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CANALICULAR AND SINUSOIDAL DISPOSITION OF BILIRUBIN MONO- AND DIGLUCURONIDES IN SANDWICH-CULTURED HUMAN AND RAT PRIMARY HEPATOCYTES

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ABSTRACT:

Due to cholestasis or adverse drug effects, the excretion of bilirubin conjugates can decrease; therefore, the level of bilirubin (B) and bilirubin glucuronides (BGs) increases in the serum with the concomitant shift of bilirubin di- versus monoglucuronide (BDG/BMG) equilibrium. The aim of this study was to utilize the collagen-sandwich culture of hepatocytes as an in vitro model for studying B conjugation and canalicular versus sinusoidal disposition of BGs. Canalicular and sinusoidal efflux of BMG and BDG obtained in sandwich-cultured rat primary hepatocytes was compared with that measured in human hepatocyte cultures. The BMG and BDG were separated by high-performance liquid chromatography and identified by mass spectrometry. The biliary excretion index (BEI) was estimated by measuring disposition of BGs into standard and Ca^{2+}, Mg^{2+}-free medium. Significantly more BGs were excreted into the canalicular networks than into the medium in 96-h sandwich culture of both human and rat hepatocytes (BEI, 62.5 and 80.6, respectively). The BDG/BMG ratio in the medium versus that in the canalicular networks was 0.55/1.48, which is similar to the serum/bile values (0.6/1.5) observed in vivo by Mesa et al. [Mesa VA, De Vos R, and Fevery J (1997) *J Hepatol* 27:912–916]. In contrast, the BEI for *p*-nitrophenol glucuronide was 5.2. The low BEI value is in agreement with empirical observations, which suggest that molecules with low molecular weight are preferably excreted by the kidney. In conclusion, sandwich-cultured primary hepatocytes provide a useful in vitro method to differentiate between sinusoidal and canalicular disposition of BGs. Since the normal BDG/BMG ratio changes in hyperbilirubinemia, this model could be used to predict drug effects leading to hyperbilirubinemia.

Bilirubin (B), the breakdown product of heme, is formed by degradation of hemoproteins. This toxic compound is cleared from circulation by the liver into the bile canaliculus mainly as bilirubin mono- (BMG) and diglucuronides (BDG) produced by UDP-glucuronosyltransferase (UGT1A1) (Burchell et al., 1997) via active transport processes (Jedlitschky et al., 1997). Hyperbilirubinemia can be classified into two types depending on whether B is conjugated or not. Unconjugated hyperbilirubinemia can be caused by mutations in UGTs as observed in Gilbert's and Crigler-Najjar syndromes in human and in Gunn rats, an animal model for these syndromes. Conjugated hyperbilirubinemia, such as Dubin-Johnson syndrome, is caused by genetic defects in the canalicular BG transport mediated by MRP2 (König et al., 2003; Higuchi et al., 2004) or dysfunction of MRP2, e.g., due to cholestasis (Kamisako et al., 2000). The cholestatic expression pattern of sinusoidal and canalicular transporters highly differs from normal; consequently, the kinetics of B uptake and efflux is altered (Rippin et al., 2001). Besides the genetic reasons, drugs can also cause both kinds of hyperbilirubinemia either by competing for UGTs or transporters, or by causing cholestasis. In both kinds of hyperbilirubinemia the concentration and the duration of intracellular storage of B increase in hepatocytes, resulting in a change in the amount and proportion of B, BMG, and BDG both in the bile and in the serum.

Many reports have described the role of mrp1, mrp2, and mrp3 in the transport of BGs under both normal and cholestatic conditions (Jedlitschky et al., 1997; Mesa et al., 1997; Keppler and König, 2000). However, these studies were all conducted either in vivo or using rather simplified systems such as membrane vesicles or expressed transporters. Primary hepatocytes cultured in a sandwich configuration have been shown to be a special in vitro model in which cells reestablish their polarity; consequently, the transport proteins are expressed specifically on the surface of the canalicular or sinusoidal membrane domain (Liu et al., 1999a; LeCluyse et al., 2000). The morphology resembles the natural state and allows differentiation of the canalicular efflux from the sinusoidal efflux of compounds. This suggests that sandwich cultures of hepatocytes can model their in vivo elimination into the bile and the serum, respectively. The sandwich culture of primary hepatocytes has been extensively characterized for use in estimating the biliary clearance of drugs in vitro. Following a substrate uptake period, extracellular Ca^{2+} depletion disrupts the tight junctions between adjacent cells; the content of the canalicular net-

