

Fig. 3. Human CYP3A4 protein and activity levels in the mouse liver following Ad vector administration

Human CYP3A4 protein levels in the mouse liver following Ad vector administration were evaluated by Western blot (A) and immunohistochemical analysis (B). Human CYP3A4 activity was determined by the rate of formation of 6β-hydroxydexamethasone (6βOH) (C) and 1'-hydroxymidazolam (D) in mouse liver microsomes. Ad-E4-122aT-hCYP3A4 was intravenously administered to mice at a dose of 3 × 10¹⁰, 1 × 10¹¹, and 2 × 10¹¹ VPs/mouse *via* the tail vein. The livers microsomes were prepared 2 days after Ad vector administration. White bars indicate 200 μm. Data represent the mean ± S.D. (*n* = 3). **p* < 0.05, and ***p* < 0.01, compared with the liver microsomes of mice treated with Ad-E4-122aT-GFP. Human liver microsomes (HLMs) were used as a positive control.

122aT-hCYP3A4. Human CYP3A4 protein levels in the livers of mice treated with 2 × 10¹¹ VPs of Ad-E4-122aT-hCYP3A4 were comparable to those in the human liver microsomes. Human CYP3A4 expression levels were also evaluated by immunohistochemical staining of mouse liver sections following Ad vector administration. There were human CYP3A4-positive cells throughout the liver sections of mice treated with 2 × 10¹¹ VPs of Ad-E4-122aT-hCYP3A4 (Fig. 3B). These results indicate that 2 × 10¹¹ VPs of Ad-E4-122aT-hCYP3A4 conferred to the mouse liver the ability to express human CYP3A4 protein levels comparable to those in human liver microsomes.

Next, to examine whether the human CYP3A4 protein expressed in the livers of Ad-E4-122aT-CYP3A4-administered mice was functional, mouse liver microsomes were prepared, and human CYP3A4 activities in the liver microsomes were evaluated 2 days after Ad vector administration. The concentration of 6β-hydroxydexamethasone (6βOH) was measured for the determination of human CYP3A4 activity in the liver microsomes. Metabolism of dexamethasone to 6βOH is catalyzed mainly by human CYP3A4.²⁸⁾ The rate of 6βOH formation by human liver microsomes is much higher than that in mouse liver microsomes.²⁹⁾ As expected, only a small amount of 6βOH was formed in the liver microsomes of PBS- and Ad-E4-122aT-GFP-administered mice (Fig. 3C). There was no increase in the formation of 6βOH in the livers of mice treated with 3 × 10¹⁰ VPs of Ad-E4-122aT-CYP3A4 despite the significant expression of human CYP3A4 in the liver microsomes. However, the formation of 6βOH was approximately 4- and 7-fold increased in the liver microsomes of mice treated with Ad-E4-122aT-CYP3A4 at doses of 1 × 10¹¹ and 2 × 10¹¹ VPs/mouse, respectively. In particular, the levels of 6βOH formed in the liver microsomes of mice administered Ad-E4-122aT-CYP3A4 at a dose of 2 × 10¹¹ VPs/mouse were similar to those formed in humans. We also evaluated human CYP3A4 activity by measuring the metabolism of midazolam in the liver microsomes of mice treated with Ad-E4-122aT-CYP3A4. Midazolam is commonly used as a probe for CYP3A activity in humans, and is metabolized by CYP3A to its primary metabolite, 1'-hydroxymidazolam.³⁰⁾ The formation of 1'-hydroxymidazolam was slightly but significantly increased (1.3-fold) in mice treated with Ad-E4-122aT-CYP3A4 at a dose of 2 × 10¹¹ VP/mouse, but not in mice treated with Ad-E4-122aT-CYP3A4 at doses of 1 × 10¹¹ or 3 × 10¹⁰ VPs/mouse, compared with the mice receiving Ad-E4-122aT-GFP (Fig. 3D).

