EMT induced by extended functional inhibition of CDH1 was gradual and led to the reprogramming of tumor cells.[42]

TGFβ1 may exhibit tumor suppressive or oncogenic properties. It is a major inducer of EMT, and several authors reported that TGFβ1 treatment led to an EMT in liver cells.[43,44] During the retrodifferentiation, HepaRG-tdHep exhibited a late TGFβ1 signature that was shown to be associated with a poor prognosis in HCC.[17] Moreover, exposure of HepaRG-tdHep to TGFβ1 induces the expression of EMT markers. TGFβ1 also increased IL6 at both the transcription and protein levels. Numerous studies have reported the existence of crosstalk between the TGFβ and IL6 pathways,[45-47] and Yamada et al.[47] have demonstrated that this crosstalk contributes to the gain in malignancy, including chemoresistance and EMT. Using recombinant TGFβ1 and STAT3 inhibitors, we demonstrated that IL6 is responsible for the decrease in hepatic-gene expression, whereas TGFβ1 regulates EMT markers; both signaling pathways associated with NFκB activation resulted in retrodifferentiation of HepaRG-tdHep. Using a large-scale analysis, we observed a strong upregulation of several factors in IL17 signaling pathways (CXCL10, CXCL2, or CCL1) associated with early TNF-NFκB, IL6, and TGFβ induction. Among these factors, CXCL10 produced by hepatocytes and sinusoidal cells is increased in the serum and liver of patients with chronic HCV.[48] IL17 is known to coordinate local tissue inflammation through the induction of inflammatory cytokines such as IL6, TNFα, and IL1β, which in turn promote Th17 cell expansion favoring tumor progression in cooperation with TGFβ1.[33] These findings suggest that tumor hepatocyte retrodifferentiation is accompanied by a cytokine release amplification loop and therefore a modulation of the local environment that favors tumor immune escape. Interestingly, using a connectivity map approach, we selected an inhibitor of histone deacetylase, TSA, that blocks the dedifferentiation followed by cell-cycle activation of HepaRG-tdHep. This strengthens the role of TSA reported previously to abrogate TGFβ1-induced EMT in hepatocytes or the migration of HepaRG-tdHep in an inflammatory microenvironment.[22]

Evidence is provided that embryonic stem cell-like signatures are present in poorly differentiated cancers.[25] Correlations have also been shown between the properties of tissue stem cells and cells that undergo EMT.[13,49] Consistent with these findings, we have previously shown that HepaRG progenitors expressed several oval cells, hematopoietic stem cells, and lymphoid and myeloid markers.[20] We also demonstrated in this study that the population of retrodifferentiated HepaRG-tdHep expressed stem/progenitor markers. Moreover, we identified in the gene profiles of progenitor cells derived from HepaRG-tdHep a significant enrichment of gene signatures related to stemness[25] and a poor-prognosis HCC subclass corresponding to a TGFβ signature resulting in invasive/disseminative and proliferative progenitor phenotypes.[8,24] Taken together, these observations suggest that retrodifferentiation and proliferation of hepatocytes may cooperate in the development of liver tumors.[20]

Although the HepaRG cell line displays high plasticity, its karyotype is pseudo-diploid and subnormal.[18] Cells are not carrying mutations in genes recurrently altered in HCC tumors such as tumor protein p53 (TP53), β-catenin (CTNNB1), AT rich interactive domain 1A (ARID1A) or ARID2 (Zucman-Rossi and Dubois-Pot-Schneider, pers. commun.). Consistent with its tumor origin, HepaRG cells exhibit a mutation pattern of HCC primarily associated with viral infections such as the NLR family pyrin domain containing 1 (NLRP1) and transformation/transcription domain-associated protein (TRRAP). Moreover, our observations are clinically relevant. First, it has been reported that liver biopsies and sera of HCV-infected patients contain elevated levels of inflammatory cytokines such as IL6, TNFα, CXCL10, and TGFβ2.[35,48] Second, the GSEA approach emphasized the presence of a signature of HCC proliferative and S1 subclasses that corresponds to HCC with reduced frequencies of CTNNB1 mutations.[8,24] Third, the HCC S1 subclass, which contained mostly moderate or poorly differentiated tumors associated with a high risk of early recurrence, was mainly related to EMT associated with an activation of TGFβ and WNT signaling pathways. Therefore, the behavior of HepaRG-tdHep may reflect that of some tumor hepatocytes from HCV-associated HCC. Taken together, our data suggest that cancer progenitor cells (or metastasis progenitors) may derive from tumor hepatocytes in an inflammatory environment frequently associated with HCV-related HCC. Moreover, targeting the epigenetic modulation induced during tumor hepatocyte retrodifferentiation open new therapeutic perspectives.