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ABBREVIATIONS: B, bilirubin; BMG, bilirubin monoglucuronide; BDG, bilirubin diglucuronide; BG, bilirubin glucuronide; pNP, *p*-nitrophenol; pNPG, *p*-nitrophenol glucuronide; MRP, multidrug resistance-associated protein; UGT, UDP-glucuronosyltransferase; HBSS, Hanks' balanced salt solution; HPLC, high-performance liquid chromatography; BEI, biliary excretion index; Q1 scan, the first quadrupole is scanning.

works appears in the extracellular medium and can be analyzed (Liu et al., 1999b).

Many authors have proved the advantages of utilizing primary cultures of hepatocytes maintained in a collagen sandwich configuration in studying the hepatobiliary disposition of compounds mainly by using probe substrates, such as rhodamine 123, and different fluorescent mrp2 and mrp3 substrates (Liu et al., 1999b; Annaert et al., 2001; Kostrubsky et al., 2003). We used sandwich culture of primary rat and human hepatocytes with the aim of determining the role of basolateral and canalicular transport processes in B and BG disposition. In subsequent studies we are planning to investigate drug effects on B metabolism and transport. Drug interactions either with UGTs or transport proteins can alter biliary excretion of B, leading to cholestasis and hyperbilirubinemia in vivo. Sandwich culture of hepatocytes seems to be a suitable in vitro experimental model for studying these processes.

Materials and Methods

Preparation of Human and Rat Primary Hepatocytes. Human liver tissues were obtained by qualified medical staff from Semmelweis University of Budapest as rejected donor livers. Permission of the Local Research Ethics Committee was obtained to use human tissues. All studies involving human tissue followed the tenets of the Declaration of Helsinki. Hepatocytes were prepared by a three-step perfusion procedure. Human liver samples were flushed first with Ca^{2+}-free Earle's balanced salt solution containing EGTA, then with the same buffer without chelating agent, and finally, with Earle's balanced salt solution containing Ca^{2+} and type IV collagenase (prepared from *Clostridium histolyticum*; Sigma-Aldrich, St. Louis, MO). Perfusions were carried out at 37°C, pH 7.4 as described by Bayliss and Skett (1996). Rat hepatocytes were prepared from male Wistar rats (200–250 g) (Charles River, Budapest, Hungary) by in situ liver collagenase perfusion according to the method of Seglen (1976). Cell viability (>90%) was determined by trypan blue exclusion. All procedures were approved by the Institutional Animal Care and Use Committee.

Sandwich Culture of Primary Human and Rat Hepatocytes. Hepatocytes were plated at a density of 1.8×10^5 cells/cm^2 in 30-mm dishes precoated with 0.15-ml rat tail collagen type I solution (1.6 mg/ml) in Williams Medium E containing 5% fetal calf serum, 100 nM insulin, 2.5 μg/ml amphotericin B, 0.1 mg/ml gentamicin, 30 nM Na_2SeO_3, and 0.1 μM dexamethasone (Sigma-Aldrich). Calf serum and amphotericin B were present for the first 24 h and then omitted. Cells were maintained at 37°C in a humidified atmosphere of 95% air-5% CO_2. Four hours after plating, and every day thereafter, the medium was changed to Williams Medium E supplemented with insulin, gentamicin, dexamethasone, and Na_2SeO_3. At 24 h after plating, the medium was aspirated and cells were overlaid with 200 μl of ice-cold, neutralized rat tail collagen type I solution (1.5 mg/ml, pH 7.4) to achieve sandwich configuration. Then, 45 min after overlay, 1.5 ml of warm Williams Medium E supplemented with insulin, gentamicin, dexamethasone, and Na_2SeO_3 was placed on the top of the gelled collagen layer. Rat tail collagen type I was isolated by the method of Koebe et al. (1994).