Next, in order to examine the duration of human CYP3A4 activities in the mouse liver following Ad vector administration, human CYP3A4 activities in the mouse liver were assessed at 2, 4, 6, 8, and 14 days after administration of 2 × 10¹¹ VPs of Ad-E4-122aT-hCYP3A4. The highest human CYP3A4 activity was observed at 4 days after administration; the formation rate of 6βOH in the liver microsomes on day 4 was slightly but significantly higher than that in human liver microsomes (Fig. 4). Human CYP3A4 activities in the mouse liver were maintained for at least 8 days after Ad-E4-122aT-hCYP3A4 administration. On day 14, although the formation rate of 6βOH was reduced by 50% compared with that on day 4, the level of human CYP3A4 activity in the mouse liver microsomes was still 63% of that in the human liver microsomes. These results indicate that human CYP3A4 activity comparable to that in human liver microsomes is maintained in the mouse liver for at least 8 days following intravenous administration of Ad-E4-122aT-hCYP3A4.

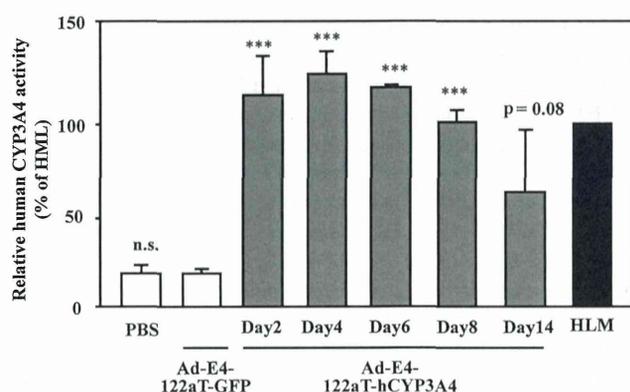


Fig. 4. Duration of human CYP3A4 activities in the mouse liver following Ad vector administration

Human CYP3A4 activities in the mouse liver following Ad vector administration were assessed by measuring the formation rate of 6β OH in mouse liver microsomes. Ad-E4-122aT-hCYP3A4 was intravenously administered to mice at a dose of 2×10^{11} VPs/mouse *via* the tail vein. The liver microsomes were prepared on the indicated days after Ad vector administration. Values are presented as the mean \pm S.D. ($n = 3$). *** $p < 0.001$, compared with mice treated with Ad-E4-122aT-GFP. Human liver microsomes (HLMs) were used as a positive control.

***In vivo* pharmacokinetics profiles of human CYP3A4 substrates in mice over-expressing human CYP3A4:** Next, in order to examine whether Ad vector-mediated over-expression of human CYP3A4 in the mouse liver alters the *in vivo* pharmacokinetics of human CYP3A4 substrates, dexamethasone and midazolam were orally administered to mice treated with Ad-E4-122aT-CYP3A4 at a dose of 2 mg/kg. Subsequently, the blood concentrations of dexamethasone, midazolam, and their metabolites were measured. The AUC of 6β OH in mice treated with Ad-E4-122aT-GFP was decreased by 72%, compared with that for the mice receiving PBS (Table 1), probably due to the decline in endogenous metabolic activity in the liver by transduction with a high titer of the Ad vector in the liver, although there was no apparent difference in the formation rate of 6β OH between the liver microsomes of mice receiving PBS and those receiving Ad-E4-122aT-GFP, as shown in Figure 3C. The AUC of 6β OH was 1.4-fold and 2.7-fold elevated in mice treated with Ad-E4-122aT-hCYP3A4 at a dose of 3×10^{10} VPs/mouse and 2×10^{11} VPs/mouse, respectively, compared with the mice receiving Ad-E4-122aT-GFP (Fig. 5B and Table 1). The $T_{1/2}$ of dexamethasone and the C_{max} of 6β OH in mice receiving Ad-E4-122aT-hCYP3A4 were largely lower and higher than those in mice receiving Ad-E4-122aT-GFP, respectively (Table 1). These results indicate that Ad vector-mediated over-expression of human CYP3A4 in the mouse liver alters the *in vivo* pharmacokinetics of human CYP3A4-specific metabolites in mice. On the other hand, the AUCs for midazolam and the primary metabolite of midazolam, 1'-hydroxymidazolam, were not significantly different between the mice treated with Ad-E4-122aT-hCYP3A4 and Ad-E4-122aT-GFP (Figs. 5C, 5D and Table 2).