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References

1. El-Serag HB, Rudolph KL. Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. Gastroenterology 2007;132:2557-2576.
2. Bismuth H, Majno PE. Hepatobiliary surgery. J Hepatol 2000;32:208-224.
3. Holczbauer A, Factor VM, Andersen JB, Marquardt JU, Kleiner DE, Raggi C, et al. Modeling pathogenesis of primary liver cancer in lineage-specific mouse cell types. Gastroenterology 2013;145:221-231.
4. Chiba T, Kita K, Zheng YW, Yokosuka O, Saisho H, Iwama A, et al. Side population purified from hepatocellular carcinoma cells harbors cancer stem cell-like properties. HEPATOLOGY 2006;44:240-251.
5. Ma S, Chan KW, Hu L, Lee TK, Wo JY, Ng IO, et al. Identification and characterization of tumorigenic liver cancer stem/progenitor cells. Gastroenterology 2007;132:2542-2556.
6. Coulouarn C, Derambure C, Lefebvre G, Daveau R, Hiron M, Scotte M, et al. Global gene repression in hepatocellular carcinoma and fetal liver, and suppression of dudulin-2 mRNA as a possible marker for the cirrhosis-to-tumor transition. J Hepatol 2005;42:860-869.
7. Lee JS, Heo J, Libbrecht L, Chu IS, Kaposi-Novak P, Calvisi DF, et al. A novel prognostic subtype of human hepatocellular carcinoma derived from hepatic progenitor cells. Nat Med 2006;12:410-416.
8. Hoshida Y, Nijman SM, Kobayashi M, Chan JA, Brunet JP, Chiang DY, et al. Integrative transcriptome analysis reveals common molecular subclasses of human hepatocellular carcinoma. Cancer Res 2009;69: 7385-7392.
9. Zhang L, Theise N, Chua M, Reid LM. The stem cell niche of human livers: symmetry between development and regeneration. HEPATOLOGY 2008;48:1598-1607.
10. Sell S. Cellular origin of cancer: dedifferentiation or stem cell maturation arrest? Environ Health Perspect 1993;101(Suppl 5):15-26.
11. Fan B, Malato Y, Calvisi DF, Naqvi S, Razumilava N, Ribback S, et al. Cholangiocarcinomas can originate from hepatocytes in mice. J Clin Invest 2012;122:2911-2915.
12. Thiery JP. Epithelial-mesenchymal transitions in tumour progression. Nat Rev Cancer 2002;2:442-454.
13. Polyak K, Weinberg RA. Transitions between epithelial and mesenchymal states: acquisition of malignant and stem cell traits. Nat Rev Cancer 2009;9:265-273.
14. Dean M, Fojo T, Bates S. Tumour stem cells and drug resistance. Nat Rev Cancer 2005;5:275-284.
15. Xu J, Lamouille S, Derynck R. TGF-beta-induced epithelial to mesenchymal transition. Cell Res 2009;19:156-172.
16. Chiappini F. Circulating tumor cells measurements in hepatocellular carcinoma. Int J Hepatol 2012;2012:684802.
17. Coulouarn C, Factor VM, Thorgeirsson SS. Transforming growth factor-beta gene expression signature in mouse hepatocytes predicts clinical outcome in human cancer. HEPATOLOGY 2008;47:2059-2067.
18. Gripon P, Rumin S, Urban S, Le Seyec J, Glaise D, Cannie I, et al. Infection of a human hepatoma cell line by hepatitis B virus. Proc Natl Acad Sci U S A 2002;99:15655-15660.
19. Aninat C, Piton A, Glaise D, Le Charpentier T, Langouet S, Morel F, et al. Expression of cytochromes P450, conjugating enzymes and nuclear receptors in human hepatoma HepaRG cells. Drug Metab Dispos 2006;34:75-83.
20. Cerec V, Glaise D, Garnier D, Morosan S, Turlin B, Drenou B, et al. Transdifferentiation of hepatocyte-like cells from the human hepatoma HepaRG cell line through bipotent progenitor. HEPATOLOGY 2007;45: 957-967.
21. Parent R, Marion MJ, Furio L, Trepo C, Petit MA. Origin and characterization of a human bipotent liver progenitor cell line. Gastroenterology 2004;126:1147-1156.
22. Coulouarn C, Corlu A, Glaise D, Guenon I, Thorgeirsson SS, Clement B. Hepatocyte-stellate cell cross-talk in the liver engenders a permissive inflammatory microenvironment that drives progression in hepatocellular carcinoma. Cancer Res 2012;72:2533-2542.
23. Laurent V, Glaise D, Nubel T, Gilot D, Corlu A, Loyer P. Highly efficient SiRNA and gene transfer into hepatocyte-like HepaRG cells and primary human hepatocytes: new means for drug metabolism and toxicity studies. Methods Mol Biol 2013;987:295-314.
24. Chiang DY, Villanueva A, Hoshida Y, Peix J, Newell P, Minguez B, et al. Focal gains of VEGFA and molecular classification of hepatocellular carcinoma. Cancer Res 2008;68:6779-6788.
25. Ben-Porath I, Thomson MW, Carey VJ, Ge R, Bell GW, Regev A, et al. An embryonic stem cell-like gene expression signature in poorly differentiated aggressive human tumors. Nat Genet 2008;40:499-507.
26. Sana TR, Janatpour MJ, Sathe M, McEvoy LM, McClanahan TK. Microarray analysis of primary endothelial cells challenged with different inflammatory and immune cytokines. Cytokine 200521;29:256-269.
27. Dasu MR, Hawkins HK, Barrow RE, Xue H, Herndon DN. Gene expression profiles from hypertrophic scar fibroblasts before and after IL-6 stimulation. J Pathol 2004;202:476-485.
28. Bruzzese F, Leone A, Rocco M, Carbone C, Piro G, Caraglia M, et al. HDAC inhibitor vorinostat enhances the antitumor effect of gefitinib in squamous cell carcinoma of head and neck by modulating ErbB receptor expression and reverting EMT. J Cell Physiol 2011;226:2378-2390.
29. Lu Y, Cuevas B, Gibson S, Khan H, LaPushin R, Imboden J, et al. Phosphatidylinositol 3-kinase is required for CD28 but not CD3 regulation of the TEC family tyrosine kinase EMT/ITK/TSK: functional and physical interaction of EMT with phosphatidylinositol 3-kinase. J Immunol 1998;161:5404-5412.
30. Zeng R, Yao Y, Han M, Zhao X, Liu XC, Wei J, et al. Biliverdin reductase mediates hypoxia-induced EMT via PI3-kinase and Akt. J Am Soc Nephrol 2008;19:380-387.
31. Kaimori A, Potter JJ, Choti M, Ding Z, Mezey E, Koteish AA. Histone deacetylase inhibition suppresses the transforming growth factor beta1-induced epithelial-to-mesenchymal transition in hepatocytes. HEPATOLOGY 2010;52:1033-1045.
32. Shankar S, Nall D, Tang SN, Meeker D, Passarini J, Sharma J, et al. Resveratrol inhibits pancreatic cancer stem cell characteristics in human and KrasG12D transgenic mice by inhibiting pluripotency maintaining factors and epithelial-mesenchymal transition. PLoS One 2011;6: e16530.
33. Capece D, Fischietti M, Verzella D, Gaggiano A, Cicciarelli G, Tessitore A, et al. The inflammatory microenvironment in hepatocellular carcinoma: a pivotal role for tumor-associated macrophages. Biomed Res Int 2013;2013:187204.
34. Budhu A, Forgues M, Ye QH, Jia HL, He P, Zanetti KA, et al. Prediction of venous metastases, recurrence, and prognosis in hepatocellular carcinoma based on a unique immune response signature of the liver microenvironment. Cancer Cell 2006;10:99-111.
35. Nakagawa H, Maeda S, Yoshida H, Tateishi R, Masuzaki R, Ohki T, et al. Serum IL-6 levels and the risk for hepatocarcinogenesis in chronic hepatitis C patients: an analysis based on gender differences. Int J Cancer 2009;125:2264-2269.
36. Abdel-Razzak Z, Loyer P, Fautrel A, Gautier JC, Corcos L, Turlin B, et al. Cytokines down-regulate expression of major cytochrome P-450 enzymes in adult human hepatocytes in primary culture. Mol Pharmacol 1993;44:707-715.
37. Berasain C, Castillo J, Perugorria MJ, Latasa MU, Prieto J, Avila MA. Inflammation and liver cancer: new molecular links. Ann N Y Acad Sci 2009;1155:206-221.
38. Libermann TA, Baltimore D. Activation of interleukin-6 gene expression through the NF-kappa B transcription factor. Mol Cell Biol 1990; 10:2327-2334.
39. He G, Yu GY, Temkin V, Ogata H, Kuntzen C, Sakurai T, et al. Hepatocyte IKKbeta/NF-kappaB inhibits tumor promotion and progression by preventing oxidative stress-driven STAT3 activation. Cancer Cell 2010;17:286-297.
40. Techasen A, Namwat N, Loilome W, Bungkanjana P, Khuntikeo N, Puapairoj A, et al. Tumor necrosis factor-alpha (TNF-alpha) stimulates