Bilirubin Glucuronide and *p*-Nitrophenol Glucuronide Efflux Experiments. The 24-h, 72-h, and 96-h sandwich cultures of rat and the 96-h sandwich culture of human hepatocytes were incubated with 25 μM B (Merck, Darmstadt, Germany) for 60 min at 37°C in a humidified atmosphere of 95% air-5% CO_2. B (60 mM) was dissolved in dimethyl sulfoxide/1.0 M NaOH (88:12) as a stock solution and diluted to 3 mM in Williams Medium E containing 20 mg/ml BSA. pNP (Reanal Finechemical Co., Budapest, Hungary) was added to the medium at a 0.1 mM concentration; the incubation lasted for 20 min. Biliary excretion of BG and pNP glucuronide was measured by the method of Liu et al. (1999c). Briefly, cells were loaded with B and pNP for 60 and 20 min, respectively, in Williams Medium E as described above. Then the medium was removed, and cells were rinsed twice with 2 ml of warm HBSS and incubated with 0.5 ml of standard or Ca^{2+}, Mg^{2+}-free HBSS for 20 min. The Ca^{2+}, Mg^{2+}-free HBSS was supplemented with 0.5 mM EDTA. Aliquots of the incubation medium (withdrawn at the end of the substrate loading phase) and that of the standard and Ca^{2+}, Mg^{2+}-free HBSS were stored at −80°C until analysis. The accumulation of conjugates in the canalicular networks was expressed by the biliary excretion index (BEI) values. BEI was calculated as follows: $BG_{Ca^{2+},\ Mg^{2+}\text{-free HBSS}} - BG_{\text{standard HBSS}} / BG_{Ca^{2+},\ Mg^{2+}\text{-free HBSS}} \times 100$. All experiments were performed with hepatocytes from three independent preparations. The number of replicates within each experiment was three.

Bilirubin Glucuronide Formation by Human and Rat Hepatocyte Suspension. Freshly isolated human and rat hepatocytes were suspended at a density of 2×10^6 cells/ml in warm HBSS and incubated with 25 μM B for 60 min at 37°C. After incubation, the cells were centrifuged at 50*g* and the supernatant was stored at −80°C until analysis.

Bilirubin Glucuronide Formation by Human and Rat Hepatic Microsomes. For the preparation of hepatic microsomes, livers were homogenized in 0.1 M Tris-HCl buffer (pH 7.4) containing 1.15% KCl and 1 mM EDTA. Microsomes were prepared by differential centrifugation according to the method of van der Hoeven et al. (1974). Microsomal protein concentrations were determined by the method of Lowry et al. (1951) using bovine serum albumin as standard. The microsomal BG formation was measured according to the method of Burchell (1981). The incubation mixture contained 0.4 mM B, 4 mM UDP-glucuronic acid (Sigma-Aldrich), and 0.4 mg of protein in a final volume of 400 μl. After 10 min of incubation at 37°C, the reaction was stopped by adding 400 μl of ice-cold methanol. Samples were centrifuged at 10,000*g* for 10 min and the supernatant was stored at −80°C until HPLC analysis. BG was confirmed by β-glucuronidase cleavage of the conjugates in separate incubations by the addition of 1000 U of β-glucuronidase (from bovine liver) in sodium acetate buffer (pH 4.5) in the presence or absence of 10 mM D-saccharic acid-1,4-lactone (Sigma-Aldrich). Following incubation at 37°C for 2 h, the reaction was stopped by adding ice-cold methanol, samples were centrifuged at 10,000*g* for 10 min, and the supernatant was stored at −80°C until HPLC analysis.

HPLC Analysis of Bilirubin and *p*-Nitrophenol Conjugates. The amount of conjugates was determined by HPLC analysis. Samples were filtered through a filter with 0.45-μm pore size, and 100 μl of sample was applied to HPLC. Chromatography was performed on a Merck/Hitachi HPLC system using an L-6200 A pump, an L-4250 UV detector, and a D-6000 A interface with a 100 × 4.6 mm Chromolith Performance RP 18e (Merck) chromatographic column for analytical separation. To separate BG, a gradient elution was used. The solvents were composed of A (75% 0.01 M sodium phosphate buffer, pH 3.2, containing 150 μl/l triethylamine and 25% acetonitrile) and B (20% sodium phosphate buffer, pH 3.2, containing 1.5 ml/l triethylamine and 80% acetonitrile). After 1 min of isocratic elution with solvent A, the gradient started reaching 10% of B in 6 min. Following separation of the conjugates, the column was rinsed of remaining substrate as well as other hydrophobic components by using a gradient reaching 100% solvent B, and then the column was re-equilibrated. BMG and BDG were monitored at 450 nm; the flow rate was 3 ml/min. pNP-glucuronide was analyzed isocratically using 93% 0.01 M sodium phosphate buffer, pH 3.2, 7% acetonitrile as a mobile phase. The flow rate was 2 ml/min, and the effluent was monitored with a spectrophotometric detector at 305 nm. Conjugates were quantified using calibration curves prepared with pNPG and B as standards.

