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Characterization of Chemically Induced Hepatotoxicity in Collagen Sandwiches of Rat Hepatocytes

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It has been shown that hepatocytes cultured in a collagen sandwich configuration maintain cell viability, morphology, and drug metabolizing activities for several weeks. The purpose of this study was to characterize chemically induced general toxicity in this system by exposing hepatocytes to eight different hepatotoxic compounds. Cell function and viability was measured by analyzing the secretions of urea and albumin and the release of lactate dehydrogenase. Significant decreases in urea and albumin secretions were detected after treatments with 32 nM aflatoxin B_1 and 1 mM doses of cadmium and the alkylating agents N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and methyl methane sulfonate (MMS). However, no significant toxicity could be measured following exposures to 5 mM carbon tetrachloride, 1 mM N, N-dimethylformamide (DMF), 1 mM vinyl acetate, and 1 mM acetaminophen. Western blots of cell lysates showed that hepatocytes maintained CYP1A, 2B, 3A2 but gradually lost CYP2E1, which is the main metabolic enzyme for acetaminophen, carbon tetrachloride, and DMF. The metabolites of acetaminophen were identified using liquid chromatography and electrospray mass spectrometry. It was determined that the hepatocytes converted most of the acetaminophen to the glucuronide and sulfate metabolites and only formed a small amount of the glutathione adduct. This research shows that the collagen sandwich culture system can only be used selectively for detecting hepatotoxicity and for identifying major metabolites of xenobiotic compounds.

Key Words: **toxicity; collagen; sandwich; hepatocytes; aflatoxin; acetaminophen.**

Cultured hepatocytes rapidly lose liver-specific functions, which limits their use for studying drug metabolism and toxicity. This phenomenon is thought to be due to the lack of proper extracellular matrix, cell-cell contacts, and humoral factors in the *in vitro* systems, which are known to regulate gene expression in the liver (Ben Ze′ev *et al.*, 1988; Gomez-Lechon *et al.*, 1998; LeCluyse *et al.*, 1996). Providing extracellular matrix proteins such as collagen (Dunn *et al.*, 1991; Ezzell *et al.*, 1993; Kern *et al.*, 1997) or complex substrata such as Matrigel (Hamilton *et al.*, 2001; Moghe *et al.*, 1997a) has been shown to enhance hepatic function *in vitro* especially when cells are cultured in a sandwich configuration. Extracellular matrix in a sandwich form provides anchorage for the cells on the top and bottom (Dunn *et al.*, 1991; Moghe *et al.*, 1997b), and it also stores signaling molecules such as heparan-sulfate proteoglycans secreted by the cells (Babu and Sudhakaran, 1991).

Research has shown that hepatocytes in a collagen sandwich maintain drug metabolism (Bader *et al.*, 1998; Kern *et al.*, 1997; LeCluyse *et al.*, 2000; Rotem *et al.*, 1995), morphology (Ezzell *et al.*, 1993; Moghe *et al.*, 1996), and bile secretion (Liu *et al.*, 1999). Furthermore, hepatocytes in this system maintain transcriptional activity for albumin at least as high as freshly isolated hepatocytes and significantly higher than hepatocytes cultured on a single layer of collagen (Dunn *et al.*, 1992). Collagen sandwiches and collagen-Matrigel sandwiches are also inducible for both CYP1A2 and CYP3A4 in systems with human hepatocytes (LeCluyse *et al.*, 2000). It has been demonstrated that both human and rat hepatocytes in collagen sandwiches express CYP3A and CYP1A activity, as well as glucuronosyltransferase, sulfotransferase, and glutathione *S*-transferase activities (Kern *et al.*, 1997).

Bader *et al.* (1994) used collagen sandwich cultures of rat and human hepatocytes to study the metabolism of the drug urapidil. They found that the metabolites produced corresponded to the *in vivo* metabolites in rats and humans, suggesting that collagen sandwiches could be used to predict the metabolites of xenobiotics. In order to monitor the multiple enzyme activities simultaneously, Kern *et al.* (1997) examined the metabolism of testosterone in rat and human hepatocytes and found that most of the *in vivo* metabolites were also produced in the sandwich. Interestingly, the CYP2C11 metabolites were found at higher levels than in freshly isolated hepatocytes, suggesting that the activity of this enzyme was restored in the sandwich system. This was a remarkable result, since the activity of this enzyme is thought to be regulated *in vivo* by gonadal and pituitary hormones, and is normally lost

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in cell culture. While most metabolic enzymes were preserved in the sandwich, the enzyme levels of rat hepatocytes fluctuated significantly after isolation, whereas there was only a slight decrease in the enzyme activities of human hepatocytes, suggesting that human cells might be more suitable for *in vitro* studies.

In spite of the large body of literature examining the viability and the drug-metabolizing capacity of hepatocytes in collagen sandwiches there are very limited studies on the reliability of this system in toxicological studies. De Smet *et al.* (2000) studied the biotransformation of trichloroethylene (TCE) in collagen gel sandwiches and found that TCE and its metabolites caused significant toxicity. These results suggest that hepatocytes in the sandwich system maintained CYP1A and CYP2B and possibly CYP2E1, the enzymes responsible for metabolizing TCE.

This study analyzes the response of hepatocytes in a collagen sandwich to a variety of hepatotoxins. Aflatoxin B_1 was the model compound for developing toxicity assays since this compound was found to be toxic at nanomolar doses and its toxicity could be detected with a variety of methods. The direct compounds, which do not need bioactivation, were N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), methyl methane sulfonate (MMS), and cadmium. The indirect toxins, which require biotranformation for toxicity, were acetaminophen, carbon tetrachloride, vinyl acetate, and N,N-dimethylformamide. Experiments were compared across several isolations to determine the reproducibility of toxicity in the collagen sandwich. The cells were most susceptible to the direct toxins and aflatoxin B_1 and resistant to acetaminophen, carbon tetrachloride, vinyl acetate, and dimethylformamide. The maintenance of several CYP450s in the culture has been examined and it was found that CYP1A1, 2B1/2, and 3A2 increase with culture time but CYP2E1 gradually decreases and is undetectable by day 7. The metabolism of acetaminophen was further investigated using liquid chromatography and mass spectrometry. It was determined that the cells convert acetaminophen primarily to the sulfate and glucuronide metabolites and only produce a small amount of the glutathione adduct, which is a detoxification product of the toxic N-acetylbenzoquinone metabolite (NABQI).