the epithelial-mesenchymal transition regulator Snail in cholangiocarcinoma. Med Oncol 2012;29:3083-3091.
41. Yadav A, Kumar B, Datta J, Teknos TN, Kumar P. IL-6 promotes head and neck tumor metastasis by inducing epithelial-mesenchymal transition via the JAK-STAT3-SNAIL signaling pathway. Mol Cancer Res 2011;9:1658-1667.
42. Andersen H, Mejlvang J, Mahmood S, Gromova I, Gromov P, Lukanidin E, et al. Immediate and delayed effects of E-cadherin inhibition on gene regulation and cell motility in human epidermoid carcinoma cells. Mol Cell Biol 2005;25:9138-9150.
43. Caja L, Bertran E, Campbell J, Fausto N, Fabregat I. The transforming growth factor-beta (TGF-beta) mediates acquisition of a mesenchymal stem cell-like phenotype in human liver cells. J Cell Physiol 2011;226: 1214-1223.
44. Zeisberg M, Yang C, Martino M, Duncan MB, Rieder F, Tanjore H, et al. Fibroblasts derive from hepatocytes in liver fibrosis via epithelial to mesenchymal transition. J Biol Chem 2007;282:23337-23347.
45. Yamamoto T, Matsuda T, Muraguchi A, Miyazono K, Kawabata M. Cross-talk between IL-6 and TGF-beta signaling in hepatoma cells. FEBS Lett 2001;492:247-253.
46. Liton PB, Li G, Luna C, Gonzalez P, Epstein DL. Cross-talk between TGF-beta1 and IL-6 in human trabecular meshwork cells. Mol Vis 2009;15:326-334.
47. Yamada D, Kobayashi S, Wada H, Kawamoto K, Marubashi S, Eguchi H, et al. Role of crosstalk between interleukin-6 and transforming growth factor-beta 1 in epithelial-mesenchymal transition and chemoresistance in biliary tract cancer. Eur J Cancer 2013;49: 1725-1240.
48. Selimovic D, El-Khattouti A, Ghozlanh, Haikel Y, Abdelkader O, Hassan M. Hepatitis C virus-related hepatocellular carcinoma: an insight into molecular mechanisms and therapeutic strategies. World J Hepatol 2012;4:342-355.
49. Chaffer CL, Weinberg RA. Cancer cell of origin: spotlight on luminal progenitors. Cell Stem Cell 2010;7:271-272.

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Short Communication

Validation of uPA/SCID Mouse with Humanized Liver as a Human Liver Model: Protein Quantification of Transporters, Cytochromes P450, and UDP-Glucuronosyltransferases by LC-MS/MS[S]

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ABSTRACT

Chimeric mice with humanized liver (PXB mice) have been generated by transplantation of urokinase-type plasminogen activator/severe combined immunodeficiency mice with human hepatocytes. The purpose of the present study was to clarify the protein expression levels of metabolizing enzymes and transporters in humanized liver of PXB mice transplanted with hepatocytes from three different donors, and to compare their protein expressions with those of human livers to validate this human liver model. The protein expression levels of metabolizing enzymes and transporters were quantified in microsomal fraction and plasma membrane fraction, respectively, by means of liquid chromatography–tandem mass spectrometry. Protein expression levels of 12 human P450 enzymes, two human UDP-glucuronosyltransferases, eight human ATP binding cassette (ABC) transporters, and eight human solute carrier transporters were determined. The variances of protein expression levels among samples from mice humanized with hepatocytes from all donors were significantly greater than those from samples obtained from mice derived from each individual donor. Compared with the protein expression levels in human livers, all of the quantified metabolizing enzymes and transporters were within a range of 4-fold difference, except for CYP2A6, CYP4A11, bile salt export pump (BSEP), and multidrug resistance protein 3 (MDR3), which showed 4- to 5-fold differences between PXB mouse and human livers. The present study indicates that humanized liver of PXB mice is a useful model of human liver from the viewpoint of protein expression of metabolizing enzymes and transporters, but the results are influenced by the characteristics of the human hepatocyte donor.