Mass Spectrometric Analysis of Bilirubin Conjugates. Mass spectrometric experiments were run on an Applied Biosystems/MDS Sciex (Foster City, CA) API-2000 tandem mass spectrometer in TurboIonSpray mode. Two PerkinElmer Series 200 HPLC pumps (PerkinElmer Life and Analytical Sciences, Boston, MA) were coupled to the system. The samples were acquired in normal Q1 scan and in multiple reaction monitoring scan modes. In the Q1 scan measurements the instrument was set in the mass range of 500 to 1000, using a scan time of 1 s. In multiple reaction monitoring mode, 937 to 475 and 761 to 475 transitions were monitored using a scan time of 150 ms each. The collision energy was 30 eV in both transiting channels. The samples were injected into water/acetonitrile, 1:1, at a flow rate of 0.2 ml/min. Analyst 1.4 software (Applied Biosystems) was used for controlling the acquisitions and for data processing.

Statistics. Data are reported as mean ± S.D. Statistically significant differences were evaluated with a two-tailed Student's *t* test. In all cases, $p < 0.05$ was deemed significant.

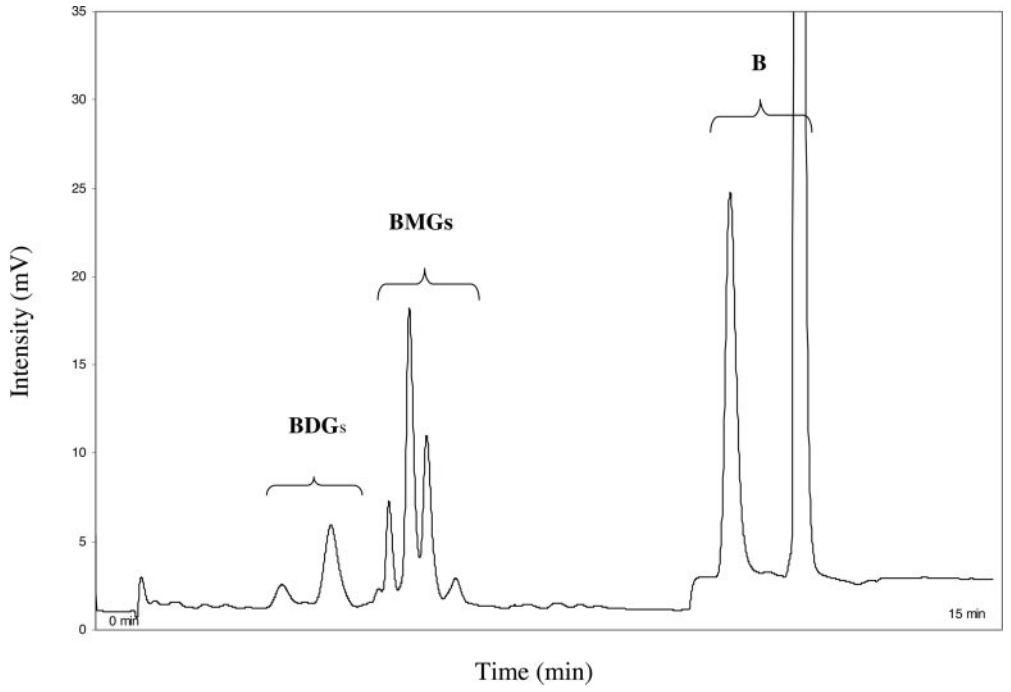

FIG. 1. A representative elution profile of the bilirubin conjugates from the supernatant of freshly prepared human hepatocytes incubated with 25 μM bilirubin for 60 min at 37°C. The elution peaks of bilirubin mono- and diglucuronides as well as the residual unconjugated bilirubin are indicated.

