博士（生命科学）　木　本　絵　美

学位論文題名

Characterization and application of primary human hepatocytes as a tool to assess hepatic drug disposition

（初代培養ヒト肝細胞の薬物動態学的特性と肝動態評価に関する研究）

学位論文内容の要旨

INTRODUCTION

The liver is the primary organ for xenobiotic elimination wherein the hepatic sinusoidal drug transport process includes passive diffusion and/or uptake mediated by hepatic uptake transporters. Based on the concept of extended clearance, drug uptake is the rate limiting step if passive diffusion into the liver is significantly lower than intrinsic clearance mediated by metabolism and/or biliary elimination, and therefore affects drug pharmacokinetics (PK) and tissue disposition. Transporter proteins have recently been recognized to play a critical role in maintaining body homeostasis and drug safety and as such are now a pivotal part of the submission and approval process for new drug applications. Early profiling for transporter drug-drug interactions (DDIs) reduces the risks of drugs and allows pharmaceutical companies to bring safer, more efficacious therapies to patients. *In vitro* studies using transporter-expressed cell lines have been fundamental to identify the transporters that contribute to drug disposition and predict potential DDIs. However, the key challenge continues to revolve around the ability to accurately predict human drug disposition from *in vitro* data for drugs that are predominately eliminated by transporter-mediated processes. Human hepatocyte *in vitro* systems are widely accepted as a valuable tool to screen new chemical entities and to investigate metabolic liability, gene induction, and toxicity. Sandwich-cultured human hepatocytes (SCHH), which repolarize to develop intact bile pockets and provide a three dimensional orientation and proper localization of transporters, represent an alternative *in vitro* model to assess hepatic disposition. Simultaneous assessment of all the processes occurring in SCHH and mechanistic application of the data generated are currently lacking. Despite the utility of hepatocytes, the characterization of each *in vitro* system remains an active area of research with respect to the advancement of hepatic drug disposition and DDI predictions. Furthermore, the gaps encountered when modeling results from *in vitro* and *in vivo* observations still represent a black box. As a result, quantitative techniques are critical to establish scaling factors for PK predictions to understand drug disposition and adverse effects involving transporters as well as to account for PK variations across different populations. It is therefore prudent to optimize current *in vitro* systems to feed into innovative prediction methods and modeling tools that will allow a better understanding of transporter impact with respect to drug disposition. The aim of this thesis was to investigate the characterization and application of primary human hepatocytes, with the incorporation of new protein quantification techniques, as a tool to assess the hepatic drug disposition of traditional organic anion-transporting polypeptide (OATP) substrates and beyond.

1. Characterization of Digoxin Uptake in SCHH

Digoxin is a drug that is commonly used to treat congestive heart failure. Circulating digoxin levels (maximal concentration of ~1.5 ng/ml) require careful monitoring, and patients are susceptible to DDI-mediated cardiotoxicity. An increase in digoxin plasma exposure caused by inhibition of multidrug resistance 1 (MDR1) has been reported. Digoxin was also reported to be actively transported into human hepatocytes by the OATP1B3, thereby suggesting that the inhibition of OATPs may result in a clinically relevant DDI similar to that observed for MDR1. Although several studies in rats have shown that oatps contribute to the disposition of digoxin, further characterization in human systems has not been fully described. The purpose of this study was to investigate the hepatic uptake mechanisms of digoxin using SCHH and transporter-expressing cells. Digoxin uptake in SCHH involves both a saturable (carrier-mediated) process and a passive (nonsaturable) process. At low concentrations, the saturable component exhibited an apparent Km of 2.39 μM and a Vmax of 4.49 pmol/min/mg protein. The calculated passive diffusion clearance was 1.25 μL/min/mg protein3. The uptake of [^{3}H]digoxin in SCHH was not inhibited by a variety of substrates or inhibitors for OATP1B1, OATP1B3, OATP2B1, organic anion transporter 2, organic cation transporter 1, and monocarboxylate transporter 8. Cytochalasin B, which inhibits glucose transporters, did not significantly inhibit digoxin uptake, whereas the flavonoids quercetin and rutin inhibited uptake by ~50%. Nonlabeled digoxin inhibited [^{3}H]digoxin uptake by ~50%. Studies with OATP-transfected human embryonic kidney cells or oocytes also indicated that digoxin is not a substrate of OATP1B1, OATP2B1, or OATP1B3.