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Introduction

Species differences in drug metabolism and transport in the liver between humans and experimental animals are a critical issue during drug development. To overcome this problem, chimeric mice with humanized liver (PXB mice; PhoenixBio Co., Ltd., Hiroshima, Japan) have been generated by transplantation of human hepatocytes into albumin enhancer/promoter-driven urokinase-type plasminogen activator/severe combined immunodeficiency ($uPA^{+/+}$/SCID) mice; in these mice approximately 80% of the hepatocytes are human (Tateno et al., 2004). PXB mice generate human-specific metabolites (Inoue et al., 2009; Kamimura et al., 2010; Yamazaki et al., 2010; De Serres et al., 2011), and pregnane X receptor (PXR)–dependent induction of metabolizing enzymes was observed when the mice were treated with a human PXR ligand (Hasegawa et al., 2012). Therefore, the liver of PXB mice is considered to be potentially useful as a model of human liver for studies of drug metabolism.

The uptake of most drugs from circulating blood into the liver at the sinusoidal membrane of hepatocytes involves active transport. The drugs subsequently undergo biotransformation by intracellular enzymes such as cytochrome P450 (P450) and UDP-glucuronosyltransferase (UGT), and the parent drug or its metabolites are eventually excreted from the hepatocytes by canalicular and/or sinusoidal transporter proteins. Therefore, expression analyses of metabolic enzymes and transporters in the liver of PXB mice are essential to validate the model. For example, it has been established by means of quantitative PCR and quantitative immunoblot analyses that PXB mice with a high replacement ratio express eight human P450s and human phase II enzymes, including three UGTs, at levels similar to those in human liver (Katoh et al., 2004,2005). Gene expression of the human ATP binding cassette (ABC) transporters and human solute carrier (SLC) transporters was also confirmed in humanized liver (Nishimura et al., 2005; Kikuchi et al., 2010). However, protein expression of drug transporters has not yet been quantitatively analyzed in PXB mice. This is important, because we recently showed that there is a poor correlation between protein and mRNA expression levels of metabolizing enzymes (except

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ABBREVIATIONS: ABC, ATP binding cassette; BCRP, breast cancer resistance protein; BSEP, bile salt export pump; CV, coefficient of variance; γ-GTP, γ-glutamyl transpeptidase; LC-MS/MS, liquid chromatography–tandem mass spectrometry; LLOQ, lower than the limit of quantification; MRM, multiplexed multiple reaction monitoring; MDR, multidrug resistance protein; MRP, multidrug resistance-associated protein; OAT, organic anion transporter; OATP, organic anion transporting polypeptide; OCT, organic cation transporter; PXB mice, chimeric mice with humanized liver; P450R, NADPH-cytochrome P450 reductase; UGT, UDP-glucuronosyltransferases; uPA/SCID, urokinase-type plasminogen activator/severe combined immunodeficiency.

CYP3A4) and transporters in human livers; for example, the correlation coefficients (r^2) were less than 0.3 for CYP1A2, 2C9, 2A6, 2E1, UGT1A1, CYP2B7, multidrug resistance-associated protein 2 (MRP2), multidrug resistance protein 1 (MDR1), bile salt export pump (BSEP), multidrug and toxin extrusion protein 1 (MATE1), organic cation transporter (OCT1), sodium/taurocholate cotransporting polypeptide (NTCP), and organic anion transporting polypeptide (OATP)1B3 (Ohtsuki et al., 2012). Furthermore, metabolizing activities of P450s such as CYP2C9, 2C19, 2D6, and 2E1 were correlated to expression levels of protein rather than mRNA (Ohtsuki et al., 2012). In addition, human hepatocytes are transplanted to produce the PXB mice, so it is also important to consider the influence of the donor on the protein expression of metabolizing enzymes and transporters.

We have recently developed a liquid chromatography–tandem mass spectrometry (LC-MS/MS)–based protein quantification method that does not require antibodies (Kamiie et al., 2008). In this method, the target protein concentration in a sample is determined after enzymatic digestion by quantifying one or more peptide fragments specific to the target molecule. By using this method, we have measured protein expression levels of metabolizing enzymes and transporters in human and mouse livers (Kamiie et al., 2008; Kawakami et al., 2011; Ohtsuki et al., 2012). Since the target peptide is identified by mass-weight information, a single amino acid difference can be distinguished. Furthermore, the specificity, accuracy, and dynamic range of quantification by LC-MS/MS–based analysis [coefficient of variance (CV) $< 20\%$ and three-orders-of-magnitude dynamic range] are greatly superior to those in the case of immunoblot analysis (Kamiie et al., 2008; Kawakami et al., 2011; Ohtsuki et al., 2011). Therefore, this method was considered suitable for validating PXB mouse as a human liver model in terms of protein levels in the liver.

The purpose of the present study was to clarify the protein expression levels of metabolizing enzymes and transporters in liver of PXB mice transplanted with human hepatocytes from different donors by using LC-MS/MS, and to compare the protein expression levels with those of human livers to validate PXB mouse as a model of human liver.

Materials and Methods

Generation of PXB Mice. The present study was approved by the Ethics Committees of the Graduate School of Pharmaceutical Sciences, Tohoku University, and PhoenixBio Co., Ltd. The experiments in this report conformed to the guidelines established by the Animal Care Committee, Graduate School of Pharmaceutical Sciences, Tohoku University, and PhoenixBio Co., Ltd. The cryopreserved human hepatocytes from donor BD85 (black, male, 5 years old), BD72 (white, female, 10 years old), and BD87 (white, male, 2 years old) were purchased from BD BioSciences (San Jose, CA) (Supplemental Table 1). The chimeric mice with humanized liver were generated by the method described previously (Tateno et al., 2004). Briefly, uPA$^{+/+}$/SCID mice were prepared (Tateno et al., 2004), and at 3 weeks after birth they were injected with human hepatocytes through a small left-flank incision into the inferior splenic pole. The concentration of human albumin in the blood of the chimeric mice and the replacement index (RI; the rate of the replacement from mouse to human hepatocytes) were measured using latex agglutination immunonephelometry (LX Reagent "Eiken" Alb II; Eiken Chemical, Tokyo, Japan) and anti-human specific cytokeratin 8 and 18 antibody (Cappel Laboratory, Cochranville, PA), respectively (Supplemental Table 1). There was a good correlation between the human albumin concentration and the RI (Tateno et al., 2004). In this study, the chimeric mice used were 13–14 weeks of age.