MATERIALS AND METHODS

Cell culture. Twenty-four well plastic plates (Becton Dickinson) were coated with 110 μl of collagen solution. The collagen solution consisted of 2 mg/ml collagen type I (Cohesion Technologies Inc., Palo Alto, CA) dissolved in water. The solution also contained 10% 10× phosphate buffered saline, which included 20 g/l glucose and 37 g/l sodium bicarbonate. The pH of the collagen was measured every time a collagen solution was prepared and was found to be 7.4.

Hepatocytes were isolated from male Fisher 344 inbred rats (Taconic, Germantown, NY) with a protocol described in Powers *et al.* (2002) and were immediately plated on collagen-coated 24-well dishes. The cell viability, measured with trypan blue exclusion, was typically 87–91%. The cells were incubated in Hepatocyte Growth Medium (Block *et al.*, 1996) with the following modifications: purified bovine serum albumin was omitted, the concentration of niacinamide was 0.305 g/l, and the concentrations of $ZnCl_2$, $ZnSO_4$, $CuSO_4$, and $MnSO_4$ were 0.0544 mg/l, 0.075 mg/l, 0.02 mg/l, and 0.0025 mg/l respectively. Furthermore, the medium was supplemented with 20 ng/ml epidermal growth factor (EGF).

The oxygen tension in the incubator was 20% and the carbon dioxide pressure was 5%. Cells were plated at a density of 100,000/well in a 24-well plate with 300 μl of medium. One day after plating the medium was removed and 55 μl of collagen solution was pipetted on top of cells to form the second layer of collagen. The collagen gelled within 1 h in the incubator and new medium was pipetted on the cells.

Assays for Measuring Cell Viability

XTT and alamar blue. The reduction of the tetrazolium salts XTT (2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilidem) and Alamar Blue were measured using standard colorimetric and fluorescence based kits from Sigma Aldrich and Biosource International (Camarillo, CA) respectively. These salts were added directly to cultures as described in the company protocols and the absorbance and the fluorescence of the medium were assayed after incubating the compounds with the cells for 24 h. XTT was measured at 450 nm and Alamar Blue was measured at excitation and emission wavelengths of 555 nm and 600 nm respectively.

Albumin secretion. Albumin secretion was measured by enzyme-linked immunosorbent assay (ELISA) using sheep IgG fraction against rat albumin (ICN Pharmaceuticals, Costa Mesa, CA) and horseradish peroxidase-conjugated goat anti-rat IgG (Accurate Chemical, Westbury, NY). The absorbance was measured at 450 nm with a Spectramax 250 microplate spectrophotmeter (Molecular Devices Corp., Sunnyvale, CA). Medium samples had to be diluted 20–50-fold before measurement, and the exact concentrations were calculated after comparison with the absorbances of albumin standards (ICN Pharmaceuticals, Costa Mesa, CA).

Urea secretion. Urea was measured with a colorimetric assay (Sigma, Kit #640-A). Urea was hydrolyzed by urease to form ammonia, which formed indophenol, after reaction with phenol nitroprusside and alkaline hypochlorite. Medium samples between 5 and 15 μl were incubated for 15 min at 37°C with 40 μl of urease, followed by 20 min of room temperature incubation with 80 μl each of nitroprusside and hypochlorite. The absorbances of samples were promptly measured at 570 nm, as indophenol only keeps its color for 1–2 h.

Lactate dehydrogenase. Lactate dehydrogenase was measured with Promega Kit # G7890. Briefly, this assay is based on the observation that dying cells do not maintain membrane integrity and release cytoplasmic contents, including enzymes such as lactate dehydrogenase, into the medium. Medium samples of 50 μl were removed from each well and combined with 50 μl of substrate solution containing lactate, rezaurin, diaphorase, and NAD^+ and incubated at room temperature in the dark for 10 min. After the reaction was terminated with 25 μl stop solution, the fluorescence was assessed immediately at excitation of 555 nm and emission at 600 nm. In order to quantitate cell death as a percentage the release of lactate dehydrogeanse from control cells and lysed cells was also measured. Cells were lysed according to the Promega protocol by adding 6 μl of lysis solution to the cells for 20 min before collecting samples. The assumption was that when the cells were treated with the lysis solution 100% of the cells would die and all of the lactate dehydrogenase would leak into the medium. Flourescent signal due to leakage from control cells was subtracted from the signal from toxin-treated and lysed cells in order to correct for normal cell death during culture time. Thus, the percentage of cells that died due to exposure to a toxin was estimated as $100 \times (X - C)/(L - C)$, where X is the signal from toxin-treated cells, C is the signal from control cells, and L is the signal from lysed cells.

Western blots for P450s 1A, 2B, 3A, and 2E. Cells were lysed in RIPA buffer containing 50 mM Tris-HCl, 0.5% deoxycholic acid, 0.1% SDS, and 1%

Nonidet P40. Protein concentrations were determined using the Pierce BCA protein binding kit, and absorbance was measured at 570 nm. Aliquots of 10 μg of protein were run on a 10 or 15% polyacrylamide gel and transferred to a polyvinyldifluoride membrane filter. The membranes were treated with 5% blotting grade milk for 1 h to block non-specific binding, rinsed, and incubated with a panel of antibodies against different antigens. Immune complexes were detected with an enhanced chemiluminescence substrate (Perkin Elmer), and exposed to Kodak MR film.

