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Cultured human adult hepatocytes: a new model for drug metabolism studies

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Drugs and other chemicals are metabolized by various pathways which are classified into two groups: phase I reactions corresponding to oxidations, catalyzed for instance by cytochrome P-450 dependent mono-oxygenases, reductions and hydrolyses. and phase II reactions corresponding to conjugations.

Enzyme activities of drug biotransformation vary with both age and species. Some characteristic differences exist between human and common laboratory mammalian liver. Human foetal liver, but not foetal liver from various experimental animals, is able to catalyze oxidations whereas, in contrast with rat foetal liver in late gestation, it does not perform glucuronidation of certain substrates [1]. Comparison of cytochrome P-450-dependent monooxygenase activities in adult human and rat liver microsomes revealed significant differences [2].

Various experimental models are used to study drug metabolism. Experiments *in vivo* allow the determination of overall biotransformation. To investigate hepatic metabolism *in vitro*, different systems have been used, including subcellular fractions, isolated and cultured hepatocytes. The majority of studies have dealt with liver microsomes. It is a convenient model to study reactions located in the endoplasmic reticulum, such as oxidation or glucuronidation, but it needs addition of cofactors and does not include enzymes which have a cytoplasmic localization like sulphotransferase [3]. Such disadvantages are overcome with isolated hepatocytes. This model is more physiological because transport across the cell membrane and competitions between all the metabolic reactions are still present. However, freshly isolated hepatocytes have a short life limited to 3–6 hr [4] and, therefore, the best model for long-term studies on drug metabolism should be cultured liver cells.

Both phase I and phase II enzyme activities have been demonstrated in cultured hepatocytes from laboratory animals [5]. Very few data are available on cultured human liver cells and they deal mainly with foetal cells [6, 7], since human adult samples are difficult to obtain and since no technique allowing isolation of large numbers of parenchymal cells was developed. We have recently been able to overcome these difficulties by perfusing a selected area of human liver. The procedure allows isolation of 1–10×10^8 viable hepatocytes [8].

However, until now the use of cultured hepatocytes for drug metabolism studies was limited by the rapid loss of cytochrome P-450 content. Indeed, when they are maintained under conventional culture conditions, adult hepatocytes exhibit rapid phenotypic changes, leading to a strong decrease or the disappearance of highly differentiated functions. Thus, these cells lose, within 48 hr of culture, as much as 80% of their cytochrome P-450 content [9]. This decline may be slightly delayed by supplementing the culture medium with various components, such as hormones [10] or isonicotinamide [11]. However, the loss of highly differentiated functions may be prevented or corrected under peculiar culture conditions. We have found that adult rat [12] or human [13] hepatocytes co-cultured with an undifferentiated rat liver cell line remain able to secrete high levels of albumin for several weeks.

The purpose of this study was to investigate drug biotransformation activities exhibited by cultured and co-cultured human adult hepatocytes. Biotransformation of ketotifen* (Zaditen®) was selected for investigation because in man it has been shown to be metabolized by various typical pathways.

Materials and methods

Cell isolation and culture. Hepatocytes from two male kidney transplantation donors aged 23 and 25 years, respectively, were used. These cells were obtained by perfusion with collagenase of a selected area of the liver according to a method previously described [8]. Cell yield was 4 and 10×10^8 hepatocytes and cell viability 70% and 85%, respectively, for the two liver dissociations. For conventional cultures, isolated hepatocytes were seeded in 3.5 ml of Ham F_{12} medium containing 10% foetal calf serum at a density of 2.5×10^6 cells per 25 cm^2 polystyrene Falcon flask. After 4 hr and every day thereafter the medium, to which 7×10^{-5} M hydrocortisone hemisuccinate was added, was renewed.

Human adult hepatocytes were also co-cultured with rat epithelial cells according to a procedure described in detail elsewhere for rat and human hepatocytes [12, 13]. Briefly, human hepatocytes were first seeded at a density of 1.5×10^6 cells per flask in 3.5 ml of Ham F_{12} medium containing 10% foetal calf serum. After 3 hr, when the cells had attached, the medium was discarded and 2×10^6 rat liver epithelial cells were added in an equal vol. of fresh medium. This medium, supplemented with 3.5×10^{-6} M hydrocortisone hemisuccinate, was renewed every day thereafter. The epithelial cell line was derived from a 10-day-old rat liver [14].

Incubation of human hepatocytes with ketotifen. In man, ketotifen is biotransformed by several metabolic pathways, namely *N*-demethylation, *N*-oxidation, *N*-glucuroconjugation giving the *N*-quaternary ammonium derivative and reduction of the keto group followed by *O*-glucuronidation [15].