Results

HPLC Analysis and Identification of Bilirubin Glucuronides. Figure 1 shows a typical HPLC chromatogram of B, and BMG and BDG. The multiple peaks for BMG and BDG represent the isomers of each compound due to the endo and exo position of the vinyl groups reflecting the three isoforms of the parent B (IXα, XIIIα, and IIIα) (Odell et al., 1990; Brower et al., 2001). The identity of the conjugates was confirmed by treatment with β-glucuronidase enzyme. The peak of all glucuronides disappeared due to enzymatic cleavage, whereas D-saccharic-acid-1,4-lactone inhibited the hydrolysis; consequently, all peaks were preserved (data not shown). BDG and BMG were collected according to their peaks observed during HPLC, and aliquots of the samples containing the separated conjugates were applied to mass spectrometric analysis. The *m/z* for BMG was 761.2 and that for BDG was 936.9. The calibration curve was prepared using B as standard. Since there is no significant difference between the absorbance spectra of B and BMG or BDG (Wu, 1983; Brower et al., 2001), this calibration was used for the determination of BMG and BDG concentration.

BG and pNPG Biliary Excretion Index Determination in Sandwich-Cultured Human and Rat Hepatocytes. B and pNP were incubated with sandwich-cultured rat and human hepatocytes after 4 days of culture. After washing out the medium containing the substrates, the cells were incubated in standard or Ca^{2+}, Mg^{2+}-free HBSS. The amount of conjugates disposed into the medium was determined by HPLC analysis. Significantly more BG was observed in Ca^{2+}, Mg^{2+}-free HBSS than in the standard medium both in human and rat hepatocyte cultures (270% and 500% over the standard medium, respectively), showing that BG is predominantly excreted into the canalicular networks (Fig. 2). In contrast, although the amount of pNPG was significantly different in Ca^{2+}, Mg^{2+}-free HBSS from that observed in the standard medium (84.6 $\pm$ 1.1 versus 80.2 $\pm$ 1.1 pmol/10^6 cells/min), the difference was much smaller than that in the case of BGs. The BEI for BGs was 62.5 in human and 80.6 in rat sandwich-cultured hepatocytes and 5.2 for pNPG in rat hepatocytes.

The Ratio of BDG to BMG in the Medium and in the Canalicular Networks. We compared the BDG/BMG ratio in the medium after a 60-min period of B loading with that measured in the canalicular networks (Fig. 3). Sandwich-cultured rat hepatocytes were incubated with B for 60 min, and the BG content of the medium was analyzed. Cells were rinsed twice with warm HBSS and 0.5 ml of standard, or Ca^{2+}, Mg^{2+}-free HBSS was added. The BG content of the canalicular networks was determined by the difference of BG concentration obtained in the Ca^{2+}, Mg^{2+}-free HBSS and standard HBSS. The proportion of BDG and BMG in the medium at the end of the B loading phase was 35.5 $\pm$ 3% and 64.5 $\pm$ 3%, respectively. The proportion of BDG and BMG in the canalicular networks turned out to be 59.7 $\pm$ 2% and 40.3 $\pm$ 3%, respectively. These values were compared with the in vivo data published by Mesa et al. (1997), who observed 38 $\pm$ 30% of BDG and 62 $\pm$ 10% of BMG in rat serum, and 60 $\pm$ 4% of BDG and 38 $\pm$ 4% of BMG in rat bile. The BDG/BMG ratio observed in the medium of sandwich-cultured rat hepatocytes and in the serum was almost the same (0.55 and 0.6, respectively), whereas the reciprocal relationship was obtained for the conjugates in the canalicular networks (1.48) just as in the bile (1.5).

Shift of the Equilibrium Among Conjugates toward BDG during Culturing. In the medium the ratio of BDG to BMG produced by freshly isolated human hepatocytes was significantly different from that obtained with rat hepatocytes (36.5/63.5 and 6.9/93.1, respectively), suggesting that human hepatocytes produce more BDG than do rat hepatocytes (Fig. 4). The equilibrium among conjugates in the medium of both human and rat hepatocyte cultures shifted toward BDG during culturing. The amount of BDG after 96 h of culture was about 2.5-fold greater than that of BMG in the medium of human hepatocyte cultures. In contrast, the medium of rat hepatocyte cultures contained significantly less BDG than BMG.

