Current Topics

Recent Advances in the Understanding of Nuclear Receptors- and Drug-Metabolizing Enzymes-Mediated Inter-Individual Differences

Review

Functional Interaction between Cytochrome P450 and UDP-Glucuronosyltransferase on the Endoplasmic Reticulum Membrane: One of Post-translational Factors Which Possibly Contributes to Their Inter-Individual Differences

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Cytochrome P450 (P450) and uridine 5'-diphosphate (UDP)-glucuronosyltransferase (UGT) catalyze oxidation and glucuronidation in drug metabolism, respectively. It is believed that P450 and UGT work separately because they perform distinct reactions and exhibit opposite membrane topologies on the endoplasmic reticulum (ER). However, given that some chemicals are sequentially metabolized by P450 and UGT, it is reasonable to consider that the enzymes may interact and work cooperatively. Previous research by our team detected protein-protein interactions between P450 and UGT by analyzing solubilized rat liver microsomes with P450-immobilized affinity column chromatography. Although P450 and UGT have been known to form homo- and hetero-oligomers, this is the first report indicating a P450-UGT association. Based on our previous study, we focused on the P450-UGT interaction and reported lines of evidence that the P450-UGT association is a functional protein-protein interaction that can alter the enzymatic capabilities, including enhancement or suppression of the activities of P450 and UGT, helping UGT to acquire novel regioselectivity, and inhibiting substrate binding to P450. Biochemical and molecular bioscientific approaches suggested that P450 and UGT interact with each other at their internal hydrophobic domains in the ER membrane. Furthermore, several *in vivo* studies have reported the presence of a functional P450-UGT association under physiological conditions. The P450-UGT interaction is expected to function as a novel post-translational factor for inter-individual differences in the drug-metabolizing enzymes.

Key words drug metabolism; protein-protein interaction; inter-individual difference; cytochrome P450; uridine 5'-diphosphate-glucuronosyltransferase; endoplasmic reticulum

1. INTRODUCTION

Drug metabolism is a defense mechanism against a large number of chemical compounds, including drugs and endogenous substrates. Hydrophobic compounds are difficult to eliminate from the body; however, they can be eliminated by drug-metabolizing enzymes that catalyze several types of reactions, such as oxidation, reduction, hydrolysis, and conjugation, in which the hydrophobic compounds are converted into polar metabolites. Most drug-metabolizing enzymes are localized to the liver, and more than 70% of drugs undergo such drug-metabolizing reactions.^{1,2)} Cytochrome P450 (P450, CYP) and uridine 5'-diphosphate (UDP)-glucuronosyltransferase (UGT) play crucial roles in phases I and II of drug metabolism, catalyzing oxidation and glucuronidation, respectively.^{1,3)} Large inter-individual differences are recognized by their hepatic expression level and *in vivo* catalytic activity of drug-metabolizing enzymes.⁴⁻⁸⁾ To elucidate such inter-individual differences, a large number of studies have been conducted and some molecular mechanisms have been

established, including the identification of single nucleotide polymorphisms (SNPs) and gene induction by nuclear receptors.^{3,9-11)} Although these mechanisms can explain some of the inter-individual differences in drug metabolism, they have yet to be fully elucidated, including the discrepancy between hepatic expression level and clearance of drug-metabolizing enzymes. CYP3A4 is one of the major P450 isoforms involved in 30–50% of drug metabolism.¹²⁾ Although its hepatic expression levels vary by up to 40 times, the difference in *in vivo* CYP3A4-mediated clearance is as much as 10 times.^{7,13)} SNPs that can affect the catalytic activity of CYP3A4 are very rare; therefore, there is likely another mechanism that should be placed at the post-translational step.^{11,14)}

Protein-protein interactions are necessary to maintain homeostasis in the body. Through these interactions, proteins can operate diverse functions, including acting as a switch for enzymatic reactions, transducing signals, maintaining the cytoskeleton, and scaffolding other proteins. It has been reported that over 80% of proteins form complexes with others to function.¹⁵⁾ Drug-metabolizing enzymes also form homo- and het-