2. Characterization of OATP Expression and Functional Activity in Human Hepatocytes

As shown in chapter 1, digoxin is taken up into SCHH by a passive process and a carrier-mediated process that does not involve OATP1B1, OATP2B1, or OATP1B3. However, SCHH indeed maintains the functional activity of these transporters, which play an important role in the uptake of several drugs. Since the substrate specificities of OATP1B1, 1B3, and 2B1 are broad and overlapping, the contribution of each isoform to the overall hepatic uptake is of concern when assessing transporter-mediated DDIs or genetic polymorphism impact in the clinic. Herein, we quantitatively measured OATP proteins in cryopreserved hepatocytes, SCHH, and the liver, and examined the relationship with functional uptake of OATP substrates in an effort to identify the OATP isoform(s) contributing to the hepatic uptake of pitavastatin, which is known to be selectively distributed to the liver and eliminated in bile in an unchanged form. The modulation of OATP expression in SCHH was found to be lot-dependent. However, OATP protein measurements averaged from 5 lots of SCHH were comparable to that of suspended hepatocytes. All three OATP transporters in suspended hepatocytes and SCHH were significantly lower than those in the liver. The SCHH uptake clearance (CLuptake) of pravastatin was 177% of that in pre-culture for Hu4163, thereby suggesting a link between uptake activity and OATP1B1 expression as OATP1B1 expression was elevated to a similar extent. In light of the expression trends detailed above, the results indicate that OATP1B1 is the major isoform contributing to pravastatin transport. Also in line with the expression trends, the uptake of CCK-8 decreased by approximately 50% post-culture due to the reduction in OATP1B3. In contrast, the SCHH CLuptake of rosuvastatin was maintained at a comparable level to suspension, which may reflect the impact of the overall OATP expression levels post-culture. In suspended hepatocytes, OATP1B1 appeared to show a positive trend with respect to the uptake of pitavastatin, which suggests a selective contribution of OATP1B1 to pitavastatin transport and thus an OATP quantitative protein expression–activity relationship (QPEAR). While the passive diffusion of rosuvastatin in SCHH was consistent across hepatocyte lots, the passive diffusion of pitavastatin varied over a broad range (>4-fold) in suspended hepatocytes and was inversely correlated with transporter-mediated uptake, presumably due to cell membrane alterations imparted by cryopreservation.

3. Modulation of Cytochrome P450 Activity in SCHH

From the results detailed in chapter 2, the aforementioned SCHH model is suggested to be a feasible tool to characterize transporter-mediated and passive *in vitro* uptake parameters. SCHH have also been widely used for *in vitro* assessments of biliary clearance, however, the modulation of metabolism enzymes has not been fully evaluated in this system. The present study was therefore undertaken to determine the activity of cytochrome P450 (CYP) 1A2, 2C8, 2C9, 2C19, 2D6, and 3A and to evaluate the impact of 1-aminobenzotriazole (ABT) on hepatic uptake and biliary excretion in SCHH. The SCHH maintained integrity and viability as determined by lactate dehydrogenase release and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assays conducted over the culture period. Although all assessed P450 activity decreased in day 2 SCHH, the extent of the decrease and the subsequent rebound in activity varied across the different isoforms. Day 5 CYP1A2 activity was approximately 2.5-fold higher than day 1 activity, whereas the CYP3A and CYP2C9 activities were 90 and 60% of the day 1 levels, respectively. In contrast, the initial CYP2C8, CYP2C19, and CYP2D6 activity losses did not rebound over the 5-day culture period. Furthermore, ABT was not found to have an effect, whether directly or indirectly as a P450 inactivator, with respect to the hepatic transport of rosuvastatin, atrovastatin, and midazolam in SCHH.

CONCLUSION

The following conclusions can be drawn on the basis of this research:

- Important aspects of digoxin uptake in human hepatocytes have been highlighted and the results are consistent with a significant passive component (40–50%) and an active component that does not involve typical hepatic drug transporters, such as OATP1B1, OATP1B3, OATP2B1, OAT2, and OCT1.
- SCHH maintains OATP protein expression and membrane integrity and, if feasible when considering research goals, would be considered a superior tool for the characterization of *in vitro* transport parameters without the complication of membrane leakage.
- Quantitative protein expression-activity relationship (QPEAR) emphasizes the importance of protein quantification for each lot of hepatocytes intended for *in vivo* extrapolation.
- Due to the differential modulation of P450 activity, SCHH may not be considered a suitable tool for metabolic stability assessments with compounds predominantly cleared by certain P450 enzymes.