The Ratio of BDG to BMG Formed by Human and Rat Hepatic Microsomes. The UGT1A1 activities in the liver microsomes of five human donors were measured as total BG formation and compared with that obtained in the liver microsomes pooled from 14 donors

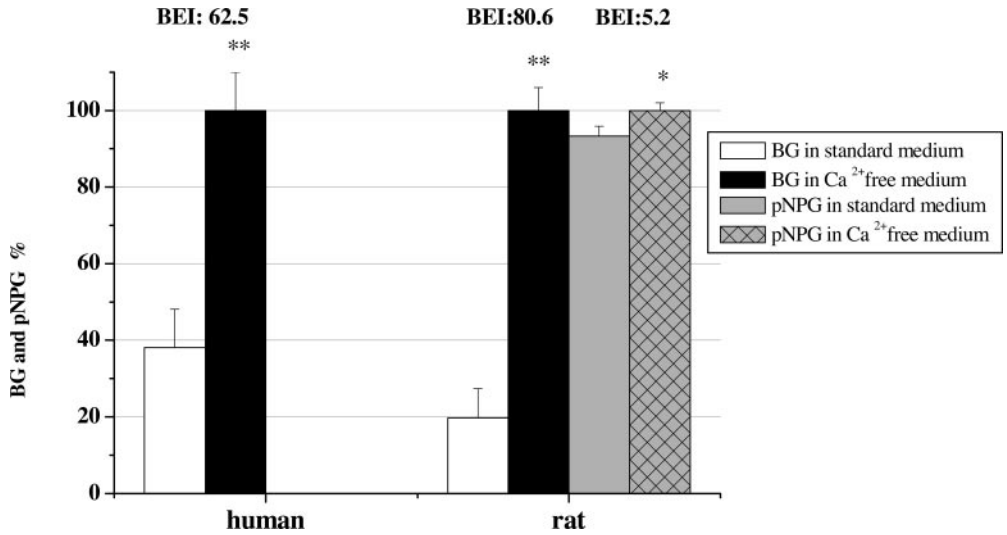

FIG. 2. BG and pNPG disposition into standard and into Ca^{2+}, Mg^{2+}-free HBSS in sandwich cultures of primary human and rat hepatocytes. Data are expressed as percentage of conjugates excreted into the Ca^{2+}, Mg^{2+}-free medium. The BEIs of BG and pNPG are reported above the individual bars. Means of three independent experiments ± S.D. *, $p < 0.05$, for standard versus Ca^{2+}, Mg^{2+}-free HBSS; **, $p < 0.01$, for standard versus Ca^{2+}, Mg^{2+}-free HBSS.

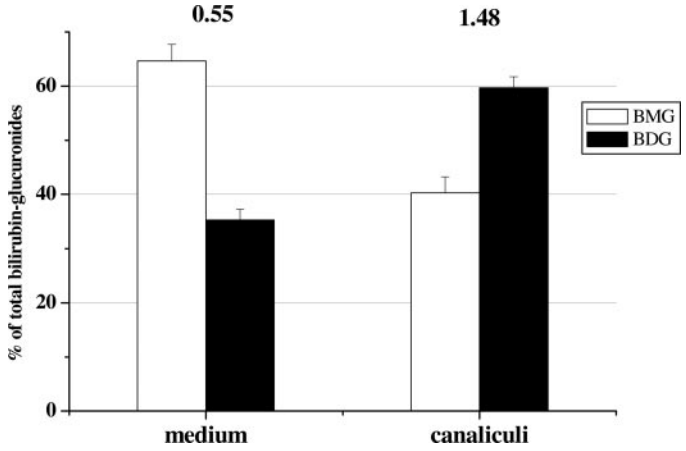

FIG. 3. The proportion of BDG and BMG in the medium and in the canalicular networks of 96-h sandwich culture of rat hepatocytes. Data are expressed as percentage of total BGs. The BDG/BMG ratio is indicated above the bars. Means of three independent experiments ± S.D.