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ero-oligomers (dimers, tetramers, or a higher form) within the same kind of enzyme: P450,^{16–19)} UGT,^{20–22)} sulfotransferase,²³⁾ and glutathione *S*-transferase (GST).²⁴⁾ Among them, P450 and UGT oligomerizations have been well studied. Researchers have utilized a reconstituted system with purified P450s from animal liver and estimated P450 oligomerizations.^{16,25,26)} UGT oligomerization has also been suggested by studies that attempted to purify the enzyme from animal tissues,^{27–29)} and the use of recombinant proteins has greatly advanced research on oligomerization.^{30–33)} Interestingly, enzymatic capabilities were altered in isoform-specific and functional interactions rather than in non-specific aggregations. The detailed information about P450^{16,17)} and UGT^{34,35)} oligomerizations and their effects on each enzyme is available in the literature. In this review, we focus on the P450-UGT association, a protein–protein interaction between the two drug-metabolizing enzymes.

2. P450 AND UGT FORM A FUNCTIONAL COMPLEX ON THE ENDOPLASMIC RETICULUM (ER) MEMBRANE

Our team first reported the P450–UGT interaction two decades ago,³⁶⁾ but it is still believed that the different kinds of enzymes work separately. Keeping the dogma in mind, we summarize our findings that indicate the presence of P450-UGT and its effects on enzymatic capability of P450 and UGT.

2.1. Prediction and Detection of the P450–UGT Complex Both P450 and UGT are important drug-metabolizing enzymes located in the ER membrane. However, they catalyze different kinds of reactions (oxidation categorized as phase I *versus* glucuronidation categorized as phase II), and their membrane topologies are inverted relative to each other; P450 and its electron donors, reduced form of nicotinamide adenine dinucleotide phosphate (NADPH)-cytochrome P450 reductase (CPR) and cytochrome *b*₅ face the cytosol. In contrast, the major part of UGT orients in the luminal side, and C-terminal short peptides following transmembrane region are only in the cytosolic side^{37,38)} (Fig. 1). These differences may strengthen the “general concept;” however, several compounds are known to be sequentially metabolized by P450 and UGT (Fig. 1), including codeine. A portion of the clinical cough medicine is first demethylated and converted to morphine by CYP2D6, and the 3-hydroxy group that is generated is a target of UGT2B7 in the human liver. Given their sequential

metabolism, it is reasonable to consider that P450 and UGT interact with each other and optimize each other's function to maximize the rate of the cooperative reaction. Iyanagi *et al.* proposed the cooperation of P450 and UGT on the ER membrane when they cloned and characterized rat UGT1A6 in the mid-1980s, but to the best of our knowledge, we presented protein–protein interactions between P450 and UGT for the first time.³⁹⁾

Our team detected physical binding between P450 and UGT by affinity chromatography in 2000.³⁶⁾ Taura *et al.* treated Sprague-Dawley rats with phenobarbital and prepared liver microsomes from them. The microsomes were solubilized with sodium cholate and centrifuged. The supernatant was collected as a source of prey proteins. As a bait protein, purified rat CYP1A1 was immobilized on a Sepharose 4B column. Then, sodium cholate-solubilized microsomes were applied onto the CYP1A1-affinity column, and the binding proteins were eluted with buffer using an NaCl gradient. Following immunoblotting, UGT and microsomal epoxide hydrolase (mEH) were collected in the high NaCl fraction, similar to CPR, while ER chaperones, including protein disulfide isomerase and calnexin (CNX), were not. These results suggest the presence of CYP1A1–UGT and CYP1A1–mEH interactions in the rat liver, and their affinity was close to that of the P450–CPR complex. Following this study, Ishii *et al.* and Takeda *et al.* added evidence for P450–UGT interaction in rat liver microsomes (RLM) and human liver microsomes (HLM) by co-IP.^{40–42)} In fact, the UGT in the P450–UGT complex is catalytically active.⁴⁰⁾ Other groups also reported supporting data with HLM.^{43,44)} To investigate isoform-specificity in P450–UGT association, we also utilized heterologous expression systems in mammalian and insect cells and reported further interactions between human isoforms using several biochemical approaches, such as GST-overlay assay,^{41,42,45)} chemical cross-linking,⁴²⁾ and histidine (His)-tag pull-down assays.^{46,47)} Further details of the reported P450–UGT association are presented in Table 1.