These SCHH characterizations and proteomic techniques are fundamental to obtain empirical scaling factors for incorporation into physiologically based pharmacokinetic (PBPK) models that are applied in the prediction of human PK and transporter-mediated drug disposition, which is necessary to avoid certain types of DDIs that can cause serious toxicity in patients.

学位論文審査の要旨

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Characterization and application of primary human hepatocytes as a tool to assess hepatic drug disposition

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博士学位論文審査等の結果について（報告）

肝臓は薬物の代謝や排泄といった解毒の役割を担う重要な臓器である．経口投与された薬物は，肝臓への取り込み動態が全身の動態や組織移行に影響を与える．そのため近年，薬物動態や安全性におけるトランスポーターの重要性がクローズアップされてきており．医薬品開発の早期段階から，トランスポーターが関与する薬物間相互作用(DDIs)を評価する事により，より安全でかつ有効な薬を患者に届ける事が可能となる．したがって,トランスポーターを強制発現させた細胞は薬物とトランスポーターの関与を評価する上で有用な *in vitro* 試験のツールとなる．しかしながら，トランスポーターで排泄される薬物は，従来の *in vivo 試験*からの予測ではタンパクの種差を無視できない事もあり予測が困難である．また，*in vitro* データを用いてヒト体内薬物動態を正確に予測するには，十分な *in vitro in vivo correlation (IVIVC)*や *extrapolation (IVIVE)*が検討されておらず，医薬品開発において非常に重要な課題でとなっている．そこで本研究では，トランスポーターがその動態に関与する薬物の，ヒトでの薬物動態や DDI の予測の信頼性を向上させ，肝の *in vitro* 評価系を確立することを目的とし，ヒト肝細胞を用いてその薬物動態学的特性と肝動態評価について解析した．

1. Characterization of Digoxin Uptake in SCHH

OATP1B3 の基質となることが報告されている digoxin の sandwich-cultured human hepatocytes (SCHH)における肝取り込み機構を評価した．その結果，digoxin の SCHH での肝取り込みは，トランスポーターを介したものと受動拡散の両方の関与が示唆されたが，OATP1B1, 1B3, 2B1 による取り込みではないことが明らかとなった．また，SCHH は OATP に関与する薬物の取り込みを評価するモデルとして有用であることも示された．

2. Characterization of OATP Expression and Functional Activity in Human Hepatocytes

SCHH が OATP の機能活性を保持することが示されたことから，肝組織と in

vitro 評価系(遊離肝細胞と SCHH)での OATP 発現量を評価し, pitavastatin の肝取り込みにおける上記三つの OATP isoforms との関与を検討した. その結果, 三つの isoforms とも、肝組織での発現レベルは遊離肝細胞や SCHH よりも高いことが示された. また, SCHH において, 選択的基質である pravastatin と CCK-8 の肝取り込みは, それぞれ OATP1B1 と 1B3 の発現変動と相関していることが示された. 遊離肝細胞において, pitavastatin の肝取り込みは OATP1B1 と正の相関傾向を示した. 以上の結果からも, SCHH は OATP の細胞膜上での発現状態を良く保持していることが示唆された.

3. Modulation of Cytochrome P450 Activity in SCHH

SCHH における cytochrome P450 (CYP) 1A2, 2C8, 2C9, 2C19, 2D6, 3A の活性と薬物の肝取り込みや胆汁排泄における 1-aminobenzotriazole (ABT)の影響を検討した. その結果, SCHH 培養初日と比較し 5 日目において, CYP1A2 活性は約 250%, CYP3A と 2C9 の活性はそれぞれ 90%と 60%の回復を, CYP2C8, 2C19, 2D6 の活性は活性回復を示さなかった. この CYP1A2 のアップレギュレーションは, 長期培養によるストレスによるものでないことが示唆された. さらに, ABT は SCHH における P450 の活性を抑制するが, rosuvastatin, atrovastatin, midazolam の肝輸送には影響を与えなかった.

以上, 本研究は, SCHH により得られた薬物の肝動態データとプロテオミクスで得られたタンパク発現量の scaling factor をモデルに組み込むことでトランスポーターが関与する薬物のより最適なヒト体内動態や DDI の正確な予測を可能とする新知見を得たものであり, より安全な医薬品開発に貢献するところ大なるものがある. よって著者は, 北海道大学博士 (生命科学) の学位を授与される資格あるものと認める.