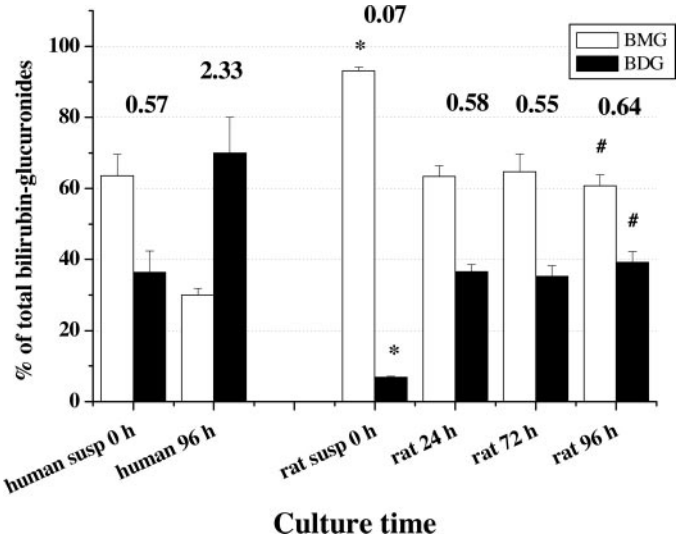

FIG. 4. Change in the proportion of BMG and BDG in the medium of rat and human hepatocyte suspensions and in primary hepatocyte cultures is presented. Data are expressed as percentage of total BGs. The BDG/BMG ratio is indicated above the bars. Means of three independent experiments ± S.D. *, $p < 0.01$, for human hepatocyte suspension versus rat hepatocyte suspension; #, $p < 0.01$, for 96-h primary culture of human versus rat hepatocytes.

(Fig. 5). The ratio of BDG to BMG was also determined. The average of the UGT1A1 specific activities measured in the five livers was similar to that obtained with the pooled microsomes, 0.603 ± 0.232 and 0.670 nmol/mg protein/min, respectively; however, almost 3-fold differences were observed concerning the individual values. The UGT1A1 activity of the pooled liver microsomes prepared from five male Wistar rats was higher than that of the human microsomes (0.978 nmol/mg protein/min). The ratio of BDG to BMG formed by the pooled human microsomes was higher than that formed by rat microsomes (0.10 and 0.04, respectively).

Discussion

During isolation by collagenase perfusion, hepatocytes lose their polarity; consequently, the membrane-specific expression of transport proteins disappears. Previous studies using hepatocytes in sandwich culture have shown that the cells in this configuration reestablished dynamically in 3 days after seeding bile canalicular networks between adjacent cells re-formed (LeCluyse et al., 2000; Chandra et al., 2001). Several studies have demonstrated that three membrane domains were distinct after 5 days of culture; in addition, canalicular transport proteins, such as mdr1, mrp2, and bsep were localized to the specific membrane surface (Luttringer et al., 2002). Liu et al. (1999a) demonstrated that long-term sandwich-cultured hepatocytes represent a useful in vitro model for studying biliary disposition of compounds. Extracellular Ca^{2+} depletion disrupted the tight junctions between adjacent cells: the content of the canalicular networks appeared in the extracellular medium and could be studied (Liu et al., 1999b); consequently, the BEI for a compound could be determined.

The aim of our work was to use the sandwich configuration of primary hepatocytes as an in vitro model in studying the basolateral versus canalicular disposition of bilirubin conjugates. Bilirubin conjugates are eliminated through basolateral (mrp1, mrp3) and canalicular (mrp2) transporters as well (Jedlitschky et al., 1997; Keppler and König, 2000; König et al., 2003), although under physiological conditions. the bilirubin conjugates are primarily excreted from the body

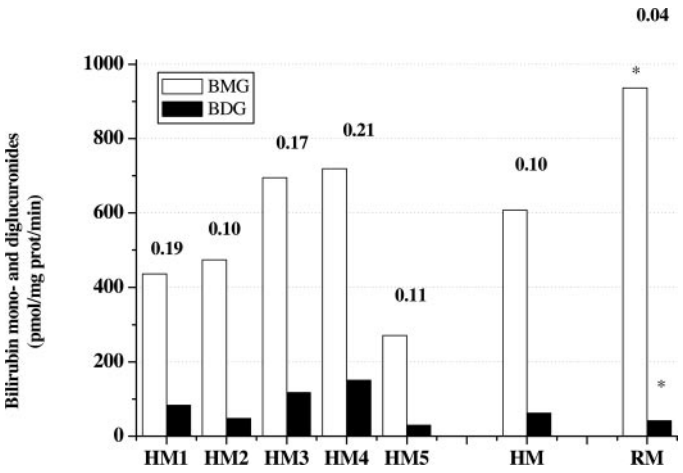

FIG. 5. Formation of BMG and BDG by human and rat hepatic microsomes. Bilirubin-conjugating enzyme activity data of hepatic microsomes from five individual human donors are compared with the enzyme activity of hepatic microsomes pooled from 14 human donors, and with hepatic microsomes pooled from five Wistar rats. The BDG/BMG ratio is indicated above the bars. *, $p < 0.05$, for pooled hepatic human microsomes versus pooled hepatic rat microsomes. HM, human microsomes; RM, rat microsomes.