LC-MS/MS–Based Protein Quantification Analysis. Microsomal and plasma membrane fractions of liver were prepared as described previously (Ohtsuki et al., 2012). For details, see Supplemental Data. Protein quantitation of the target molecules was simultaneously performed by means of high-performance (HP)LC-MS/MS for metabolizing enzymes or nanoLC-MS/MS for transporters with multiplexed multiple reaction monitoring (multiplexed MRM) as described previously (Ohtsuki et al., 2011; Shawahna et al., 2011; Uchida et al., 2011). Protein expression levels were determined by quantifying specific target peptides produced by trypsin digestion (Supplemental Table 2). Absolute amounts of each target peptide were determined by using an internal standard peptide, which is a stable isotope-labeled peptide with an amino acid sequence identical to that of the corresponding target peptide. Details of the quantification procedure are given in Supplemental Data.

One specific peptide was selected for quantification of each target protein and measured at four different MRM transitions. The amount of each peptide was determined as an average of three or four MRM transitions from one sample. In cases where signal peaks of fewer than three transitions were obtained, the amount of peptide in the sample was defined as under the limit of quantification. The absolute expression amount of CYP3A4 was calculated from the quantitative data obtained for a peptide generated from both CYP3A4 and CYP3A43 by subtracting the value obtained for a peptide that is specific for CYP3A43. Since CYP3A43 was under the limit of quantification in all samples, quantification values obtained with CYP3A4/43 peptides were used as those of CYP3A4.

For the comparison of protein expression levels between humanized liver of PXB mice and human liver, the data for microsomal fraction of 17 human liver biopsies were taken from our previous publication (Ohtsuki et al., 2012).

Statistical Analysis. Statistical significance of differences among donors was determined by one-way analysis of variance followed by the Bonferroni test using Origin 9 software (OriginLab Corp., Northampton, MA).

Results

Protein Expression Levels of Metabolizing Enzymes in Microsomal Fraction of PXB Mouse Liver. Protein expression levels of 12 human P450 enzymes, human NADPH-cytochrome P450 reductase (P450R), Na^+/K^+ ATPase, and γ-glutamyl transpeptidase (γ-GTP) were determined in liver microsomal fraction of PXB mice with transplanted hepatocytes from three different donors (Table 1). Two human UGT enzymes were determined in hepatocytes from two different donors. Na^+/K^+ ATPase and γ-GTP are membrane markers, and both the human and mouse molecules were quantified. The coefficients of variance of their quantification values were 14.5% and 14.1%, respectively, among 11 samples, and the values were not significantly different among the three donors. This suggests that the purity of the microsomal fraction was similar in all cases.

CYP2E1, P450R, and UGT2B7 were expressed most abundantly at 51.8, 31.6, and 55.0 pmol/mg protein of microsomal fraction, respectively (Table 1). The highest CV among samples was 76.1% for CYP2A6. The average of %CV of all samples was 43.4%. This is significantly greater than those of the individual donors ($P < 0.05$), which were 26.5%, 23.9%, and 23.0% for BD85, BD72, and BD87, respectively, suggesting that differences among the donors contribute substantially to the variances of expression levels of the target proteins in all samples. CYP2C9, 2C8, 2A6, 2C19, 2D6, 2B6, and P450R showed significant differences in protein expression levels among donors ($P < 0.05$). In addition, CYP3A5 and 3A7 were determined in all five samples from donor BD85, but were not detected or were detected in only 1 sample from the other donors. CYP2D6 was detected in only one sample from donor BD72, but was detected in all samples from BD85 and BD87.

Protein Expression Levels of Transporters in Plasma Membrane Fraction of PXB Mouse Liver. Protein expression levels of seven human ABC transporters, eight human solute carrier transporters, Na^+/K^+ ATPase, and γ-GTP were determined in plasma membrane fraction of PXB mouse liver, since these drug transporters function at the plasma membrane (Table 2). Na^+/K^+ ATPase and γ-GTP are membrane markers, and the CVs of their quantification values were 29.4% and 40.1%, respectively, among 11 samples. The quantified values were not significantly different among the three

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TABLE 1

Protein expression levels of metabolizing enzymes in microsomal fraction of humanized liver of PXB mice

Unit of protein expression is pmol/mg protein of microsomal fraction.

Donor	ALL (*n* =11)				BD85 (*n* = 5)				BD72 (*n* = 3)				BD87 (*n* = 3)			
	Mean	S.D.	%CV	*n*	Mean	S.D.	% CV	*n*	Mean	S.D.	%CV	*n*	Mean	S.D.	%CV	*n*
CYP2E1	51.8	9.7	18.7	11	51.7	9.4	18	5	52.9	7.8	15	3	50.9	15.2	30	3
CYP3A4	27.8	12.4	44.5	11	26.7	11.1	41	5	24.8	10.7	43	3	32.4	18.9	58	3
CYP2C9*	21.7	14.2	65.5	11	9.71	1.00	10	5	28.6	10.6	37	3	34.8	14.4	41	3
CYP1A2	20.7	6.4	30.9	11	23.3	5.1	22	5	16.5	7.1	43	3	20.4	7.7	38	3
CYP2C8*	15.8	6.3	39.8	11	10.2	1.8	18	5	16.9	1.9	11	3	24.1	2.1	9	3
CYP2A6*	10.2	7.8	76.1	11	2.93	0.40	14	5	12.4	1.6	13	3	20.2	3.3	16	3
CYP2C19*	8.84	6.25	70.8	11	4.90	1.15	24	5	16.6	7.2	43	3	7.59	2.99	39	3
CYP2D6*	7.07	2.94	41.6	9	6.77	1.64	24	5	1.23			1	9.51	1.75	18	3
CYP3A7	6.32	3.31	52.3	5	6.32	3.31	52	5	LLOQ				LLOQ			
CYP3A5	5.56	4.09	73.5	6	6.56	3.66	56	5	LLOQ				0.579			1
CYP4A11	5.04	1.97	39.1	9	4.85	1.99	41	5	2.29			1	6.26	1.24	20	3
CYP2B6*	1.90	0.91	47.6	11	1.68	0.49	30	5	1.08	0.27	25	3	3.11	0.45	15	3
P450R*	31.6	18.7	59.3	11	12.4	1.7	14	5	43.7	4.6	11	3	51.2	1.7	3	3
UGT2B7	55.0	20.8	37.8	6	N.D.				43.0	11.4	26	3	67.0	22.7	34	3
UGT1A1	19.0	2.4	12.5	6	N.D.				19.7	3.5	18	3	18.4	0.5	3	3
Na^+/K^+ ATPase	10.4	1.5	14.5	11	9.94	2.14	22	5	10.5	0.4	4	3	11.1	0.9	8	3
γ-GTP	2.64	0.37	14.1	11	2.57	0.33	13	5	2.64	0.55	21	3	2.74	0.37	13	3

n, Number of determined samples; LLOQ, lower than the limit of quantification; N.D., not determined.