2.2. Effects of the P450–UGT Interaction on UGT Capability Our team focused on the CYP3A4–UGT2B7 interaction since both CYP3A4 and UGT2B7 are major isoforms in the human liver and play important roles in drug metabolism.^{48,49)} Takeda *et al.* examined the effect of CYP3A4 on UGT2B7 activity and reported some interesting findings. UGT2B7 is a UGT isoform responsible for morphine glucuronidation in humans, and the UGT isoform gener-

Fig. 1. Postulated Topology of P450 and UGT on the Endoplasmic Reticulum Membrane and Sequential Drug Metabolism Catalyzed by These Enzymes

CPR, NADPH-cytochrome P450 reductase; *b*₅, cytochrome *b*₅; e[−], electron; NADPH, reduced form of nicotinamide adenine dinucleotide phosphate; UDP, uridine diphosphate; UDPGA, UDP-glucuronic acid; RH, substrate; R-OH, metabolite formed by P450 and substrate of UGT; R-O-GlucA, glucuronide generated by UGT. (Color figure can be accessed in the online version.)

Table 1. P450–UGT Interactions Detected Using Several Biochemical Approaches

P450 isoform	P450 source	UGT isoform	UGT source	Methods	References
CYP1A1	Purified from RLM	Rat UGTs	RLM	Affinity chromatography	36)
CYP3A4	HLM	UGT2B7	HLM	Co-IP	41–43)
CYP3A4	HLM	UGT1A1	HLM	Co-IP	43)
		UGT1A6			
CYP3A4	Recombinant	UGT2B7	Insect cell microsomes	Overlay assay Cross-linking	41,42)
CYP1A2	RLM	Rat UGTs	RLM	Co-IP	40)
CYP2B2					
CYP2C11/13					
CYP3A2					
CYP3A4	Recombinant	UGT1A7	Insect cell microsomes	Overlay assay	45)
CYP1A2	HLM	UGT2B7	HLM	Co-IP and tandem MS	44)
CYP3A4					
CYP3A4	Insect cell microsomes	UGT2B7	Insect cell microsomes	Co-expression and His-tag pull down assay	46)
CYP3A1	Insect cell microsomes	UGT2B3	Insect cell microsomes	Co-expression and His-tag pull down assay	60)
CYP3A4	COS-1 lysates	UGT1A9	COS-1 lysates	Co-expression and His-tag pull down assay	47)

Co-IP, co-immunoprecipitation; HLM, human liver microsomes; MS, mass spectrometry; RLM, rat liver microsomes.

ates less morphine-6-glucuronide (M-6-G) than morphine-3-glucuronide (M-3-G). They then constructed UGT2B7-stably expressing COS-1 cells, and the microsomes were utilized as a UGT source. Purified CYP3A4 (expressed in *Escherichia coli*) was mixed with solubilized UGT2B7 microsomes, and the generation of the M-3-G form decreased significantly. In contrast, M-6-G formation was enhanced in a CYP3A4-dependent manner. Such alterations were not observed in the absence of a detergent. These results suggest that the P450–UGT interaction not only elevates UGT catalytic ability but also alters its regioselectivity.⁴¹⁾ Similarly, we reported that UGT oligomerization also alters the regioselectivity of morphine glucuronidation in guinea pig and mouse UGT isoforms.^{33,50,51)} Ramsden *et al.* reported the same result: when CYP3A4 was knocked down in hepatocyte-derived cells, UGT2B7-catalyzed azidothymidine glucuronidation was enhanced, which supports CYP3A4-mediated suppression of UGT2B7 at the cellular level.¹⁹⁾ Takeda *et al.* also suggested that the elevation of UGT activity depends on P450 isoforms. In addition to CYP3A4, CYP1A2, and CYP2C9, each of them was expressed in *E. coli* and purified, were added to solubilized UGT2B7 microsomes, and their effects on M-3-G formation were compared.⁵²⁾ CYP1A2 and CYP2C9 (especially CYP1A2) markedly reduced UGT2B7-catalyzed M-3-G formation compared to CYP3A4. Furthermore, Ishii *et al.* suggested that the effects of CYP3A4 are different among UGT isoforms and variants.⁴⁵⁾ They constructed a co-expression system of CYP3A4 and UGT1As with a baculovirus-insect cell system and compared UGT1A activity in the absence and presence of CYP3A4. Several allelic variants of UGT1A1 have been reported, and UGT1A1*6 is one such variant. UGT1A1*6 has a single mutation, G71R, and the point mutation results in low catalytic activity.⁵³⁾ Although co-expression of CYP3A4 had little effect on UGT1A6-mediated serotonin glucuronidation, UGT1A1*1 (wild-type (WT)), and UGT1A1*6-catalyzed glucuronidation was significantly activated by CYP3A4. Several polymorphisms in UGT1A7 have also been reported.⁵⁴⁾ In