Discussion

In this study, we used an Ad vector to generate a human CYP3A4-expressing animal that could be used for the evaluation of drug metabolism in humans. For this purpose, a human CYP3A4-expressing Ad vector (Ad-E4-122aT-hCYP3A4) was generated

Table 1. Pharmacokinetic parameters estimated for dexamethasone in Ad vector-administered mice

Dexamethasone	$T_{1/2}$ (h)	T_{max} (h)	C_{max} (ng/mL)	AUC _{last} (ng h/mL)	AUC _{inf,Obs} (ng h/mL)
PBS	6.8	0.17	783	2,709	5,087
Ad-E4-122aT-GFP (2×10^{11} VP/mouse)	5.4	1.0	799	2,443	3,903
Ad-E4-122aT-hCYP3A4 (3×10^{10} VP/mouse)	4.5	0.17	779	2,327	3,461
Ad-E4-122aT-hCYP3A4 (1×10^{11} VP/mouse)	3.0	0.17	987	1,853	2,276
Ad-E4-122aT-hCYP3A4 (2×10^{11} VP/mouse)	3.5	0.17	855	1,624	2,092
6β OH-dexamethasone	$T_{1/2}$ (h)	T_{max} (h)	C_{max} (ng/mL)	AUC _{last} (ng h/mL)	AUC _{inf,Obs} (ng h/mL)
PBS	3.9	1.0	9.00	37.3	54.2
Ad-E4-122aT-GFP (2×10^{11} VP/mouse)	3.8	1.0	8.00	28.1	39.1
Ad-E4-122aT-hCYP3A4 (3×10^{10} VP/mouse)	4.1	1.0	8.00	37.4	55.0
Ad-E4-122aT-hCYP3A4 (1×10^{11} VP/mouse)	4.4	0.17	11.0	40.5	59.6
Ad-E4-122aT-hCYP3A4 (2×10^{11} VP/mouse)	4.4	0.17	28.0	74.3	106

using a novel Ad vector that exhibits a reduction in Ad vector-mediated hepatotoxicity. A single administration of Ad-E4-122aT-hCYP3A4 to mice at dose of 2×10^{11} VPs/mouse achieved levels of human CYP3A4 activity in the liver microsomes of the Ad-CYP3A4-administered mice that were similar to those in human liver microsomes. In addition, pharmacokinetic experiments revealed that the AUC of 6β OH was 2.7-fold elevated in the Ad-E4-122aT-hCYP3A4-administered mice, compared with the mice receiving a control Ad vector. To the best of our knowledge, this is the first report demonstrating the production of mice possessing hepatic activity of human CYP3A4 at levels similar to those in the human liver by the intravenous administration of an Ad vector expressing human CYP3A4, although several studies have reported that various types of human CYP activities are up-regulated in cultured cell lines, including HepG2 cells, by transduction with Ad vectors expressing human CYP enzymes.^{31,32)}

An Ad vector was used for over-expression of human CYP3A4 in the mouse liver in this study. An Ad vector was considered suitable for this purpose because Ad vectors are known to have strong hepatotropism. Several groups, including ours, have demonstrated that more than 90% of the injected dose of an Ad vector accumulates in the liver within several minutes following intravenous administration into mice.^{16,33,34)} Although transgenic animals expressing human CYP enzymes have been developed,¹⁰⁻¹²⁾ there remain several concerns with the resulting strains; the integration site and integrated copy numbers of the transgene cannot be adequately controlled, leading to unpredictable expression in transgenic mice. On the other hand, Ad vector-mediated over-expression of human CYP enzymes in the livers of experimental animals has several advantages for evaluating CYP-mediated drug metabolism. First, human CYP enzyme activities in the liver can be easily controlled by altering the injected doses of an Ad vector. It is well known that CYP-mediated drug metabolism exhibits considerable interindividual variation, due in part to genetic polymorphisms. For example, in the case of human CYP2D6, which is known to be highly polymorphic, more than 100

