$ 56	614 $\pm$ 86	1971 $\pm$ 262	1299 $\pm$ 293	10.0 $\pm$ 0.8**	8.5 $\pm$ 0.6**
5 mM-lactate	292 $\pm$ 19*	469 $\pm$ 36	544 $\pm$ 44	618 $\pm$ 68*	4565 $\pm$ 309	3710 $\pm$ 336	9.3 $\pm$ 1.0**	10.5 $\pm$ 0.5**
10 mM-lactate	264 $\pm$ 21*	420 $\pm$ 47*	478 $\pm$ 30	462 $\pm$ 124*	6463 $\pm$ 129	5703 $\pm$ 123	9.6 $\pm$ 1.0**	10.8 $\pm$ 0.4**

ratio in the medium was similar at 5 h and 7.5 h after addition of lactate, and was higher in incubations with lactate than in controls (Table 4). It was also higher in the co-cultures than in pure parenchymal cultures, presumably because of the higher [lactate] (Table 3). The rate of glucose output increased with increasing [lactate] (results not shown). The rate of glucose output (expressed as C_3 units) was lower than the rate of alanine uptake in the absence of glucagon, but higher in the presence of glucagon. In the presence of glucagon, the rate of alanine uptake, expressed as a percentage of glucose output, was $88 \pm 5\%$ in the absence of added lactate and $35 \pm 5\%$ in incubations with 10 mM-lactate, suggesting that the relative contribution of alanine to gluconeogenesis decreases with increasing lactate availability. The activity of lactate dehydrogenase in the medium showed a decreasing trend with increasing [lactate] (control 111 ± 40 , 2 mM-lactate 70 ± 18 , 5 mM-lactate 66 ± 11 , 10 mM-lactate 56 ± 5 munits/ml; means $\pm$ S.E.M., $n = 4$), but the differences were not statistically significant.

Conclusions

The following conclusions are drawn from this study. (1) Pyruvate kinase activity, assayed without or with fructose 1,6-bisphosphate, can be used to quantify epithelial-cell density in co-cultures and the L isoenzyme of the parenchymal hepatocytes. (2) Leakage of lactate dehydrogenase by pure parenchymal cultures and co-cultures can be used to assess the relative preservation of parenchymal cells in co-culture. (3) The capacity for ketogenesis and the activity of carnitine palmitoyl-transferase decline less rapidly in co-cultures than in pure parenchymal cultures. (4) In parenchymal hepatocyte/epithelial co-cultures, the epithelial cells, which have a high glycolytic rate, provide the parenchymal cells with a continual supply of lactate, which is maintained at a physiological concentration (millimolar range). Lactate is utilized by the parenchymal hepatocytes in co-cultures, in part for gluconeogenesis, but probably also for glycogenesis and lipogenesis. (5) Alanine uptake by parenchymal hepatocytes is lower in co-cultures than in pure parenchymal cultures, which lack an exogenous supply of lactate. Studies with pure parenchymal cultures showed that increasing lactate availability decreases the metabolism of alanine and the production of urea and ammonia. These findings together suggest that, in co-cultures of parenchymal and epithelial cells, the lactate produced by the epithelial cells is utilized by the parenchymal cells in preference to alanine. Utilization of amino acids, as opposed to lactate, as gluconeogenic, glycogenic, oxidative or lipogenic substrates is associated with additional metabolic demands for disposal of amino groups. In most conventional media used for hepatocyte culture, amino acids are the only available exogenous substrate apart from glucose. In early culture hepatocytes may be in net gluconeogenic rather than glycolytic flux, consequently amino acids are used as gluconeogenic

substrates. The present study suggests that hepatocytes in co-culture are exposed to a more physiological substrate milieu because of the continual availability of lactate. This suppresses amino acid utilization. The continual provision of lactate by the epithelial cells, which mimics the physiological environment, may contribute to the improved preservation of hepatocyte function in co-culture.

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