this study, the effects of CYP3A4 on three UGT1A7 variants, UGT1A7*1 (WT), 1A7*2, and 1A7*3, were measured. While co-expression of CYP3A4 enhanced UGT1A7*1 and 1A7*2 activities, UGT1A7*3 was significantly suppressed by P450. The difference between UGT1A7*2 and 1A7*3 is a single residue, W208R. Although the role of the residue remains unclear, it may function as a switch in CYP3A4 effects. This study raised the possibility that genetic factors affect UGT activity via the functional P450–UGT association.

Most UGTs contain several *N*-glycosylation sites, namely asparagine-X-serine/threonine in their sequences, and the post-translational modification affects their glucuronidation ability.^{55–58)} However, rat UGT2B3 does not have any potential *N*-glycosylation sites.⁵⁹⁾ To examine the role of the glycosylation chain of UGT in association with P450, Nakamura *et al.* selected rat CYP3A1 and UGT2B3 as targets, and first generated a UGT2B3(S316N) mutant in which a potential glycosylation site mimicking UGT2B2 was introduced.⁶⁰⁾ UGT2B3 WT and the S316N mutant were expressed in insect cells, and the prepared microsomes were used as sources of enzymes. Glycosylation of the mutant was confirmed by immunoblotting and EndoH treatment. The result of the His-tag pull-down assay indicated that both UGT2B3 WT and the S316N mutant could form a complex with co-expressed CYP3A1, but the effect of CYP3A1 on their glucuronidation activity was the opposite; co-expression of CYP3A1 suppressed UGT2B3 WT-catalyzed glucuronidation of 4-methylumbelliferone but enhanced it in the S316N mutant. This finding suggests that *N*-glycosylation of UGT functions as a regulator of functional association with CYP3A. Deglycosylation of UGT2B3(S316N) with EndoH when co-expressed with CYP3A1 enhances glucuronidation activity. Therefore, once the P450–UGT complex was formed, the removal of the carbohydrate chain of UGT did not affect UGT function. Although, it has been suggested that glycosylation of UGT plays an important role in the P450–UGT association, further studies on other UGT isoforms possessing original *N*-glycosylation sites are necessary.

Taura *et al.* analyzed the functional P450-UGT association from a different point of view. As described above, some metabolites formed by P450 are further glucuronidated by UGT. Hence, P450 can function as a substrate-donor/transporter for UGT, and Taura *et al.* suggested the presence of intermediate transfer from P450 to UGT.⁶¹⁾ α -Naphthoflavone is an inhibitor of CYP1A1 but an allosteric activator of CYP3A4.⁶²⁾ They measured 3-hydroxy-benzo(a)pyrene [3-OH-B(a)P] glucuronidation with 3-methylcholanthrene-treated rat liver microsomes with and without permeability. Interestingly, α -naphthoflavone inhibited [3-OH-B(a)P] glucuronidation in intact microsomes, but this inhibition disappeared when the microsomes were permeabilized. If α -naphthoflavone directly binds to and inhibits rat UGT(s), this inhibition should have been marked in the permeabilized one. Thus, there are three modes of action for α -naphthoflavone: 1) α -naphthoflavone binds to CYP1As and inhibits the transfer of [3-OH-B(a)P] to UGTs; 2) α -naphthoflavone affects and dissociates the CYP1A-UGT complex, which attenuates the substrate transfer; and 3) the combination of possibilities 1 and 2. Although its molecular effect remains unclear, α -naphthoflavone may be a key tool for further analysis of the P450-UGT interaction.

