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SHORT COMMUNICATIONS

Presence of phase I and phase II drug metabolizing enzymes in cultured human foetal hepatocytes

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In contrast with foetal liver of various laboratory animal species, human foetal liver is able to metabolize drugs via cytochrome P-450 dependent enzymes but not to perform glucuronidation of most substrates [1, 2].

However, because of evident difficulties in the obtention of such a material, only limited definitive information is still available on drug biotransformation by human foetal liver [1, 2]. Most of the studies were performed with subcellular fractions of liver tissue. Only a few have dealt with isolated or cultured liver cells which represent an approach closer to the *in vivo* situation since enzymes may operate under physiological conditions, e.g. with physiological concentrations of cofactors [2].

Early cultures from human foetal liver were obtained either with explants which gave a constant heterogeneous cell population [3] or with incompletely purified hepatocyte populations isolated by enzymatic digestion [4, 5]. Pelkonen *et al.* [5] reported that fibroblastic cells derived from 9 to 14-week-old foetuses exhibited a benzo[*a*]pyrene metabolizing system for 4–11 days in primary cultures; however the activity of this system was low and these cells were likely of non-parenchymal origin [6, 7]. Nau *et al.* [8] used organ cultures and parenchymal cells isolated by dispase treatment from 5 to 20 week-old foetuses and demonstrated their capability to exhibit first order kinetics in drug metabolism for up to 3 days. The highest yields of isolated hepatocytes have been obtained with the use of collagenase as a dissociating agent [9]. Rollins *et al.* [10] showed oxidation of acetaminophen and its conjugation with glutathione and sulphate but not with glucuronic acid by freshly collagenase-dissociated hepatocytes from human foetuses aged 19 and 22 weeks, respectively. By using an arginine-free medium containing hydrocortisone, as previously reported for foetal rat liver [9, 11], we have been able to cultivate collagenase-treated human foetal hepatocytes without significant fibroblastic proliferation and to demonstrate maintenance of specific markers up to 15 days [12]. In this communication we present evidence that hepatocytes isolated according to this procedure from a human foetus in late gestation exhibit *in vitro* various drug metabolizing enzyme activities including glucuronidation.

Materials and methods

Hepatocyte isolation and culture. Hepatocytes were obtained from a 7.5-month-old foetus. The cells were isolated by digestion of slices of the liver with a solution containing 0.025% collagenase and 5 mM $CaCl_2$ buffered with HEPES (*N*-2 hydroxyethylpiperazine-*N'*-2-ethane sulphonic acid, from Sigma) pH 7.6 at 37° under gentle stirring [12]. Before enzymatic digestion, liver fragments were washed three times with HEPES buffer.

Freshly isolated hepatocytes were seeded in Falcon flasks in an arginine-free Ham F_{12} medium supplemented with 10% foetal calf serum and 10^{-5} M hydrocortisone hemisuccinate (Roussel, France). The medium was renewed 4 hr after seeding and every day thereafter.

Light and electron microscopy. Living cultures were examined under phase-contrast microscopy. For electron microscopic studies, hepatocyte cultures were fixed in 2.5% glutaraldehyde buffered with sodium cacodylate 0.1 M for 3 min at 4°, postfixed in 1% osmium tetroxide solution buffered with sodium cacodylate, dehydrated in graded ethanols and embedded in Epon [13]. Ultrathin sections were doubly stained with uranyl acetate and lead citrate.

Analysis of the drug metabolites appearing in the medium. Two molecules, whose metabolic pathways are well known, were used: guanfacine (Estulic®), an antihypertensive drug [14], and ketotifen (Zaditen®), a new type of antianaphylactic agent active after oral administration [15]. In adult humans, guanfacine is metabolized mainly by ring hydroxylation then glucuro-conjugation. The major metabolite isolated from urine is the *O*-glucuronide of 3-hydroxyguanfacine [16]. Ketotifen is biotransformed by *N*-demethylation, by *N*-glucuroconjugation giving the *N*-quaternary ammonium derivative and by reduction of the keto group followed by *O*-glucuroconjugation.* These two drugs, therefore, represent a series of metabolic routes of more general interest and are used as metabolic activity indicators.

The drugs were added 12 hr after cell seeding at a concentration of 100 μg/ml/2 × 10^6 cells. The medium was harvested 24 hr thereafter and the metabolites were analysed by gas-chromatography. For guanfacine, parent drug was analysed after derivatisation with hexafluoroacetylacetone as previously described [17]. The 3-hydroxyguanfacine was determined before and after incubation with β-glucuronidase. This metabolite was analysed after an additional methylation step.† For ketotifen, parent drug and metabolites were determined after extraction before and after incubation with β-glucuronidase.