Toxin Treatment

Preparation of toxins and exposure to cells. Aflatoxin B_1 was prepared fresh from powder (Sigma) before each experiment. The 1 mg powder in the sealed container was combined with 0.5 ml of DMSO using a syringe and needle. A sample of the solution was then removed with the syringe, diluted 200-fold with DMSO, and its absorbance was measured with a spectrophotometer at 362 nm to determine the concentration. The stock solution of aflatoxin was dissolved into DMSO at 1000 times the desired concentrations, and the final dilution was made into medium to ensure a DMSO concentration of 0.1%. N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) and acetaminophen were weighed out and dissolved in DMSO first and then in medium, maintaining the DMSO concentration 0.1% in all cultures. Methyl methane sulfonate (MMS), cadmium, vinyl acetate, and N,N-dimethylformamide (DMF) were diluted straight into the medium.

As outlined in Figure 1, the cells were washed with 2 ml/well of Hanks's Buffer (Life Technologies, Carlsbad, CA) four times one day after the preparation of the second layer of collagen. The following day, toxins were dissolved in the medium as described above and 300 μl of toxin-containing medium was added to each well. After toxic compounds were added, the medium was collected and replaced with fresh medium (not containing toxin) every two days until six days after toxin-treatment and stored at −80°C until urea and albumin analysis. Parallel cultures were maintained for assessing the release of lactate dehydrogenase 24 h after toxin treatment, and the cultures from which the samples were taken were discarded.

Metabolism of acetaminophen. Cells were treated with 1 mM acetaminophen for 24 h and the conditioned medium was stored at −80°C until analysis. Metabolites were first separated with an Agilent 1100 HPLC using a 2× 150 mm C18 column and 10 mM TFA and acetonitrile solvents at a 100 μl/min flowrate. The fractions were collected for mass spectrometry analysis to confirm the structure of the metabolites. Mass spectrometry was performed on an Agilent XCT LC-MS/MS electrospray ion trap. The LC was performed with a 300 μm × 12 cm Vydac column with solvent A as 0.1% acetic acid and 5% acetonitrile and solvent B as 0.08% acetic acid, 5% methanol, 5% water, and 90% acetonitrile at a 4 μl/min flowrate.

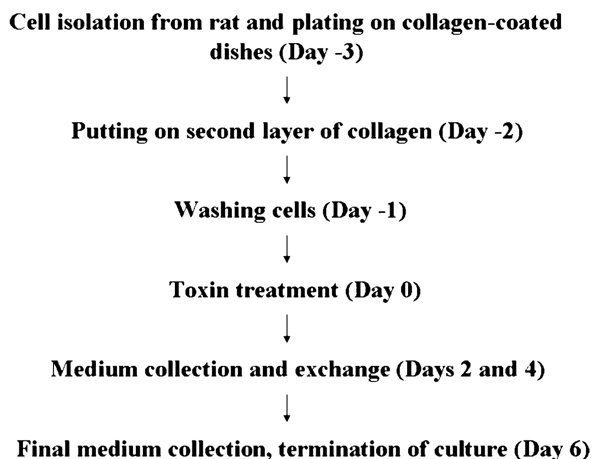

FIG. 1. Protocol for the preparation of a collagen sandwich and toxin treatment of hepatocytes.

To determine whether the glutathione adduct was produced in the cells, a standard was synthesized according to the protocol of Yan *et al.* (2000) and its retention time was determined with liquid chromatography and its structure was confirmed with mass spectrometry. The glutathione adduct was also identified in the sample by isolating the fraction with the appropriate retention time and confirming its molecular weight and fragmentation pattern with mass spectrometry.

Statistical analysis. In order to determine whether the observed toxicity was statistically significant, p values were calculated using the Student's paired t-test; p values less than 0.05 were considered statistically significant.

RESULTS

Toxicity Studies

Aflatoxin B_1 and direct-acting compounds. Cells were exposed to 16 nM, 32 nM, and 64 nM aflatoxin B_1 and the reductions of XTT and Alamar Blue were measured (Fig. 2). XTT and Alamar Blue decreased about 20% at 16 nM and 60% at 64 nm. Based on this data the LD_{50} was estimated to be 32 nM. The excretion of urea and the secretion of albumin were measured after treating the cells with 32 nM aflatoxin B_1 and it was found that urea and albumin decreased by 50 and 70% respectively compared to controls (Fig. 3A). Methyl methane sulfonate (MMS), N-methyl-N′-nitro-N-nitrosoguanidine (MNNG), and cadmium showed significant and reproducible toxicity at 1 mM doses with urea decreasing 20–40% and albumin decreasing by 80% or more. In contrast, the release of lactate dehydrogenase was more varied, indicating only 10–20% cell death for aflatoxin B_1 and MNNG and 40 and 60% cell death for MMS and cadmium respectively (Fig. 4A). Table 1 shows the values for the paired t-test values for aflatoxin, MMS, MNNG, and cadmium in the urea, albumin, and lactate

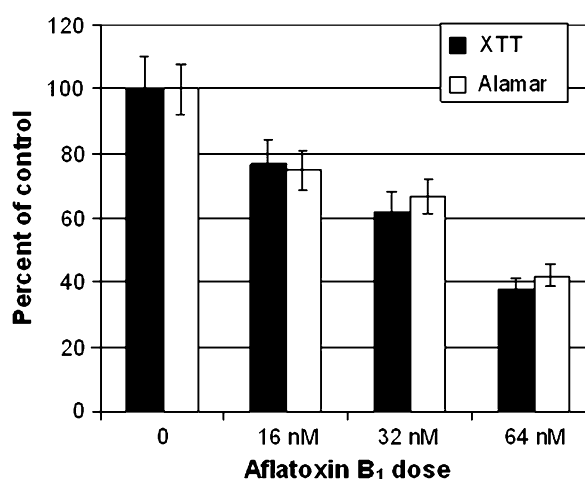

FIG. 2. Reductions of XTT and Alamar Blue (expressed as percent of the control) six days after treatment with aflatoxin B1. Reductions of XTT and Alamar Blue were measured colorimetrically and fluorescently respectively after incubating the cells for 24 h with the substrates. The sample number, n, is 2 for all measurements and the error bars show the deviations from the mean.