*Significant difference ($P < 0.05$) in protein expression levels among donors (BD85, BD72, and BD87).

donors. This suggests that the purity of plasma membrane fraction was similar among donors, although the variability appeared to be greater than that of the microsomal fraction

All human transporters, except breast cancer resistance protein (BCRP), were quantified in all 11 samples (Table 2). MDR3 and BSEP exhibited the highest expression levels among the quantified transporters at 8.68 and 7.57 pmol/mg plasma membrane protein, respectively. Human BCRP was not detected in five samples from donor BD85, and was not quantified in samples from BD72 and BD87, in accordance with a report that BCRP expression is very low in human liver plasma membrane fraction (Ohtsuki et al., 2012). The average value of %CV of all samples was 47.0%, which was similar to that of microsomal fraction (43.4%), and significantly greater than that of BD72 (25.5%, $P < 0.01$) or BD87 (25.6%, $P < 0.01$). It was also greater, though not significantly, than that of BD85 (35.4%). Therefore, differences among the donors appear to contribute substantially to the variances of protein expression levels in all samples. MDR3, BSEP, MRP2, ABCG8, OCT1, and OATP1B3 showed significantly different protein expression levels depending on the hepatocyte donors ($P < 0.05$).

Comparison of Protein Expression Levels in Liver of PXB Mice and Human. The protein expression levels of metabolizing enzymes and transporters in PXB mouse liver shown in Tables 1 and 2 were compared with those in human liver. The protein expression levels

TABLE 2

Protein expression levels of transporters in plasma membrane fraction of humanized liver of PXB mice

Unit of protein expression is pmol/mg protein of plasma membrane fraction.

Donor	ALL (*n* = 11)				BD85 (*n* = 5)				BD72 (*n* = 3)				BD87 (*n* = 3)			
	Mean	S.D.	%CV	*n*	Mean	S.D.	%CV	*n*	Mean	S.D.	%CV	*n*	Mean	S.D.	%CV	*n*
Canalicular localized transporter																
MDR3/ABCB4*	8.68	7.32	84.3	11	4.19	1.29	31	5	7.47	3.66	49	3	17.4	9.24	53	3
BSEP/ABCB11*	7.57	7.91	104	11	3.06	1.13	37	5	6.12	2.79	46	3	16.5	11.29	68	3
MDR1/ABCB1	4.71	1.78	37.7	11	4.30	1.55	36	5	3.86	1.19	31	3	6.26	2.10	34	3
MRP2/ABCC2*	2.37	0.88	37.1	11	2.06	0.91	44	5	1.89	0.19	10	3	3.36	0.34	10	3
MATE1	1.10	0.22	19.7	11	1.07	0.23	22	5	1.28	0.21	17	3	0.981	0.086	9	3
ABCG8*	0.956	0.701	73.3	11	0.417	0.106	25	5	1.89	0.62	33	3	0.921	0.132	14	3
BCRP/ABCG2	LLOQ				LLOQ				N.D.				N.D.			
γ-GTP	2.22	0.89	40.1	11	2.52	0.60	24	5	1.57	0.18	12	3	2.39	1.52	64	3
Sinusoidal localized transporter																
OCT1*	4.21	3.41	81.0	11	6.87	3.04	44	5	3.22	1.61	50	3	0.765	0.102	13	3
OAT2	4.09	1.18	28.8	11	4.89	1.08	22	5	3.76	1.06	28	3	3.09	0.49	16	3
NTCP	2.42	0.81	33.5	11	2.12	1.12	53	5	2.61	0.34	13	3	2.71	0.47	17	3
MRP6	2.29	0.51	22.2	11	2.57	0.58	23	5	1.97	0.48	25	3	2.14	0.08	4	3
OATP1B1	1.45	0.56	38.9	11	1.22	0.63	52	5	1.50	0.16	11	3	1.78	0.68	38	3
OATP1B3*	1.03	0.47	45.9	11	0.616	0.207	34	5	1.47	0.47	32	3	1.28	0.06	5	3
OATP2B1	0.578	0.241	42.5	11	0.684	0.320	47	5	0.500	0.119	24	3	0.442	0.090	20	3
Na^+/K^+ ATPase	22.6	6.65	29.4	11	23.6	9.00	38	5	22.0	1.7	8	3	21.7	7.22	33	3
Localization unknown																
ENT1	1.55	0.50	32.6	11	1.79	0.60	34	5	1.59	0.32	20	3	1.11	0.13	11	3

n, Number of determined samples; LLOQ, lower than the limit of quantification; N.D., not determined; ENT, equilibrative nucleoside transporter; NTCP, sodium/taurocholate cotransporting polypeptide.

*Significant difference ($P < 0.05$) in protein expression levels among donors (BD85, BD72 and BD87).

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determined in 17 human liver biopsies (eight males and nine females, ages 20–74) were taken from our previous report (Ohtsuki et al., 2012). In the human liver biopsies, metabolizing enzymes and transporters were quantified in both microsomal fraction and plasma membrane fraction, as in the case of PXB mice. Expression levels of molecules determined both in PXB mouse and human livers are compared in Fig. 1. The differences between PXB mouse liver and human liver for all compared metabolizing enzymes and transporters were within 4-fold, except for CYP2A6 and CYP4A11 among metabolizing enzymes, and MDR3 and BSEP among transporters. Protein expression levels of CYP2A6 and CYP4A11 in PXB mouse liver microsomal fraction were 5.50- and 4.33-fold lower than those in human liver microsomal fraction, respectively. In contrast, protein expression levels of BSEP and MDR3 in PXB mouse liver plasma membrane fraction were 5.12- and 4.62-fold greater than those in human liver plasma membrane fraction.