Results and discussion

Hepatocyte culture characteristics. Isolated foetal hepatocytes aggregated and attached to polystyrene within 2 hr, then spread over and formed monolayers of granular epithelial cells (Fig. 1). The cells were maintained up to 10 days without significant overgrowth of fibroblastic cells.

At the ultrastructural level, cultured hepatocytes exhibited a fine structure similar to that of *in vivo* hepatocytes: in particular rough endoplasmic reticulum appeared well preserved and numerous glycogen particles were present. Moreover hepatocytes formed structures comparable to bile canaliculi (Fig. 2).

Drug metabolic pathways. The metabolites of guanfacine and ketotifen found in cultured human foetal hepatocytes are displayed in Fig. 3. For guanfacine, the two metabolites investigated, 3 hydroxyguanfacine and the glucuronide of 3-hydroxyguanfacine were identified. However, unlike in adults, the glucuronide was found in small proportions (38% instead of 95% of total hydroxylated compounds) and the major metabolite produced was the free hydroxylated derivative. The hydroxylated compounds represented about 50% of those produced by adult rat hepatocytes cultured in similar conditions (not shown). Our observations indicate that human foetal hepatocytes are able to oxidise guanfacine via the cytochrome P-450 enzymatic

* Kiechel *et al.*, unpublished results.
† Guerret *et al.*, unpublished results.

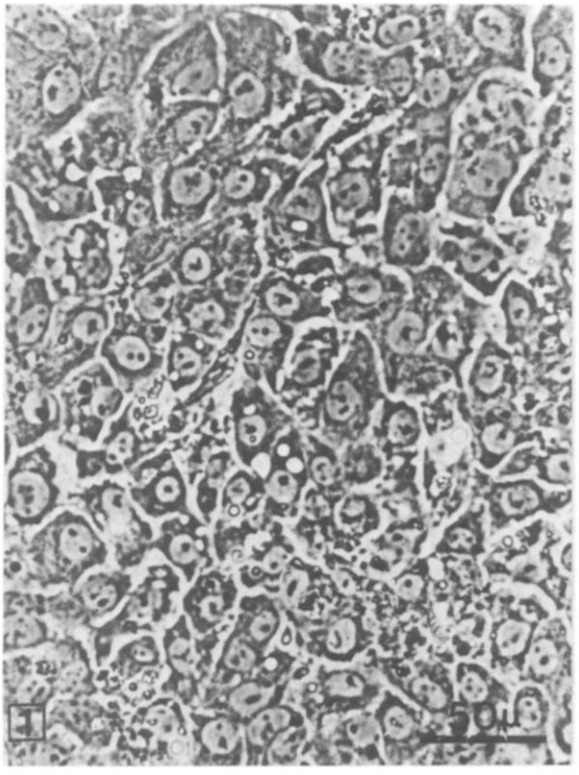

Fig. 1. Phase-contrast micrograph of human foetal hepatocytes after 4 days of culture. The cells form a monolayer of granular epithelial cells (×330).