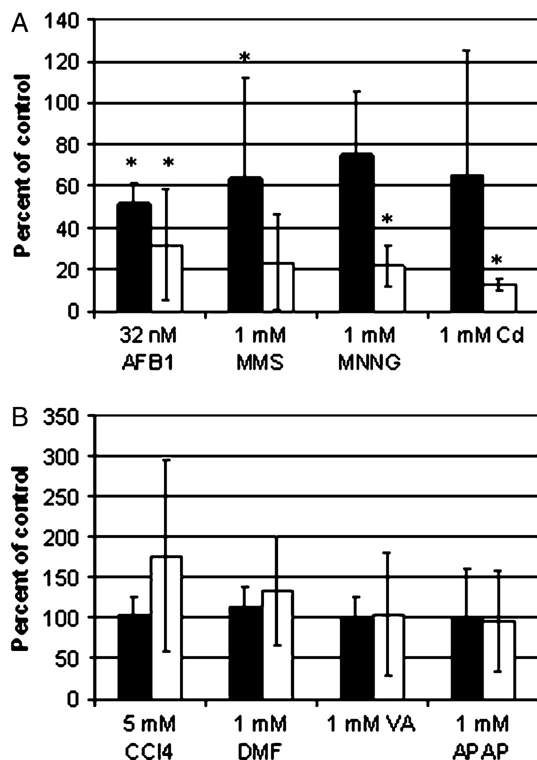

FIG. 3. Secretions of urea (in black) and albumin (in white), expressed as percent of control six days after toxin treatment. (A) Shows the compounds where toxicity was statistically significant (see Table 1), and (B) shows the compounds for which we could not detect toxicity with this approach. The sample number, n, is summarized for (A) in Table 1. For (B) n is 12 for APAP and 4 for all other compounds. The bars which show statistically significant toxicity ($p < 0.05$) have marked with (*).

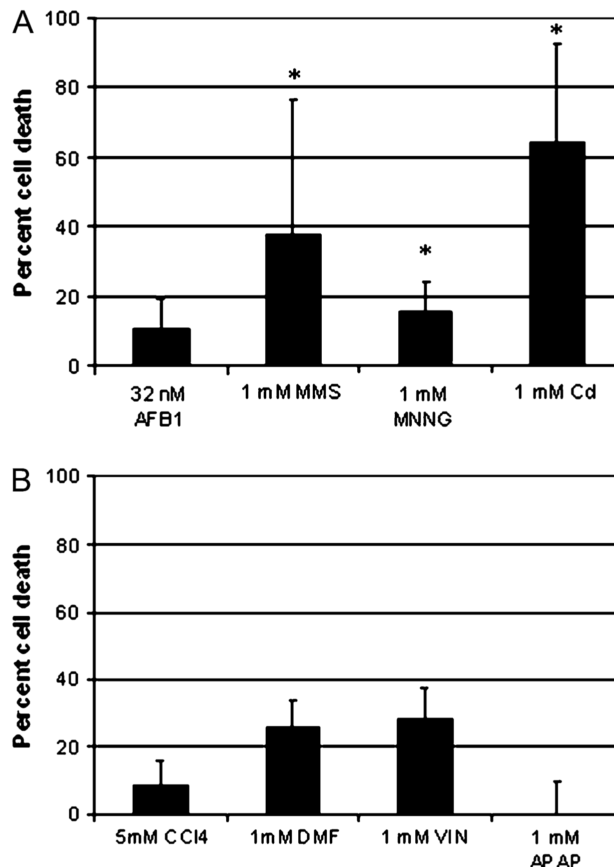

FIG. 4. Release of lactate dehydrogenase, expressed as percent cell death compared to controls, 24 h after treatment. (A) Shows the compounds where toxicity was statistically significant (see Table 1), and (B) shows the compounds for which we could not detect toxicity with this approach. The sample number, n, is summarized for (A) in Table 1. For (B) n is 12 for APAP and 4 for all other compounds. The bars which show statistically significant toxicity ($p < 0.05$) have marked with (*).

dehydrogenase assays. Most of the values were below 0.05, showing that toxicity is statistically significant within the 95% confidence interval. Although some of the p values were greater than 0.05, there were at least two assays with p values less than 0.05 for each toxin.

Acetaminophen, carbon tetrachloride, vinyl acetate, and N, N-dimethyl formamide. Cultures were exposed to 1 mM doses of acetaminophen, vinyl acetate, dimethylformamide, and a 5 mM dose of carbon tetrachloride. The secretions of urea, albumin, and the release of lactate dehydrogenase were measured six days after treatment. The secretions of urea and albumin were not statistically different from controls (Fig. 3B). According to the lactate dehydrogenase assay, a 5 mM dose of carbon tetrachloride caused about 10% cell death, and a 1 mM dose of dimethylformamide or vinyl acetate caused about 20% cell death. However, there was no increase in the release of lactate dehydrogenase after a 1 mM dose of acetaminophen (Figure 4B).

Drug Metabolism

The presence of various CYP450's proteins was measured as a function of time with Western blotting. Figure 5 shows the expression levels of CYP1A1, 2B1/2, 2E1, and 3A2 between days 2 and 7. As the figure shows, CYPs 1A, 2B1/2, and 3A2 increase with culture time while the expression of 2E1 gradually decreases and is undetectable by day 7.

TABLE 1
Paired t-Test p Values for Control and Toxin-Treated Cells in the Urea, Albumin, and Lactate Dehydrogenase Assays

	Urea	Albumin	LDH
Aflatoxin	0.0003 (20)	0.003 (8)	0.19 (6)
MMS	0.02 (15)	0.06 (7)	0.00003 (15)
MNNG	0.05 (17)	0.0004 (12)	0.011 (11)
Cd	0.21 (11)	0.008 (7)	0.000003 (11)

Note. $p < 0.05$ was considered statistically significant. The sample number (n) is listed in parenthesis for each assay.