2.3. Effect of P450-UGT Interaction on P450 Capability Next, our team started research on the reverse effect of the P450-UGT interaction: the effect of UGT on P450 ability. Research on a pair of CYP3A4 and UGT2B7 was the most advanced; therefore, we first examined the effect of UGT2B7 on CYP3A4.⁴⁶⁾ We prepared co-expression microsomes with a baculovirus-insect cell system and compared CYP3A4 activity in the absence and presence of UGT2B7 co-expression. In the case of P450, expressed active protein levels can be quantified by monitoring the difference between the spectra of the reduced form and CO-binding form.⁶³⁾ This method is

easier than measuring UGT levels, a method dependent on immunoblotting. However, there was also a difficult point: P450 needs co-expression of CPR for its activity in insect cells, and it was difficult to obtain microsomes with comparable P450/CPR ratios. Transfection was repeated to avoid misestimating the effect of UGT2B7 on CYP3A4 activity. Replicated analyses indicated that co-expression of UGT2B7 suppressed CYP3A4 activity without affecting CPR, and this suppression was observed in the range of P450/CPR ratio 10 to 0.1. This is the first evidence that the P450-UGT association affects P450 ability, and taken together with its effects on glucuronidation, we have demonstrated that P450 and UGT regulate each other's activity through the association. In this study, we also quantified UGT2B7 by immunoblotting, and such a suppression was not correlated with UGT expression, implying that not all UGT2B7 forms a complex with CYP3A4 but exists as a monomer or homo-oligomer, as described previously. Next, we aimed to determine the catalytic step of P450, which is a target of UGT2B7-mediated suppression. As shown in Fig. 2, the catalytic cycle of P450 was well characterized. In brief, substrate binding is the rate-limiting step of the cycle; thereafter, P450 receives two electrons to activate oxygen molecules, oxidizes the substrate with the activated oxygen atom, and then releases the metabolite. Electron leak pathways, including auto-oxidation and uncoupling, may result in low efficiency of P450-catalyzed oxidation and trigger generation of reactive oxygen species (ROS).^{64–66)} In addition to substrate oxidation, we assessed the effect of UGT2B7 on NADPH consumption in the presence and absence of substrate, generation of H_2O_2 (uncoupling), and substrate oxidation driven by organic hydroperoxide.⁴⁶⁾ Similar to substrate oxidation, UGT2B7-dependent suppression was significantly observed in all steps except for NADPH consumption in the absence of substrate, which sug-

Fig. 2. UGT2B7-Mediated Suppressive Effects on the Catalytic Cycle of P450

The catalytic cycle of P450, focusing on the redox status of the heme,⁶⁴⁾ is shown with slight modifications. Red circles represent the steps in which the suppressive effect was observed by co-expression of UGT2B7.⁴⁶⁾ See text for details. RH, substrate of P450; ROH, P450 metabolite. (Color figure can be accessed in the online version.)

gests that UGT2B7 suppresses the whole catalytic cycle of CYP3A4, and if so, UGT2B7 should inhibit substrate binding to P450. To further investigate this possibility, we compared substrate-binding difference spectra in the absence and presence of UGT2B7 and confirmed that co-expression of UGT2B7 tended to attenuate testosterone-induced changes in CYP3A4 spectra.⁴⁶⁾ P450 is involved in detoxification by oxidizing a large number of chemicals and elicits oxidative stress in the liver by generating ROS. CYP3A4 is a major isoform in the human liver and is known to be a major producer of ROS, such as CYP2E1.^{67–69)} Hence, UGT may suppress the whole catalytic cycle to protect the body from adverse oxidative stress.

Recently, we also reported that UGT1A9, another major isoform in the human liver, has a suppressive effect on CYP3A4 similar to UGT2B7.⁴⁷⁾ The UGT isoforms belonging to the 1A subfamily have multiple unique exons 1s, however, due to common exons 2–5, they share an identical C-terminal domain.⁷⁰⁾ Given the homology among UGT1A isoforms, the result raised the possibility that UGT1A9 as well as other UGT1As function as suppressors of CYP3A4. To further estimate the CYP3A–UGT1A association, we analyzed the interaction of rat hepatic CYP3A–UGT1A with dexamethasone, a potent inducer of CYP3A1, CYP3A2, UGT1A1, and UGT1A6 in rat liver.^{71–73)} First, we quantified hepatic protein levels of CYP3A and UGT1A by immunoblotting with rat liver microsomes as enzyme sources. Dexamethasone treat-