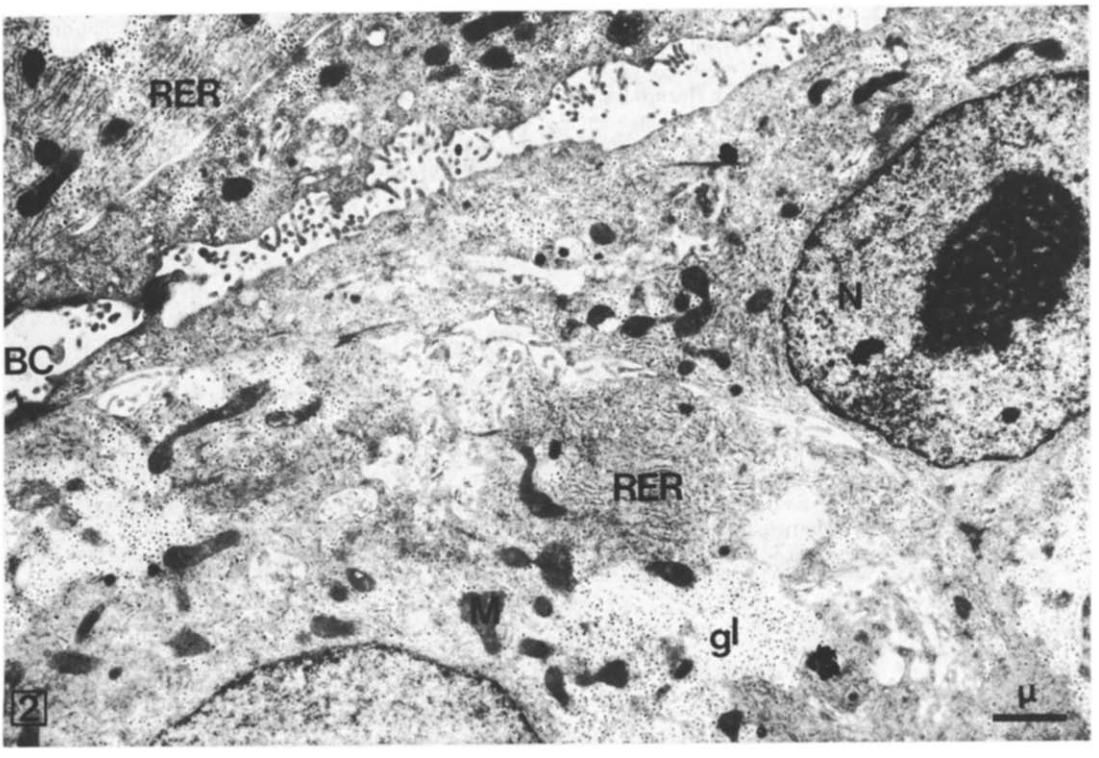

Fig. 2. Electron micrograph of human foetal hepatocytes after 4 days of culture. Note the presence of numerous glycogen particles (gl) and well developed rough endoplasmic reticulum (RER). N, nucleus; M, mitochondrion; BC, bile canaliculus (×10,500).

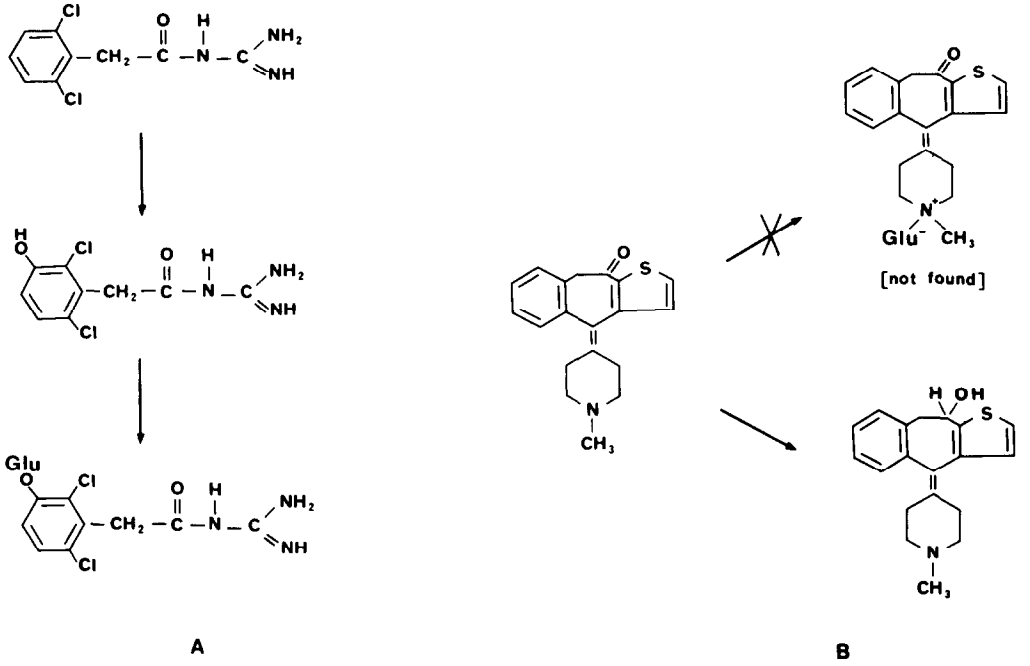

Fig. 3. Drug metabolic pathways found in cultured human foetal hepatocytes. A, guanfacine; B, ketotifen.

system and to conjugate it apparently to a lesser extent than could have been expected from *in vivo* data in adults.

For ketotifen, no glucuronidation was found. The other major metabolite identified *in vivo*, 10-hydroxypizotifen, was released in the culture medium and its structure was confirmed by comparison with an authentic synthetic sample, by retention time in gas–liquid chromatography and by mass-spectrometry. This product of the reduction of the keto group was only found in the free form.