At various times (between 24 hr and 3 weeks) after cell seeding, cultured adult hepatocytes were incubated with 3 μg/ml [^{14}C]ketotifen (sp. act. 107 μCi/mg) and 20 μg/ml of unlabelled drug per 25 cm^2 flask. At this drug concentration of 23 μg/ml of medium no toxicity occurred. Aliquots of medium were collected at 4 or 24 hr after drug addition, quickly frozen and stored at −20° until analysis.

Analytical procedures. Metabolites of ketotifen were analysed by reversed phase high performance liquid chromatography (HPLC). Their structure was confirmed by mass spectrometry.

HPLC analysis. The HPLC system consisted of two Altex 110 A pumps controlled by an Altex 421 programmer and a Berthold LB 503 continuous radioactivity detector. An analytical 300 × 4.6 mm i.d. column packed with Spherisorb 10 ODS (particle size 10 μM) was used.

Elutions were performed with a programmed multistep gradient of 0.01 M ammonium carbamate solution and methanol. The proportion of methanol–ammonium carbamate solution was 10% (v/v) at first and increased to 20%, 47.5%, 70% and 100% after 27, 42, 51 and 60 min, respectively. The total time for elution was 70 min, including the final 10 min with 100% methanol. The solvent

* Ketotifen (Zaditen®) is a new antianaphylactic agent active after oral administration.

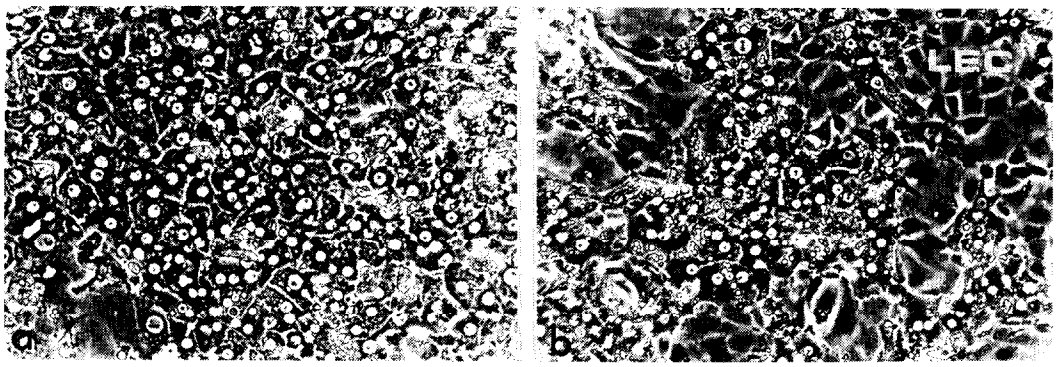

Fig. 1. Phase-contrast micrograph of adult human hepatocytes cultured alone for 2 days (a) and in co-culture for 7 days (b) (LEC, liver epithelial cells) ×150.

flow-rate was kept constant at 2 ml/min. Lyophilized samples (corresponding to 0.5–1 ml of medium) were dissolved in 300 μl of the mixture of H_2O–methanol, 50/50 (v/v). After filtration, 200 μl was injected. The radioactivity of the effluent was detected by use of a glass scintillator cell. The limit of radioactivity detection was 400 dpm which corresponded to 0.13 ng [^{14}C]ketotifen.

Retention times of the metabolites, which were similar for the two experiments, were compared with those of reference synthetic compounds. Metabolites were collected to confirm their structure by mass spectrometry.

Mass spectrometry. A Nermag R 10-10 B Sidar 11A gas chromatography–mass spectrometer supplemented by a PDP/8 computer system (Nermag, Rueil-Malmaison, France) was used. The chromatograph was equipped with a glass solid injector and a glass capillary column (30 × 0.3 mm I.D.) coated with SE 52 (Chrompack, Middleburg, The Netherlands). The spectra were taken in the electron ionization mode. The ionization voltage was 70 eV and the emission current was 250 μA.

Parent drug and metabolites were analysed before and after incubation with β-glucuronidase.

Results

Culture characteristics. Three hr after cell seeding, 60–70% of the hepatocytes were attached to the substratum. When cultured alone they formed, within 24 hr, monolayers of non-dividing granular epithelial cells which survived for 7–10 days (Fig. 1a). In co-cultures, a confluent cell monolayer was obtained within 24 hr after addition of rat liver epithelial cells. Epithelial cells did not grow thereafter and under these conditions hepatocytes survived without obvious cell loss for more than 6 weeks (Fig. 1b).