ment induced expressions of hepatic CYP3A and UGT1A by 8.7- and 4.6-fold, respectively, while the drug showed little effect on CPR (2.7-fold). From these results, we predicted that dexamethasone treatment partially attenuates hepatic CYP3A–UGT1A interaction by inducing their expressions in different ratios; a portion of the induced CYP3A should be UGT1A-free and non-suppressed, which could show higher activity than that in control rats. In accordance with this prediction, CYP3A turnover was significantly increased by dexamethasone treatment⁴⁷⁾ (Fig. 3). This is the first evidence, albeit indirect, implying the CYP3A–UGT1A functional interaction and suggests that some drugs known as inducers of P450 and UGT increase enzymatic activity of both P450 and UGT not only by simple induction but also by affecting the P450–UGT complex (Fig. 3).

2.4. Postulated P450 and UGT Domains Involved in Forming Functional Complex As described, we concluded that P450 and UGT form functional complexes that can affect their enzymatic capabilities. However, the interaction sites in the P450–UGT complex remain unknown. As a candidate CYP3A4 interaction site, Takeda *et al.* suggested its J-helix by cross-linking and Western blotting using anti-CYP3A4 antibodies with different specificities.⁴²⁾ They first cross-linked purified CYP3A4 and solubilized UGT2B7 expressed in insect cells. The CYP3A4–UGT2B7 complex was precipitated with two different antibodies toward CYP3A4, which had different epitopes, and anti-CYP3A4#1 recognized the Met145–His267

Fig. 3. Proposed Model: Dexamethasone-Mediated Attenuation of CYP3A–UGT1A Association in Rat Liver

UGT1A suppresses CYP3A in rat liver under constitutive conditions. Dexamethasone treatment induced hepatic CYP3A and UGT1A in different ratios, and the results raised the possibility that the levels of UGT-free and non-suppressed forms of CYP3A were increased. The turnover of CYP3A was actually enhanced by the treatment, supporting this possibility.⁴⁷⁾ See text for details. Dex, dexamethasone treatment; RH, substrate of CYP3A; R-OH, metabolite formed by P450.⁴⁷⁾ (Color figure can be accessed in the online version.)

region of CYP3A4, while the epitope of anti-CYP3A4#2 was mapped to Leu331-Lys342 using a peptide library. Of course, both anti-CYP3A4 antibodies could detect CYP3A4 itself in immunoblotting, but they showed quite different affinities toward the CYP3A4–UGT2B7 complex in co-IP with HLM as the enzyme source. Only anti-CYP3A4#1 could recognize and precipitate the CYP3A4–UGT2B7 complex, whereas anti-CYP3A4#2 failed. Furthermore, the result of cross-linking was consistent with this result: anti-CYP3A4#2 can detect free CYP3A4 but not CYP3A4 cross-linked to UGT2B7. This result raised the possibility that the Leu331-Lys342 region functions as an interaction site for CYP3A4 and is masked by conjugated UGT2B7 in their complex. The region Leu331-Lys342 is positioned in the *J*-helix of CYP3A4, which is predicted to be a short helix located in the cytosol.^{74,75)}

The interaction sites of UGT were also predicted by generating mutant lines and estimating their suppressive effects on CYP3A4. In a recent study, we generated truncated UGT1A9, lacking transmembrane and cytoplasmic domains (UGT1A9 Δ TM), and co-expressed the mutant with CYP3A4 and CPR by a baculovirus-insect cell system. Co-expression of UGT1A9 Δ TM significantly suppressed CYP3A4 activity similar to that of WT, and the result of the His-tag pull-down assay indicated that the truncated mutant retained the ability to form complexes with CYP3A4. In the case of UGT2B7, this Δ TM mutant also suppressed CYP3A4 activity. Taken together, the main interaction site(s) of UGT should be located in the luminal body.⁴⁷⁾ Given that P450 and UGT have quite different membrane topologies, it is reasonable to predict that their main interaction sites are hydrophobic domains close to the ER membrane, and P450 and UGT form complexes through the ER membrane. Several reports have shown that P450 binds to the ER membrane not only at the N-terminal anchor region but also through the FG loop.^{37,76,77)} Similarly, it has been suggested that UGT has an internal membrane-binding site in addition to its C-terminal transmembrane domain.^{78,79)} To support this prediction, we generated chimeric UGT2B7 in which one of the predicted internal membrane-binding sites, His183-Glu200, was replaced with that of CNX (Ala402-Cys422) and confirmed that the chimera lacked a suppressive effect on CYP3A4.⁴⁶⁾ In addition, the carboxyl-terminal region of UGT may contribute to the isoform-dependent suppression of CYP3A4.^{46,47)} Furthermore, deletion of the carboxyl-terminal of UGT2B7 or 1A9 significantly increased the K_m of