via the bile (Mesa et al., 1997). In the present study, the BEI values calculated for BG in the 96-h sandwich culture of both rat and human hepatocytes clearly show that BGs are preferably excreted into the canalicular networks, which suggests a dominant role for mrp2 in BG elimination. This finding is in good agreement with the in vitro data of Jedlitschky et al. (1997) and the in vivo data of Clarke et al., (1997). In contrast to BGs, the low BEI value for pNPG suggests that this metabolite is preferably eliminated by the basolateral transporters, which is in line with the empirical observation that small molecules are mainly excreted via the urine.

Absence of or decrease in functionally active mrp2 localized to the canalicular membrane due to either genetic disorders, e.g., Dubin-Johnson syndrome, or cholestasis prevents or diminishes the secretion of BGs into the bile. The up-regulation of mrp3 plays a compensatory role in these cases since the rate of basolateral efflux increases and prevents cell damage during impaired transport into the bile (Keppler and König, 2000). In Dubin-Johnson syndrome or Eisai transport-deficient mutant rats, the BGs are excreted from hepatocytes by mrp3, resulting in conjugated hyperbilirubinemia. Under such conditions the extended period for intracellular storage and metabolism of B may explain the shift of equilibrium of BDG to BMG toward BDG (Clarke et al., 1997; Mesa et al., 1997). The BDG/BMG ratio is different in serum and bile in vivo, 0.6 and 1.5, respectively (Mesa et al., 1997), which is in good agreement with our data (medium and canalicular networks: 0.55 and 1.48, respectively). These results suggest that sandwich culture of hepatocytes is a useful model to distinguish between canalicular and basolateral transport of BGs.

In freshly isolated rat hepatocytes, the BDG/BMG ratio was much lower than that obtained in a 24-h culture. A reason for this BDG/BMG shift might be the change in either the transporter or UGT1A1 activity after seeding. The microsomal UGT1A1 activity toward bilirubin was found to be the same in rat liver, freshly isolated hepatocytes, and hepatocytes after 5 days of culturing, regardless of the culture configuration (Richert et al., 2002). However, depending on the extracellular matrix and the medium used, expression of transport proteins varies highly during culturing. Luttringer et al. (2002) described significant changes in the mRNA level of transport proteins during culturing, even in sandwich configuration. These changes in the expression of transporters can be further modulated by the short-term regulation of the trafficking of these proteins from the intracellular pools to the membrane surface and vice versa (Kipp et al., 2001). Alterations in the regulation processes during both cell isolation and seeding might also explain the difference observed between freshly isolated hepatocytes and hepatocytes after attachment. The BDG/BMG value obtained from freshly isolated human hepatocytes is much higher than that observed with rat cells. This difference became more pronounced after 96 h of culturing, which is in agreement with reports indicating that the amount of BDG to BMG is significantly higher in human bile than in rat bile (Clarke et al., 1997; Mesa et al., 1997). Although the hepatic UGT1A1 activities of the donors in the present study were in the normal range, we observed some variability among donors concerning the BDG/BMG ratio during microsomal metabolism. An explanation might be the interindividual variations in the active transport processes across the endoplasmic reticulum membrane (Csala et al., 2004). The difference between BDG/BMG ratio obtained with freshly isolated hepatocytes from human and rat was retained on the microsomal level; as well, the BDG/BMG ratio was significantly lower in experiments with rat microsomes than with human microsomes.)

The present study demonstrates that sandwich culture of human and rat hepatocytes is a proper in vitro model for studying B conjugation and sinusoidal versus canalicular transport of BGs. The BDG/BMG ratio obtained in the medium and canalicular networks was very similar to that found previously in vivo in the serum and bile. In vitro determination of the shift in the BDG/BMG ratio brought about by drug interactions might be used for the prediction of insufficient in vivo activity of conjugating enzymes or transporters, the consequences of which can be impaired bile secretion and cholestasis.

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