Discussion

In the present study, we investigated the protein expression levels of human metabolizing enzymes in microsomal fraction and human membrane transporters in plasma membrane of humanized liver of PXB mice using LC-MS/MS–based protein quantification. In total, 11 livers of PXB mice humanized with hepatocytes from three different donors were quantified. Our results indicate that differences in the human donors contributed substantially to the variations of measured protein expression levels (Tables 1 and 2). This seems reasonable because the PXB mice were bred under controlled conditions, whereas the human donors might have been exposed to a variety of different environmental factors, such as food and drug intake, that could affect the expression of metabolizing enzymes and transporters.

Protein expression of CYP3A5 and CYP3A7 was detected in all liver samples from only one donor (BD85), and protein expression of CYP2D6 was detected in all liver samples from two donors (BD72 and BD87) (Table 1). In the previous study with PXB mice from two donors, CYP3A5 protein expression levels were very low in liver from both donors, and the reason for its low expression was genetic polymorphisms of the donor hepatocytes (Katoh et al., 2004). In our previous report, liver biopsies were clearly classified into two groups: a high-CYP3A7 group and a very low CYP3A7 group (Ohtsuki et al., 2012). Hence, the large differences in protein expression levels of these molecules might be attributable to differences in the genetic background of donor hepatocytes, although it is not known whether single-nucleotide polymorphisms were present in the transplanted human hepatocytes in our study. These results suggest that it is important to characterize the donor hepatocytes in studies with PXB mice; in principle, experiments should be performed with PXB mice humanized with hepatocytes from the same donor to ensure comparability of data.

In our previous reports, individual differences of BSEP protein expression were not large in plasma membrane fraction of human liver; the %CV was 29.7% among 17 liver biopsies (Ohtsuki et al., 2012). In contrast, %CV of BSEP protein expression was 105% in PXB liver (Table 2). A possible explanation is that BSEP expression is highly regulated in human liver, but lack of human-specific regulation in PXB mice resulted in a very high variance of protein expression. Interestingly, differences in MDR3 protein levels according to donor showed a trend similar to those of BSEP (Table 2). BSEP and MDR3 are cooperatively involved in excretion of lipids, and are regulated by FXR (Oude Elferink and Paulusma, 2007). Interspecies differences in FXR response have been reported (Cui et al., 2002; Song et al., 2013). Although further studies are necessary, it was possible that a shared regulatory mechanism of BSEP and MDR3 played some role in the interdonor differences.

The present study demonstrated that protein expression levels of most of the quantified metabolizing enzymes and transporters were within a 4-fold range from those in the same fraction of human liver reported previously (Ohtsuki et al., 2012) (Fig. 1). This range of

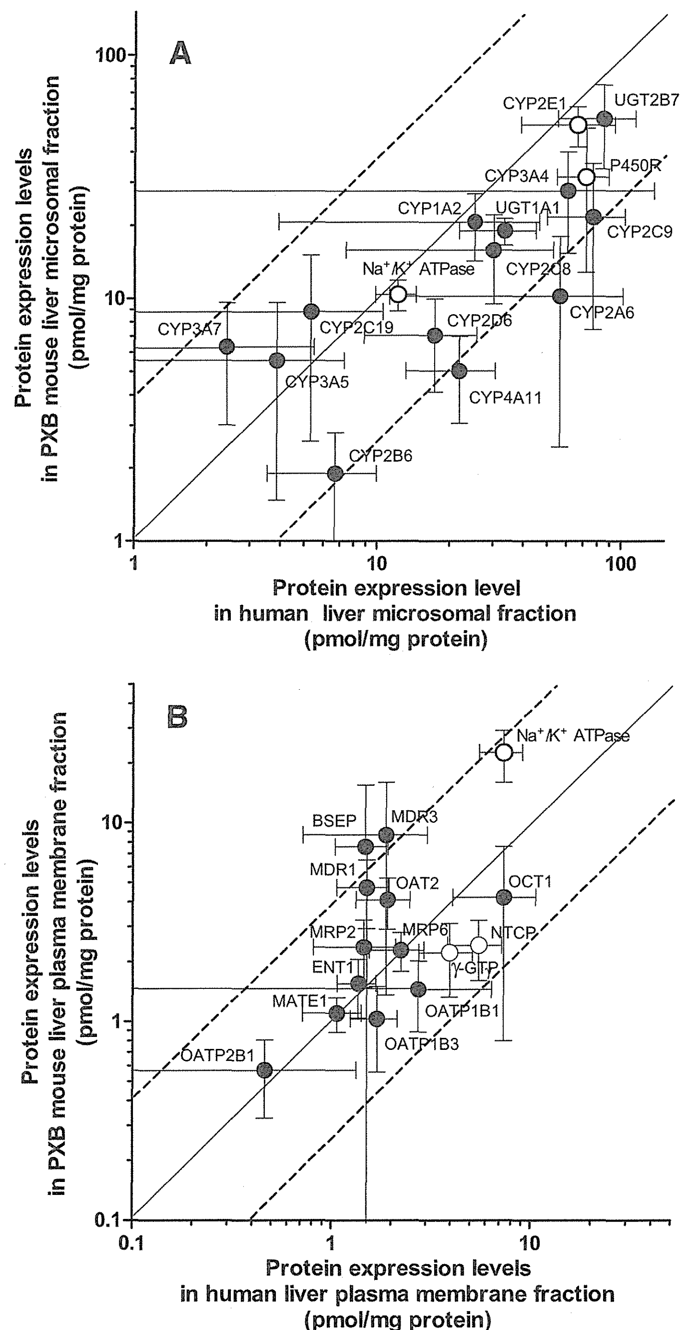

Fig. 1. Comparison of protein expression levels of metabolizing enzymes in microsomal fraction and transporters in plasma membrane fraction between humanized liver of PXB mice and human liver. Protein expression levels of metabolizing enzymes and transporters were determined in microsomal fraction (A) and plasma membrane fraction (B), respectively, of humanized liver of PXB mice, as shown in Tables 1 and 2 (mean ± S.D.). The data for microsomal fraction of 17 human liver biopsies (mean ± S.D.) were taken from our previous report (Ohtsuki et al., 2012). The solid line passing through the origin represents the line of identity, and the broken lines represent 4-fold differences. Open symbols indicate quantification values obtained with peptides that are conserved in the human and mouse proteins.