Pathways of drug metabolism. Preliminary studies showed that whatever the time of culture, glucuronides of ketotifen were not detected before 8 hr following drug addition.

Cultured human hepatocytes incubated with ketotifen between 4 and 28 hr after cell seeding provided the following metabolites in the culture media (Fig. 2). (1) Norketotifen which had the longest retention time in HPLC (74 min) and which with GLC–MS gave the characteristic molecular ion at *m/z* 295. (2) Traces of *N*-oxidized ketotifen which decomposed during GLC–MS analysis giving unchanged drug. (3) Reduced ketotifen, the major metab-

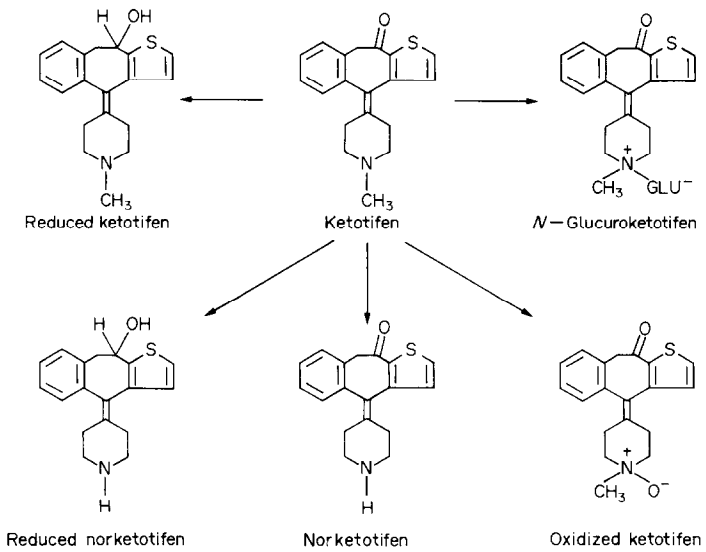

Fig. 2. Ketotifen metabolic pathways in cultured adult human hepatocytes.

olite, which had a retention time in HPLC identical to that of the synthetic reference compound obtained by reduction of ketotifen with sodium borohydride. By GLC–MS analysis, the molecular and another characteristic ions were observed at *m/z* 311 and *m/z* 293 [$M^+ - 18\ (H_2O)$], respectively. (4) Reduced norketotifen giving in GLC–MS a characteristic fragment ion at *m/z* 279 [$M^+ - 18\ (H_2O)$]. (5) Glucuronides of both unchanged drug (*N*-glucuroketotifen) and of reduced ketotifen which were also obtained in large amounts between 20 and 25 min in HPLC. They formed ketotifen and its free reduced derivative after incubation with β-glucuronidase. Small amounts of glucuronide of reduced norketotifen were also found.

These different metabolites corresponded to the occurrence of the four metabolic pathways found *in vivo* [15], *N*-demethylation, *N*-oxidation and principally ketoreduction and *N*-glucuronidation.

These two last pathways, which were the major ones, were used to estimate the metabolic activity of parenchymal cells cultured alone or associated with liver epithelial cells for 24 hr or more. A 4 hr incubation with ketotifen of cultured human hepatocytes showed that the rate of metabolism was only slightly modified for the first 4 days of conventional cultures and the first 7 days of co-cultures. In these latter, ketotifen was metabolized more actively. In older cultures, the rate of drug metabolism was strongly reduced; however, it was still much higher on day 21 of co-culture than on day 6 of conventional culture. The early differences in the rate of metabolism in relation to the age and the conditions of culture were maintained and enhanced after a 24 hr incubation in the presence of the drug. The respective percentages of unchanged, reduced and *N*-glucuronide of ketotifen obtained 4 and 24 hr after drug addition, are displayed in Table 1. In conventional cultures, the ratio of glucuronide metabolite to total metabolites (reduced and *N*-glucuronide metabolites) was 0.34–0.30 during the first 4 days. On day 6, glucuronidation activity was lost whereas reduction was still observed. In co-cultures hepatocytes actively metabolized ketotifen during the 21 day period. On day 7 the ratio of glucuronide metabolite to total metabolites was 0.69 and on day 21 it was still 0.17. At this time the percentage of reduction of total ketotifen was identical to that found in conventional cultures after 24 hr. Reduced norketotifen was not quantified but was detectable by GLC–MS during the 21 days of co-culture.

In pure control cultures of epithelial cells no biotransformation of ketotifen was detected.