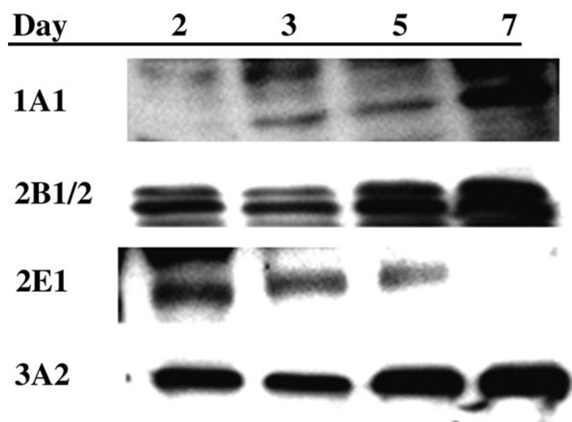

FIG. 5. Protein expression of various cytochrome P450s as a function of time in the collagen sandwich.

Metabolism of Acetaminophen

The primary metabolites of acetaminophen were measured using liquid chromatography and LC-MS/MS. The HPLC chromatogram for conditioned medium after a 24-h incubation with 1 mM acetaminophen is shown on Figure 6. The glucuronide and sulfate metabolites eluted at 5.5 and 7 min respectively and their structures were confirmed with mass spectrometry. The presence of the glutathione adduct in the 13–14 min was verified using collision induced dissociation (CID) as shown in Figure 7. The fragments corresponding to the CID spectra were identified using Chemwindows software, showing that the loss of glycine results in an m/z of 382, the loss of g-glutamic acid corresponds to an m/z of 311, and breaking the C-S bond gives rise to an ion with an m/z of 182 (structures not shown).

DISCUSSION

It has been shown by others that cells in a collagen sandwich maintain many of their Phase I and Phase II enzymes for 1–2 weeks (Bader *et al.*, 1998; Kern *et al.*, 1997; LeCluyse *et al.*, 2000; Rotem *et al.*, 1995), but there have been few studies to evaluate the collagen sandwich as a system for measuring hepatotoxicity. In this study it has been demonstrated that noninvasive markers such as urea, albumin, and lactate dehydrogenase leakage can be used to detect toxicity due to aflatoxin B1, cadmium, MMS, and MNNG. Furthermore, the metabolism of acetaminophen was examined, and it was shown that most of the parent compound is converted to the nontoxic Phase II metabolites.

Hepatocyte Growth Medium contains epidermal growth factor (EGF) in order to enhance long-term maintenance of hepatocytes. We found EGF to be essential for long-term cell survival, since removal of EGF resulted in a 50–75% decrease in viability (results not shown). However, EGF is also known to alter drug metabolism and to down-regulate many of the cytochrome P450s. Although the protein levels of CYPs 1A, 2B, and 3A increased during culture time the literature suggests that the expressions of these enzymes might be significantly lower than would be found *in vivo*. EGF has been shown to significantly decrease CYP2C11 (Ching *et al.*, 1996) and CP3A (Greuet *et al.*, 1997) activities in hepatocytes and specifically CYP1A and CYP2B activities in collagen sandwiches

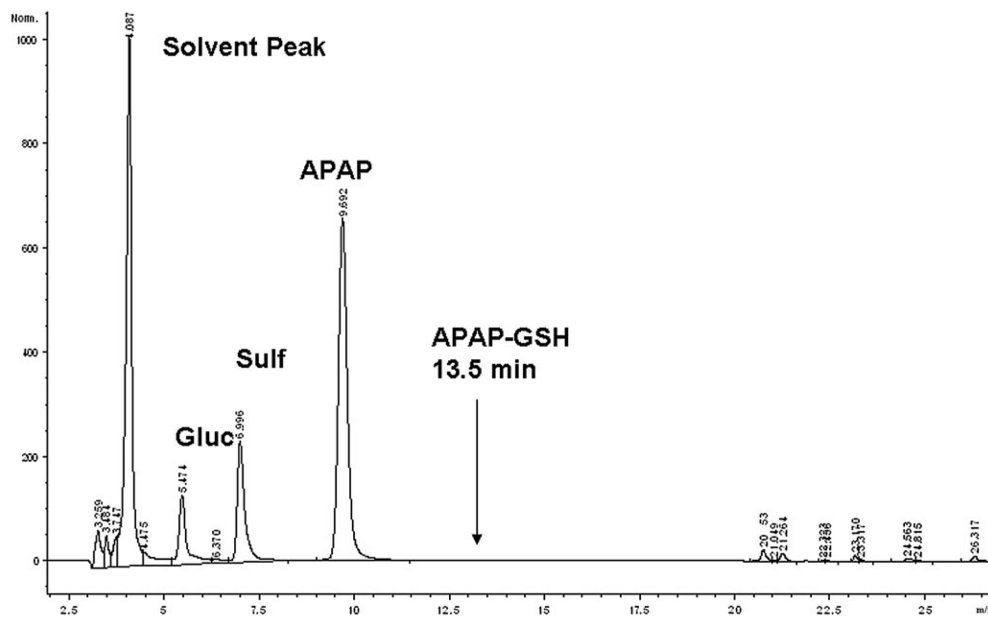

FIG. 6. HPLC chromatogram of medium from cells incubated for 24 h with 1 mM acetaminophen.