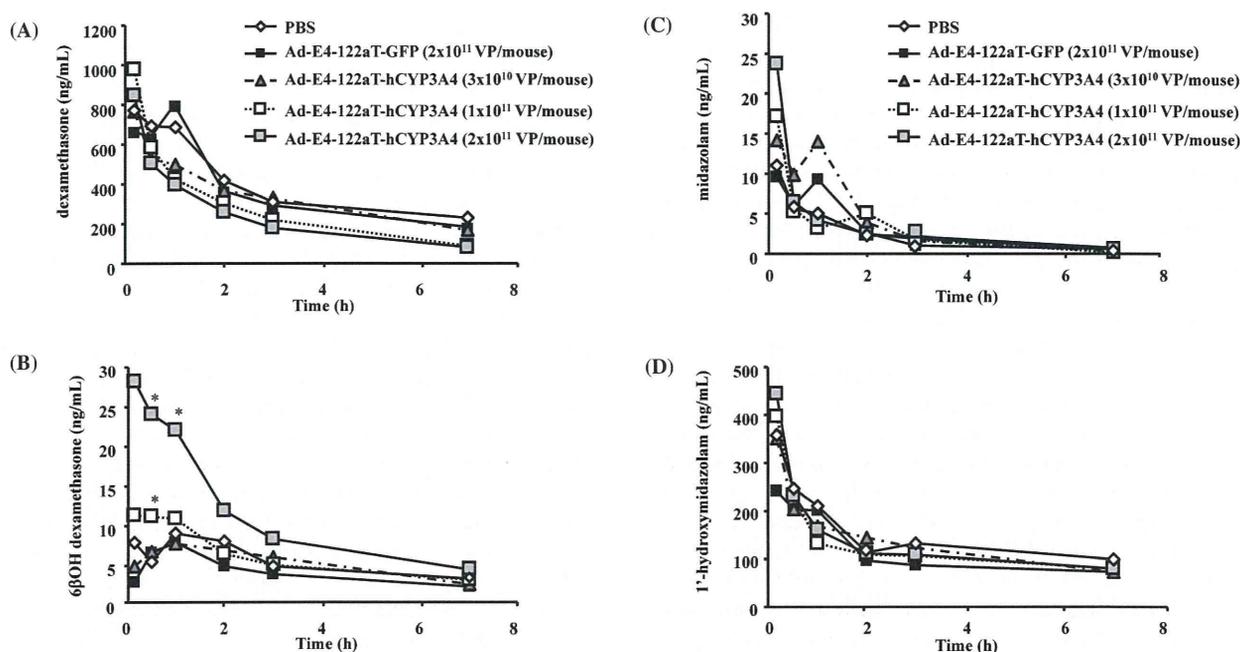


Fig. 5. *In vivo* blood clearance profiles of human CYP3A4 substrates in mice overexpressing human CYP3A4

Mice were transduced with 3×10^{10} , 1×10^{11} , and 2×10^{11} VPs of Ad-E4-122aT-hCYP3A4 or 2×10^{11} VPs of Ad-E4-122aT-GFP. Forty-eight hours after Ad vector administration, mice were orally administered dexamethasone and midazolam (2 mg/10 mL). Blood samples were taken at the indicated time points. Plasma concentration *versus* time curves of (A) dexamethasone, (B) 6 β OHD, (C) midazolam and (D) 1'-hydroxymidazolam are shown. Data represent the mean ($n = 3$). * $p < 0.05$, compared with mice treated with Ad-E4-122aT-GFP.

Table 2. Pharmacokinetic parameters estimated for midazolam in Ad vector-administered mice