These data indicate that cultured human hepatocytes derived from a 7.5-month-old foetus, exhibited phase I (oxidation, reduction) and phase II (glucuronidation) drug metabolizing enzyme activities. This is to our knowledge the first demonstration of the capability of human foetal hepatocytes to perform glucuronidation *in vitro* since Rollins *et al.* [10] were able to find only sulphoconjugation activity in freshly isolated human foetal hepatocytes. This could be due either to the age of the foetal liver which was older in our investigations or to the type of substrate used. Of course, glucuro-conjugation activity was found only with guanfacine and moreover only at a low level. However, this is in agreement with low levels of glucuronidation, if any, previously reported in the foetal liver [1, 2].

Our observations suggest that cultured hepatocytes is a suitable model for studying drug metabolism by the human foetal liver. They could serve to investigate effects of hormones in particular corticosteroids [10] and of various exogenous factors on drug metabolizing enzyme activities. The interest of hepatocyte cultures will certainly increase in the future when well defined media and more appropriate biological supports, presently under study in many laboratories, will be available.

In summary, biotransformation of two drugs, guanfacine and ketotifen, was investigated in cultured human foetal hepatocytes. Both phase I (oxidation, reduction) and phase II (glucuronidation) drug metabolizing enzymes were demonstrated in these cultured cells.

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Effects of the thyroliberin analogue CG 3703 on noradrenergic and serotoninergic transmission in rodents

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The complex behavioural response to thyroliberin (TRH) injections suggests an increased activity of different monoaminergic systems of the central nervous system (for review see [1]). Whereas an enhanced dopamine (DA) release by TRH is well documented [1], some doubts about a relevant involvement of the norepinephrine (NE) system appeared justified, since brain NE levels after TRH application remain unaltered and only slight changes in [^{3}H] NE turnover could be established [2]. Some symptoms in TRH-treated animals, particularly the "wet dog shaking" in rats, indicated an increased serotoninergic transmission [3]. However, any alteration of the 5-hydroxytryptamine (5-HT) system could so far not be verified by biochemical methods. In order to clarify the possible contribution of NE and 5-HT to the TRH syndrome, we reinvestigated the problem by means of the analogue CG 3703 (6-methyl-5-oxo-thiomorpholinyl-3-carbonyl-histidyl-prolinamide). This compound which is resistent against the TRH degrading pyroglutamate aminopeptidases from serum and tissue [4] induces a typical, but prolonged TRH syndrome at minute dosages [3, 5] and thus substantially facilitates the analysis of the biochemical basis of TRH-induced behaviour.

Materials and methods

NE was extracted from brain tissue according to Haubrich and Denzer [6] and determined by the fluorometric method of Weil-Malherbe and Bigelow [7]. NE utilisation was estimated as NE decline in α-methyl-*p*-tyrosine (α-MPT)-treated male NMRI mice of 18–22 g body wt. as described by Brodie *et al.* [8].

5-HT and 5-hydroxyindoleacetic acid (5-HIAA) were determined fluorometrically according to Curzon and Green [9]. 5-HT release was estimated by determination of 5-HIAA accumulation in probenecid-pretreated (200 mg/kg i.p.) male Wistar rats of 150–200 g body wt. 5-HT biosynthesis was evaluated by measuring 5-HT accumulation in animals pretreated with tranylcypromine (10 mg/kg i.p.). Test compound and probenecid or tranylcypromine, respectively, were administered simultaneously and the animals were sacrificed 1 hr later.

Drug effects were analysed for statistical significance by means of the two-tailed Student's *t*-test.

TRH was obtained from Serva GmbH (Heidelberg, F.R.G.). CG 3703 was synthesised [10] by Dr. E. Schwertner, Grünenthal GmbH (F.R.G.).

Results and discussion

CG 3703 did not affect the NE level of the brain at dosages up to 1 mg/kg (Table 1), whereas the ED_{50} values for this compound in various behavioural models were considerably lower [3, 5]. At 10 mg/kg, however, CG 3703 tended to reduce the NE levels in rat brain and significantly decreased the NE content of mouse brain. Mice were

Table 1. Influence of CG 3703 on the NE level in the whole brain of mice and rats

Drugs (mg/kg i.p.)	Species	nmole NE/g	±S.E.	*n*	P
Control (0)	Mouse	1.88	0.05	10	—
CG 3703 (0.5)	Mouse	1.80	0.08	5	n.s.*
CG 3703 (1.0)	Mouse	1.84	0.16	5	n.s.
CG 3703 (10)	Mouse	1.44	0.04	5	<0.001
Control (0)	Rat	2.63	0.17	10	—
CG 3703 (10)	Rat	2.24	0.13	10	<0.1

NE was measured 2 hr after drug administration.
* n.s. = no significant difference vs control.