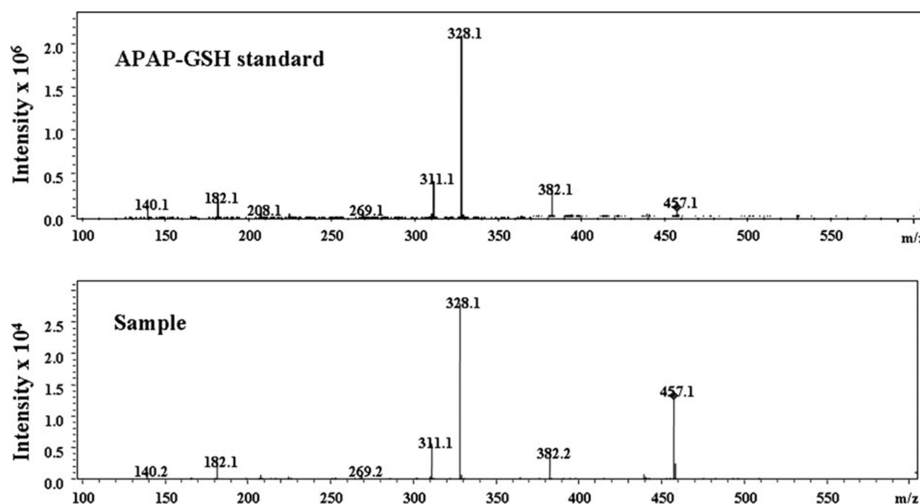

FIG. 7. Collision induced dissociation (CID) spectra of the acetaminophen-glutathione standard and the sample.

(De Smet *et al.*, 1999, 2001), which can significantly alter the toxicity of drugs and chemicals.

In order to determine the optimum cell density the activity of CYP1A and the secretions of urea and albumin were measured for two weeks at several different densities from 10,000 to 100,000 cells/cm^2. The activity of CYP1A was measured with the EROD assay as described elsewhere (Rotem *et al.*, 1995) and the secretions of urea and albumin were measured as described in the methods. It was found that at densities higher than 50,000 cells/cm^2 the activity of cytochrome P4501A plateaued and the secretion of albumin decreased slightly (data not shown).

Aflatoxin B_1 was the model compound since it was toxic at very low doses and its toxicity could be detected with several different assays. The LD_{50} for aflatoxin B_1 was determined using standard viability assays such as the reductions of XTT and Alamar Blue. The results from the XTT and the Alamar Blue assays suggested that the LD_{50} was about 32 nM. This result concurred with the urea assay, which showed that a 32 nM dose of aflatoxin B_1 resulted in a 50% decrease in the secretion of urea and a 70% decrease in the secretion of albumin.

Aflatoxin B_1 was the only compound requiring metabolic activation that significantly altered the secretions of urea and albumin in our cultures. Aflatoxin B_1 is normally oxidized by CYPs 2C11 and 3A2 (Manson *et al.*, 1997) to form the AFB1 *exo*-8,9-epoxide, the only known genotoxic product of AFB1 (Guengerich *et al.*, 1998) and the Western blots show that CYP3A2 is maintained in collagen sandwiches. Hepatocytes were resistant to the indirect compounds acetaminophen, carbon tetrachloride, vinyl acetate, and dimethyl formamide. The gradual loss of CYP2E1 could be responsible for resistance to carbon tetrachloride and acetaminophen, although other members of the CYP450 family can also metabolize these compounds. Carbon tetrachloride is activated by CYP2B1/2 and possibly CYP3A, to form the trichloromethyl radical, CCl_3* (Weber *et al.*, 2003) and acetaminophen is metabolized by CYPs 1A1/2, 2B1/2, 2C11/12 in addition to 2E1 to form the reactive N-acetylbenzoquinone imine (NABQI) (Rumack, 2002). However, CYP2E1 is the primary enzyme that metabolizes dimethylformamide so resistance to this compound is probably due to the loss of CYP2E1.

Resistance to acetaminophen can also be due to an upregulation of the detoxifying enzymes, phenolsulfotransferase, and UDP-glucuronosyltransferase, which form the sulfated and glucuronidated metabolites respectively. Rats are actually relatively immune to acetaminophen since they convert most of the compound to the sulfate and the glucuronide (Bessems and Vermeulen, 2001). The results in this study are consistent with this finding, since it was shown that hepatocytes in collagen sandwiches exposed to one millimolar acetaminophen convert most of it to the sulfate and glucuronide metabolites although a small amount of the glutathione adduct was also formed.

Hepatocytes were relatively resistant to vinyl acetate according to the urea and albumin assays, although there was an increase in the release of lactate dehydrogenase. Vinyl acetate has been shown by others to cause some intracellular acidification at doses between 10 and 1000 μM in fresh rat hepatocytes (Bogdanffy, 2002). It is possible that the hepatocytes in the sandwich have decreased carboxylesterase activity compared with fresh hepatocytes. The other possibility is that there was some toxicity at 24 h as shown by the lactate dehydrogenase assay, but the cells were able to recover from the damage by day 6 when the secretions of urea and albumin were measured.

Cadmium, MMS, and MNNG are direct toxins, thus it was expected that the hepatocytes would experience toxicity after being exposed to high doses of these compounds, even though they all act through different mechanisms. MNNG reacts with a cysteine residue (usually found on glutathione) in order to form a highly reactive electrophlic intermediate, which attacks DNA bases at random via an S_N1 reaction. Thus, exposure to

MNNG usually results in formation of electrophiles, which leads to depletion of glutathione as well as lipid peroxidation (Shertzer *et al.*, 1990). MMS is truly a direct-acting compound and reacts with the more nucleophilic sites on DNA via an S_N2 reaction to form alkylated bases (Lawley, 1972; Moffatt *et al.*, 1996). Cadmium is a heavy metal, which has been shown to cause hepatotoxicity and nephrotoxicity after chronic exposure. Cadmium has a long biological half-life in humans, and its toxicity results primarily from buildup in soft tissues and its binding to sulfhydryl groups (Rikans and Yamano, 2000).