CYP3A4 in the co-expression system.⁴⁷⁾ The interaction at the site finely tunes the substrate recognition of CYP3A4. Since the cytoplasmic *J*-helix is predicted as an interaction site for CYP3A4, it is reasonable to assume that the UGT C-terminal region, which is the only domain located in the cytosol, somehow binds to the *J*-helix.

The predicted mechanism underlying the UGT-mediated suppression of CYP3A4 is shown in Fig. 4. It has been reported that the insertion of P450 into the lipid membrane can alter its activity, and this effect is especially significant in CYP3A4.^{80,81)} Recent computer simulations have also predicted that the influx of substrate into P450 can be altered by its insertion into the lipid membrane.^{82,83)} Taken together, CYP3A4 is drawn in the ER membrane by interaction with UGTs, and this alteration of CYP3A4 position in the membrane could elicit inhibition of substrate binding followed by suppression of its catalytic activity (Fig. 4). The cytoplasmic tail of UGT forms a sub-interaction point with the *J*-helix of CYP3A4, which may affect CYP3A4 suppression in a UGT isoform-dependent manner.

3. THE ADVANTAGE OF USING A CO-EXPRESSION SYSTEM IN ANALYSIS OF THE P450–UGT INTERACTION

We examined the effect of UGT2B7 on CYP3A4 activity between the two different systems, in which CYP3A4 and UGT2B7 were co-expressed with a baculovirus-insect cell system. On the other hand, we purchased commercially available Supersomes^{TM} (Corning Life Sciences, Tewksbury, MA, U.S.A.), which expressed CYP3A4 and UGT2B7 separately and mixed them when P450 activity was measured.⁸⁴⁾ UGT2B7 significantly suppressed CYP3A4 activity in a co-expression system as described^{46,47)} but significantly increased CYP3A4 activity in a UGT2B7 microsome-dependent manner in the later system. This enhancement of CYP3A4 activity was eliminated when the mixed microsomes were pre-treated with a non-ionic detergent, *n*-octyl- β -D-glucopyranoside. CYP3A4 and UGT2B7 were present in the same membrane in a co-expression system but not in the mixed system, indicating that the former system more accurately reflects physiological conditions. In the purchased microsomes, high amounts of CYP3A4 and UGT2B7 were expressed compared to HLM. We speculated that such high amounts of CYP3A4 and UGT2B7

Fig. 4. Postulated Mechanism of UGT-Mediated Suppression of P450

P450 is drawn in the ER membrane by association with UGT, and this positional change inhibits substrate (RH) inflow into P450, which results in suppression of the total catalytic cycle of P450. It is predicted that P450 and UGT mainly interact with each other in the ER membrane, and internal membrane-associated regions work as interaction sites. Cytoplasmic domains, *J*-helix of CYP3A4, and cytoplasmic tail of UGT form a sub-interaction site, although the role remains to be elucidated.^{46,47,84)} (Color figure can be accessed in the online version.)

interact with each other in each cytoplasmic domain, J-helix, and cytosolic tail, and this non-physiological interaction triggered substrate concentration and enhanced CYP3A4 activity. The results suggest that a system closer to physiological conditions should be selected for analyzing functional P450-UGT associations to minimize the risk of misestimating the effect.