Midazolam	$T_{1/2}$ (h)	T_{max} (h)	C_{max} (ng/mL)	AUC_{last} (ng h/mL)	$AUC_{inf,obs}$ (ng h/mL)
PBS	4.7	0.17	11.0	17.4	24.2
Ad-E4-122aT-GFP (2×10^{11} VP/mouse)	2.2	0.17	10.0	18.5	21.6
Ad-E4-122aT-hCYP3A4 (3×10^{10} VP/mouse)	0.71	0.17	14.0	22.0	24.0
Ad-E4-122aT-hCYP3A4 (1×10^{11} VP/mouse)	2.2	0.17	17.0	14.4	20.8
Ad-E4-122aT-hCYP3A4 (2×10^{11} VP/mouse)	3.1	0.17	24.0	21.0	25.4
1'-Hydroxymidazolam	$T_{1/2}$ (h)	T_{max} (h)	C_{max} (ng/mL)	AUC_{last} (ng h/mL)	$AUC_{inf,obs}$ (ng h/mL)
PBS	5.6	0.17	359	977	1,763
Ad-E4-122aT-GFP (2×10^{11} VP/mouse)	12	0.17	243	743	1,962
Ad-E4-122aT-hCYP3A4 (3×10^{10} VP/mouse)	4.9	0.17	351	879	1,386
Ad-E4-122aT-hCYP3A4 (1×10^{11} VP/mouse)	11	0.17	398	816	2,044
Ad-E4-122aT-hCYP3A4 (2×10^{11} VP/mouse)	10	0.17	444	856	1,991

Mice were intravenously administered with Ad-E4-122aT-hCYP3A4. Forty-eight hours after Ad vector administration, mice received midazolam (2 mg/kg) by oral administration. Values represent the mean ($n = 3$).

allelic variants of CYP2D6 have been discovered to date (<http://www.cypalleles.ki.se/>).³⁵ Five to 10% of Caucasians are poor metabolizers, resulting in individual variation in response to many drugs due to impaired metabolism by CYP2D6.³⁶ In human CYP-possessing animal models created by Ad vector administration, certain CYP poor and extensive metabolizer phenotypes are easily

prepared by altering the Ad vector doses. Second, several types of human CYP enzymes can be simultaneously over-expressed by using an Ad vector possessing multiple human CYP genes. The combination of human CYP enzymes over-expressed in the liver can be modified by changing the human CYP genes in the Ad vector genome. Furthermore, Ad vectors are able to efficiently transduce the livers of various types of animals which are available for drug development studies, including mice, rats, dogs, and rabbits.³⁷⁻³⁹ Due to these superior properties of Ad vectors as gene delivery vehicles, we concluded that an Ad vector would be suitable for the generation of experimental animals expressing human CYP genes in the liver.

In this study, there was no significant increase in the production of 6 β OHD in the liver microsomes derived from mice treated with Ad-E4-122aT-hCYP3A4 at a dose of 3×10^{10} VPs/mouse, compared with mice treated with a control Ad vector, despite the apparent human CYP3A4 protein in the livers of mice administered Ad-E4-122aT-hCYP3A4 at a dose of 3×10^{10} VPs/mouse in the Western blotting analysis (Figs. 3A and 3C). Although there was no significant elevation of 6 β OHD formation in the liver microsomes of mice receiving 3×10^{10} VPs of Ad-E4-122aT-hCYP3A4, we consider certain levels of CYP3A4 activity to have been present in the livers of mice even at the dose of 3×10^{10} VPs/mouse of Ad-E4-122aT-hCYP3A4; however, the levels of human CYP3A4 activity generated by 3×10^{10} VPs of Ad-E4-122aT-hCYP3A4 would be much lower than the intrinsic 6 β OHD formation activity in the livers of mice treated with 2×10^{11} VPs of Ad-E4-122aT-GFP, which would account for the lack of a statistically significant increase in 6 β OHD formation in the transduced liver microsomes, compared with the liver microsomes of mice treated with a control Ad vector. In this study, we used the

rate of 6 β OHD formation as a marker of human CYP3A4 activity. Although 6 β OHD is a human CYP3A4-specific metabolite of dexamethasone, 6 β OHD is slightly formed in the mouse liver microsomes (accounting for 5.8% of total metabolites formed) due to the endogenous mouse CYP activity in the liver.²⁹⁾ In contrast, significant levels of human CYP3A4 activity were found even at 100 VPs/cell in HepG2 cells in this study. HepG2 cells, which are commonly used to evaluate hepatotoxicity induced by bioactive compounds, showed almost undetectable CYP activities.³²⁾ Thus, an Ad vector-mediated increase in human CYP3A4 enzymatic activity was clearly detected in HepG2 cells.