Figure 3A shows that albumin decreased more compared to controls than urea, suggesting that albumin might be a more sensitive marker of toxicity than urea. It has already been observed by others that albumin secretion decreases in hepatocytes in response to inflammation and thus albumin has been categorized as a "negative acute phase protein." Kang *et al.* (2002) exposed hepatocytes in collagen sandwiches to IL-1β and IL-6 and measured the expression levels of urea and albumin. Both cytokines inhibited albumin synthesis by as much as 90%, whereas urea synthesis was inhibited consistently only by IL-1β. IL-6 inhibited urea synthesis at high doses but stimulated it at low doses. Although the molecular mechanism of albumin downregulation is not completely understood, it is thought that the decrease is mediated by the hepatic nuclear factor HNF1 and the nuclear protein and IL6DEX, which is induced in the presence of IL-6 and dexamethasone during the acute phase (Whalen *et al.*, 2004). In our cultures we observed decreased albumin expression even six days after toxin treatment, suggesting that the toxic exposure caused permanent alterations within the cells, or that the necrosing cells started a chain of inflammatory processes that the cultures could not recover from.

Conclusions

This study has demonstrated that the collagen sandwich culture system can be used to detect toxicity of aflatoxin B_1 and direct acting compounds such as MMS, MNNG, and cadmium. The cells were particularly susceptible to aflatoxin B1, with the LD_{50} being around 32 nM. However, the hepatocytes were found to be resistant to the indirect toxins acetaminophen, dimethylformamide, vinyl acetate, and carbon tetrachloride. This observed resistance might be partially due to the loss of CYP2E1, the primary metabolic enzyme for acetaminophen, dimethylformamide, and carbon tetrachloride. It was also shown that most of the acetaminophen was converted to the sulfate and glucuronide metabolites although a small amount of the glutathione adduct was also formed. This study demonstrates that the collagen sandwich is a good model for studying toxicity of direct toxins and aflatoxin B_1. However, it was observed that not all of the metabolic enzymes are maintained, and therefore it is essential to monitor the activities of the relevant enzymes before using this system for hepatotoxicity studies.

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REFERENCES

Babu, P. B., and Sudhakaran, P. R. (1991). Isolation of heparan sulfate proteoglycan from beneath the monolayers of rat hepatocytes and its binding to type IV collagen. *J. Cell Biochem.* **46,** 48–53.

Bader, A., Fruhauf, N., Zech, K., Haverich, A., and Borlak, J. T. (1998). Development of a small-scale bioreactor for drug metabolism studies maintaining hepatospecific functions. *Xenobiotica* **28,** 815–825.

Bader, A., Zech, K., Crome, O., Christians, U., Ringe, B., Pichlmayr, R., and Sewing, K. F. (1994). Use of organotypical cultures of primary hepatocytes to analyze drug biotransformation in man and animals. *Xenobiotica* **24,** 623–633.

Ben Ze'ev, A., Robinson, G. S., Bucher, N. L., and Farmer, S. R. (1988). Cell-cell and cell-matrix interactions differentially regulate the expression of hepatic and cytoskeletal genes in primary cultures of rat hepatocytes. *Proc. Natl. Acad. Sci. U.S.A* **85,** 2161–2165.

Bessems, J. G., and Vermeulen, N. P. (2001). Paracetamol (acetaminophen)-induced toxicity: molecular and biochemical mechanisms, analogues and protective approaches. *Crit. Rev. Toxicol.* **31,** 55–138.

Block, G. D., Locker, J., Bowen, W. C., Petersen, B. E., Katyal, S., Strom, S. C., Riley, T., Howard, T. A., and Michalopoulos, G. K. (1996). Population expansion, clonal growth, and specific differentiation patterns in primary cultures of hepatocytes induced by HGF/SF, EGF and TGF alpha in a chemically defined (HGM) medium. *J. Cell Biol.* **132,** 1133–1149.

Bogdanffy, M. S. (2002). Vinyl acetate-induced intracellular acidification: Implications for risk assessment. *Toxicol. Sci.* **66,** 320–326.

Ching, K. Z., Tenney, K. A., Chen, J., and Morgan, E. T. (1996). Suppression of constitutive cytochrome P450 gene expression by epidermal growth factor receptor ligands in cultured rat hepatocytes. *Drug Metab. Dispos.* **24,** 542–546.

De Smet, K., Beken, S., Depreter, M., Roels, F., Vercruysse, A., and Rogiers, V. (1999). Effect of epidermal growth factor in collagen gel cultures of rat hepatocytes. *Toxicol. in Vitro* **13,** 579–585.

De Smet, K., Bruning, T., Blaszkewicz, M., Bolt, H. M., Vercruysse, A., and Rogiers, V. (2000). Biotransformation of trichloroethylene in collagen gel sandwich cultures of rat hepatocytes. *Arch. Toxicol.* **74,** 587–592.

De Smet, K., Loyer, P., Gilot, D., Vercruysse, A., Rogiers, V., and Guguen-Guillouzo, C. (2001). Effects of epidermal growth factor on CYP inducibility by xenobiotics, DNA replication, and caspase activations in collagen I gel sandwich cultures of rat hepatocytes. *Biochem. Pharmacol.* **61,** 1293–1303.

Dunn, J. C., Tompkins, R. G., and Yarmush, M. L. (1992). Hepatocytes in collagen sandwich: evidence for transcriptional and translational regulation. *J. Cell Biol.* **116,** 1043–1053.

Dunn, J. C. Y., Tompkins, R. G., and Yarmush, M. L. (1991). Long-term in vitro function of adult hepatocytes in a collagen sandwich configuration. *Biotechnol. Prog.* **7,** 237–245.

Ezzell, R. M., Toner, M., Hendricks, K., Dunn, J. C. Y., Tompkins, R. G., and Yarmush, M. L. (1993). Effect of collagen gel configuration on the cytoskeleton in cultured rat hepatocytes. *Exp. Cell Res.* **208,** 442–452.

Gomez-Lechon, M. J., Jover, R., Donato, T., Ponsoda, X., Rodriguez, C., Stenzel, K. G., Klocke, R., Paul, D., Guillen, I., Bort, R., and Castell, J. V.

(1998). Long-term expression of differentiated functions in hepatocytes cultured in three-dimensional collagen matrix. *J. Cell Physiol.* **177,** 553–562.