4. EVIDENCE FOR FUNCTIONAL P450-UGT ASSOCIATION *IN VIVO*

A number of *in vitro* studies have indicated that the functional interaction between P450 and UGT can alter their enzymatic capabilities, including enhancement or suppression of activity, helping UGT to acquire novel substrate specificity, and inhibition of substrate binding to P450. An important question is whether such a P450-UGT association is observed *in vivo*. Our recent study that analyzed the effect of dexamethasone on rat hepatic CYP3A-UGT1A interaction best answers this question.⁴⁷⁾ In addition, there are large inter-individual differences in P450 and UGT hepatic expression levels and *in vivo* catalytic activities, and some of them cannot be explained by either of the established mechanisms, including genetic factors (SNPs) and transcriptional factors (activation of nuclear receptor). These unclarified inter-individual differences are *in vivo* evidence to indicate the presence of post-translational factors, and P450-UGT interaction could work as a factor similar to P450 and UGT oligomerizations. For another case suggesting the *in vivo* P450-UGT association, Wandel *et al.* analyzed several batches of HLM and examined the relationship between CYP3A4 level and activity with midazolam as a substrate. They reported that this relationship was weak.⁸⁵⁾ Furthermore, UGT1A1 has several allelic variants (UGT1A1*6 and *28 are the major variants), and patients with mutant alleles show lower glucuronidation activity toward the substrate, such as bilirubin and SN-38.^{86–88)} Sai *et al.* demonstrated the relationship between genotype and glucuronidation activity in Japanese patients, but in their study, some patients with the mutated genotype showed comparable activity toward bilirubin and SN-38 to those observed in people with the WT genotype.^{89,90)} The presence of P450-UGT association was also suggested in clinical data. During treatment of Crigler–Najjar syndrome type II, patients are administered phenobarbital or hypericum extract (St. John's Wort), which are potent inducers of hepatic UGT1A1.^{91,92)} Nuclear receptors such as pregnane X receptor, and constitutive active/androstane receptor, play important roles in the induction of UGT1A1 and other drug-metabolizing enzymes such as CYP3A4.^{9,88)} However, as observed in our previous study, the induction ratio varied depending on the target enzyme.^{47,93,94)} Ishii *et al.* reported that CYP3A4 activated UGT1A1-mediated glucuronidation through a functional association,⁴⁵⁾ implying that phenobarbital and hypericum extract alter UGT1A1 activity *via* newly synthesizing CYP3A4, a postulated activator of the UGT, besides inducing its hepatic expression level. To date, the enhancement of glucuronidation activity resulting from the CYP3A4-UGT1A1 association is indistinguishable from that of UGT induction. However, the P450-UGT association may be one of the hidden factors of drug–drug interactions along with the established inductions by nuclear receptors. There are diverse evidences that support the functional P450-UGT association under physiological conditions, though the number

is limited to date.

5. FUTURE PERSPECTIVES

It is generally believed that Oxidation and glucuronidation operate independently during drug-metabolizing reactions. However, we performed a series of studies to provide evidence of a new paradigm. Our *in vitro* and *in vivo* data indicated that P450 and UGT form functional complexes and regulate each other. We hope that readers will reconsider their understanding of the P450–UGT interaction. To further investigate the P450-UGT association, we have to use both simple *in vitro* system and complex physiological system. The result demonstrating P450–UGT interaction was obtained with a co-expression system containing many factors because the method for purification of recombinant UGT with its full catalytic activity has not yet been established, although partial successful purification of UGT1A9 was reported.^{95,96)} As Finel and Kurkela pointed out in their review for analyzing UGT oligomerization,⁹⁷⁾ it is necessary to establish the methods and confirm the P450–UGT interaction using a purified and fully active recombinant UGT in a reconstituted system, which is one of the simplest systems utilized for P450 oligomerization research. Furthermore, the status of P450 and UGT remains unclear under physiological conditions. Does each exist as monomers, oligomers, or P450–UGT complexes? Whether their ratio can be altered by other factors, such as drug treatment, is also an important question. To answer these questions, we have to estimate the status of P450 and UGT in physiological samples such as HLM and hepatocytes. Biophysical technologies have been updated, and an increasing number of technologies are being applied to the estimation of protein–protein interactions among drug-metabolizing enzymes.^{15,98)} The combination of classic and advanced techniques has helped us to investigate the P450-UGT association. In conclusion, the P450–UGT interaction can improve our understanding of their large inter-individual differences, which can lead to safer and more personalized care.

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Conflict of Interest The authors declare no conflict of interest.

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