Discussion

The present study shows that human adult hepatocytes are able to metabolize ketotifen according to the different pathways normally found *in vivo* [15], i.e. demethylation, oxidation, reduction and glucuronidation. The main pathways were found for at least 4 days in hepatocytes cultured alone and for several weeks in hepatocytes co-cultured with another liver cell type.

A number of phenotypic changes leading to a more foetal-like state has been documented in rat hepatocytes after a few days of culture [9, 16–18]. Human adult hepatocytes cultured alone seemed to have the same behaviour. Indeed the glucuronidation pathway was selectively lost on day 6. This function, contrary to reduction, is absent or low and depends on the substrate in the foetal liver [1].

In adult hepatocytes maintained in co-culture, glucuronidation reaction was maintained much longer, for at least 3 weeks and was higher on day 7 than on day 4, and in hepatocytes cultured alone during the first 4 days. This increased drug metabolizing enzyme activity coincided with increased albumin synthesis and maximum organization of the extracellular material which was rapidly and abundantly produced in such co-cultures [12, 13]. The maintenance of the production of reduced norketotifen, which included an oxidative demethylation step, suggests that cytochrome P-450-dependent mono-oxygenases also remained active for a long time in this system. This gives further evidence that, when mixed with other liver epithelial cells, adult hepatocytes may express stable specific functions for a much longer time in culture and that both specific cell–cell interactions and an extracellular material are needed to prevent rapid phenotypic changes in cultured hepatocytes [12].

In summary, biotransformation of ketotifen, taken as a model drug, was studied in adult human hepatocytes after increasing duration of culture. The metabolic pathways found *in vivo*, namely *N*-demethylation, *N*-oxidation, reduction and glucuronidation, were exhibited in cultured cells. The two major pathways, reduction and *N*-glucuronidation, remained operative for at least 4 and 21 days in adult parenchymal cells cultured alone or in combination with rat liver epithelial cells, respectively. This indicates that human hepatocytes, maintained in an original co-culture system, may exhibit both phase I and phase II drug metabolizing enzyme activities for several weeks and suggests that this system represents a promising tool for drug metabolism studies.

Table 1. Relative percentages of unchanged and metabolized (reduced and *N*-glucuronide) ketotifen found in the medium of human adult hepatocytes cultured alone or in association with rat epithelial cells (LEC) for various times

				Metabolized ketotifen				
		Unchanged ketotifen		Reduced (R)		*N*-Glucuronide (G)		Ratio $\frac{G}{R+G}$
Cells	Time of culture (days)	a	b	a	b	a	b	b
Hepatocytes	0.17	67	25	33	48	0	25	0.34
	1	68	16	32	56	0	28	0.33
	4	63	13	37	61	0	26	0.30
	6	93	50	7	50	0	0	0.00
Hepatocytes + LEC	4	58	13	42	43	0	44	0.51
	7	51	6	49	29	0	65	0.69
	21	83	29	17	59	0	12	0.17
LEC	—	100	100	0	0	0	0	—

The cells were incubated for 4 hr (a) and 24 hr (b) with 80.5 μg of ketotifen per flask before medium collection. The values are means of two experiments. The differences between the percentages of the two experiments did not exceed 10%.

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Cholinesterase and carboxylesterase activities in soman poisoned rats treated with bispyridinium mono-oximes HI-6 and HS-6

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Traditional reactivators (oximes) used in the therapy of organophosphate poisoning have limited effect against the organophosphorus nerve agent soman [1], in accordance with their poor ability to reactivate acetylcholinesterase inhibited by soman [2, 3]. The newer bispyridinium mono-oximes HI-6 and HS-6, however, are effective in the treatment of soman poisoning in rodents [4–11], dog [12] and monkey [13], but their mechanism of action has remained unclear. Although both HS-6 and HI-6 are reported to reactivate soman inhibited acetylcholinesterase *in vitro* [2, 3], this has not been unambiguously shown to take place *in vivo*. In addition a direct inactivation of soman by the oximes [9], and/or ganglion blocking properties of HI-6 and HS-6 [14], have been suggested to be important for their antidotal effect. Recently, we found that detoxification of soman in rodents is strongly influenced by the enzyme carboxylesterase (= aliesterase = tributyrinase) which binds and, therefore, neutralizes a high proportion of soman [15–17]. Reactivation of inhibited carboxylesterases could explain the antidotal effect of those newer oximes [18]. The present investigation was undertaken to measure the effect of HI-6 and HS-6 on cholinesterase and carboxylesterase activities in different rat tissues during poisoning by soman and by tri-ortho-cresyl phosphate (TOCP), a carboxylesterase inhibitor.