Greuet, J., Pichard, L., Ourlin, J. C., Bonfils, C., Domergue, J., Le Treut, P., and Maurel, P. (1997). Effect of cell density and epidermal growth factor on the inducible expression of CYP3A and CYP1A genes in human hepatocytes in primary culture. *Hepatology* **25,** 1166–1175.

Guengerich, F. P., Johnson, W. W., Shimada, T., Ueng, Y. F., Yamazaki, H., and Langouet, S. (1998). Activation and detoxication of aflatoxin B1. *Mutat. Res.* **402,** 121–128.

Hamilton, G. A., Jolley, S. L., Gilbert, D., Coon, D. J., Barros, S., and LeCluyse, E. L. (2001). Regulation of cell morphology and cytochrome P450 expression in human hepatocytes by extracellular matrix and cell-cell interactions. *Cell Tissue Res.* **306,** 85–99.

Kang, Y. H., Berthiaume, F., and Yarmush, M. L. (2002). Long-term stable cultures of rat hepatocytes: An in vitro model to study acute and chronic hepatic inflammation. *Tissue Eng.* **8,** 681–693.

Kern, A., Bader, A., Pichlmayr, R., and Sewing, K. F. (1997). Drug metabolism in hepatocyte sandwich cultures of rats and humans. *Biochem. Pharmacol* **54,** 761–772.

Lawley, P. D. (1972). The action of alkylating mutagens and carcinogens on nucleic acids:N-Methyl-N-nitroso compounds as methylating agents. In *Topics in Chemical Carcinogenesis* (S. Nakahara and S. Odashima, Eds.), pp. 237–256. University Park Press, Tokyo.

LeCluyse, E., Madan, A., Hamilton, G., Carroll, K., DeHaan, R., and Parkinson, A. (2000). Expression and regulation of cytochrome P450 enzymes in primary cultures of human hepatocytes. *J. Biochem. Mol. Toxicol.* **14,** 177–188.

LeCluyse, E. L., Bullock, P. L., and Parkinson, A. (1996). Strategies for restoration and maintenance of normal hepatic structure and function in long-term cultures of rat heaptocytes. *Adv. Drug Del. Rev.* **22,** 133–186.

Liu, X., LeCluyse, E. L., Brouwer, K. R., Gan, L. S., Lemasters, J. J., Stieger, B., Meier, P. J., and Brouwer, K. L. (1999). Biliary excretion in primary rat hepatocytes cultured in a collagen-sandwich configuration. *Am. J. Physiol.* **277,** G12–G21.

Manson, M. M., Ball, H. W., Barrett, M. C., Clark, H. L., Judah, D. J., Williamson, G., and Neal, G. E. (1997). Mechanism of action of dietary chemoprotective agents in rat liver: Induction of phase I and II drug metabolizing enzymes and aflatoxin B1 metabolism. *Carcinogenesis* **18,** 1729–1738.

Moffatt, P., Plaa, G. L., and Denizeau, F. (1996). Rat hepatocytes with elevated metallothionein expression are resistant to N-methyl-N′-nitro-N-nitrosoguanidine cytotoxicity. *Toxicol. Appl. Pharmacol.* **136,** 200–207.

Moghe, P. V., Berthiaume, F., Ezzell, R. M., Toner, M., Tompkins, R. G., and Yarmush, M. L. (1996). Culture matrix configuration and composition in the maintenance of hepatocyte polarity and function. *Biomaterials* **17,** 373–385.

Moghe, P. V., Coger, R. N., Toner, M., and Yarmush, M. L. (1997a). Cell-cell interactions are essential for maintenance of hepatocyte function in collagen gel but not on Matrigel. *Biotechnol. Bioeng.* **56,** 706–711.

Moghe, P. V., Ezzell, R. M., Toner, M., Tompkins, R. G., and Yarmush, M. L. (1997b). Role of beta 1 integrin distribution in morphology and function of collagen-sandwiched hepatocytes. *Tissue Eng.* **3,** 1–16.

Powers, M. J., Janigian, D. M., Wack, K. E., Baker, C. S., Beer, S. D., and Griffith, L. G. (2002). Functional behavior of primary rat liver cells in a three-dimensional perfused microarray bioreactor. *Tissue Eng.* **8,** 499–513.

Rikans, L. E., and Yamano, T. (2000). Mechanisms of cadmium-mediated acute hepatotoxicity. *J. Biochem. Mol. Toxicol.* **14,** 110–117.

Rotem, A., Matthew, H. W. T., Hsiao, P. H., Toner, M., Tompkins, R. G., and Yarmush, M. L. (1995). The activity of cytochrome P450Ia1 in stable cultured rat hepatocytes. *Toxicol. in Vitro* **9,** 139–149.

Rumack, B. H. (2002). Acetaminophen hepatotoxicity: The first 35 years. *J. Toxicol. Clin. Toxicol.* **40,** 3–20.

Shertzer, H. G., Sainsbury, M., and Berger, M. L. (1990). Importance of protein thiols during N-methyl-N′-nitro-N-nitrosoguanidine toxicity in primary rat hepatocytes. *Toxicol. Appl. Pharmacol.* **105,** 19–25.

Weber, L. W., Boll, M., and Stampfl, A. (2003). Hepatotoxicity and mechanism of action of haloalkanes: carbon tetrachloride as a toxicological model. *Crit. Rev. Toxicol.* **33,** 105–136.

Whalen, R., Voss, S. H., and Boyer, T. D. (2004). Decreased expression levels of rat liver glutathione S-transferase A2 and albumin during the acute phase response are mediated by HNF1 (hepatic nuclear factor 1) and IL6DEX-NP. *Biochem. J.* **377,** 763–768.

Yan, Z., Nikelly, J. G., Killmer, L., Jr., and Tarloff, J. B. (2000). Metabolism of para-aminophenol by rat hepatocytes. *Drug Metab. Dispos.* **28,** 880–886.