In this study, human CYP3A4 activity in the mouse liver was also evaluated by measuring the metabolism of midazolam; however, no significant increase in the formation of the primary metabolite of midazolam, 1-hydroxymidazolam, was observed in the mice receiving Ad-E4-122aT-hYCP3A4, compared with the mice treated with a control Ad vector in the *in vivo* pharmacokinetic experiments (Figs. 5C and 5D), although slight but significant elevation of 1'-hydroxymidazolam formation was found in the liver microsomes of mice receiving 2×10^{11} VP of Ad-E4-122aT-hCYP3A4 (Fig. 3D). It remains unclear why midazolam metabolism did not appear to be increased in mice receiving Ad-E4-122aT-hCYP3A4; however, the *in vitro* metabolism experiment of midazolam using liver microsomes demonstrated that similar levels of 1'-hydroxymidazolam were produced in the liver microsomes of humans and PBS-treated mice, indicating that midazolam metabolic activities in the human and mouse liver are comparable. Mouse CYP3A and CYP2C metabolize midazolam to 1'-hydroxymidazolam.⁴⁰⁾ Over-expressed human CYP3A4-mediated metabolism of midazolam might be lower than mouse endogenous CYP-mediated metabolism of midazolam. In a previous study, the human CYP3A4-transgenic mice and wild-type mice exhibited no significant difference in the metabolism of orally administered midazolam.¹⁰⁾ On the other hand, when midazolam was intravenously administered to human CYP3A4-transgenic mice that showed an approximately 2-fold higher human CYP3A4 expression in the liver than wild-type mice, a higher metabolism of midazolam was observed in the livers of human CYP3A4-transgenic mice, compared with wild-type mice.¹¹⁾ Elevation of midazolam metabolism *in vivo* might be achieved by increasing the dose of Ad-E4-122aT-hCYP3A4, since higher doses of Ad-E4-122aT-hCYP3A4 mediate higher human CYP3A4 expression in the liver than is observed in the human liver. The findings described above also suggest that human CYP expression levels in experimental animals have a major effect on drug metabolism, and that it might be difficult to provide appropriate expression levels of human CYPs in transgenic animals. On the other hand, Ad vector-mediated human CYP expression levels in the organs can be easily controlled by increasing or decreasing the dose of an Ad vector. Recently, two studies demonstrated the development of human CYP3A4-humanizing transgenic mice and showed that these mice would be useful for evaluating human CYP3A4-mediated drug metabolism.^{41,42)} The human CYP3A4-expressing mice in our present study possess mouse endogenous CYP activity, which might disrupt the evaluation of human CYP-mediated drug metabolism. Intravenous administration of an Ad vector expressing human CYP genes into mouse CYP gene-knockout mice would generate a better animal model for the evaluation of drug metabolism in humans, compared with wild-type mice receiving a human CYP gene-expressing Ad vector.

A novel Ad vector, in which four copies of sequences complementary to miR-122a, a liver-specific microRNA, are incorporated into the 3'-untranslated region of the E4 gene to suppress the leaky expression of Ad genes in the liver, was used for the over-expression of human CYP3A4 genes. We previously demonstrated that this Ad vector showed a significant reduction in the leaky expression of Ad genes in the liver and Ad vector-mediated hepatotoxicity.²¹⁾ This Ad vector was thus considered suitable for over-expression of human CYP3A4 in the liver. In order to confer to the mouse liver a level of human CYP3A4 activity comparable to that in the human liver microsomes, 2×10^{11} VPs of an Ad vector expressing human CYP3A4 was required; however, intravenous administration of a conventional Ad vector expressing human CYP3A4 at such a high dose induced a significant increase in serum ALT levels 2 days after administration (data not shown). On the other hand, Ad-E4-122aT-hCYP3A4 did not induce any apparent hepatotoxicity. This property would also be advantageous for the evaluation of human CYP3A4-mediated drug metabolism.

In conclusion, we succeeded in the development of mice exhibiting hepatic activity of human CYP3A4 at levels similar to those in the human liver by intravenous administration of a novel Ad vector expressing human CYP3A4. This novel Ad vector expressing human CYP3A4 would be a powerful tool for evaluating human CYP3A4-mediated drug metabolism in the livers of experimental animals.

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