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difference was the same as that in average expression levels among the three donors. The enzyme activities of P450 enzymes were reported to be better correlated to protein expression levels in the microsomal fraction than to mRNA expression levels (Ohtsuki et al., 2012). We compared the protein expression levels of transporters between PXB mice and human in plasma membrane fraction, where the quantified transporters function. A large species difference was reported in protein expression levels of BCRP in plasma membrane fraction: 0.419 pmol/mg protein in human liver and 8.51 pmol/mg protein in mouse liver (Kamiie et al., 2008; Ohtsuki et al., 2012). Here, human BCRP was under the limit of quantification in humanized liver of PXB mice, indicating that human hepatocytes retain the characteristic low expression of BCRP in PXB mice. Overall, our results indicate that humanized liver in PXB mice well retains the protein expression pattern of metabolizing enzymes and transporters of human liver.

Our previous report demonstrated that, in sandwich-cultured human hepatocytes, differences of protein expression levels of metabolizing enzymes and transporters compared with those in human liver were within a 5-fold range (Schaefer et al., 2012). A similar range of difference was found in liver of PXB mice in this study, though in cultured human hepatocytes, the protein expression levels of transporters tended to be greater than those in human liver. Since humanized liver retains the structure of human liver tissue (Tateno et al., 2013), our results suggest that humanized liver of PXB mice is a useful model for analyzing human hepatic drug metabolism in vivo.

In conclusion, we measured the protein expression levels of metabolizing enzymes in microsomal fraction and transporters in plasma membrane fraction of humanized liver of chimeric PXB mice. The protein expression levels of the quantified metabolizing enzymes and transporters well reflected those of the donor human liver. The protein expression in humanized liver was significantly affected by the background of each individual donor. Our results indicate that humanized liver of PXB mice is a useful model of human liver, but it is important to note that interpretation of the results obtained from PXB mice requires a knowledge of not only the value of the replacement index, but also information about the background of the hepatocyte donor.

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References

Cui J, Heard TS, Yu J, Lo JL, Huang L, Li Y, Schaeffer JM, and Wright SD (2002) The amino acid residues asparagine 354 and isoleucine 372 of human farnesoid X receptor confer the receptor with high sensitivity to chenodeoxycholate. *J Biol Chem* **277**:25963–25969.

De Serres M, Bowers G, Boyle G, Beaumont C, Castellino S, Sigafoos J, Dave M, Roberts A, Shah V, and Olson K, et al. (2011) Evaluation of a chimeric (uPA+/+)/SCID mouse model with a humanized liver for prediction of human metabolism. *Xenobiotica* **41**:464–475.

Hasegawa M, Tahara H, Inoue R, Kakuni M, Tateno C, and Ushiki J (2012) Investigation of drug-drug interactions caused by human pregnane X receptor-mediated induction of CYP3A4 and CYP2C subfamilies in chimeric mice with a humanized liver. *Drug Metab Dispos* **40**:474–480.

Inoue T, Sugihara K, Ohshita H, Horie T, Kitamura S, and Ohta S (2009) Prediction of human disposition toward S-3H-warfarin using chimeric mice with humanized liver. *Drug Metab Pharmacokinet* **24**:153–160.

Kamiie J, Ohtsuki S, Iwase R, Ohmine K, Katsukura Y, Yanai K, Sekine Y, Uchida Y, Ito S, and Terasaki T (2008) Quantitative atlas of membrane transporter proteins: development and application of a highly sensitive simultaneous LC/MS/MS method combined with novel in-silico peptide selection criteria. *Pharm Res* **25**:1469–1483.

Kamimura H, Nakada N, Suzuki K, Mera A, Souda K, Murakami Y, Tanaka K, Iwatsubo T, Kawamura A, and Usui T (2010) Assessment of chimeric mice with humanized liver as a tool for predicting circulating human metabolites. *Drug Metab Pharmacokinet* **25**:223–235.

Katoh M, Matsui T, Nakajima M, Tateno C, Kataoka M, Soeno Y, Horie T, Iwasaki K, Yoshizato K, and Yokoi T (2004) Expression of human cytochromes P450 in chimeric mice with humanized liver. *Drug Metab Dispos* **32**:1402–1410.

Katoh M, Matsui T, Okumura H, Nakajima M, Nishimura M, Naito S, Tateno C, Yoshizato K, and Yokoi T (2005) Expression of human phase II enzymes in chimeric mice with humanized liver. *Drug Metab Dispos* **33**:1333–1340.

Kawakami H, Ohtsuki S, Kamiie J, Suzuki T, Abe T, and Terasaki T (2011) Simultaneous absolute quantification of 11 cytochrome P450 isoforms in human liver microsomes by liquid chromatography tandem mass spectrometry with in silico target peptide selection. *J Pharm Sci* **100**:341–352.

Kikuchi R, McCown M, Olson P, Tateno C, Morikawa Y, Katoh Y, Bourdet DL, Monshouwer M, and Fretland AJ (2010) Effect of hepatitis C virus infection on the mRNA expression of drug transporters and cytochrome p450 enzymes in chimeric mice with humanized liver. *Drug Metab Dispos* **38**:1954–1961.

Nishimura M, Yoshitsugu H, Yokoi T, Tateno C, Kataoka M, Horie T, Yoshizato K, and Naito S (2005) Evaluation of mRNA expression of human drug-metabolizing enzymes and transporters in chimeric mouse with humanized liver. *Xenobiotica* **35**:877–890.

Ohtsuki S, Uchida Y, Kubo Y, and Terasaki T (2011) Quantitative targeted absolute proteomics-based ADME research as a new path to drug discovery and development: methodology, advantages, strategy, and prospects. *J Pharm Sci* **100**:3547–3559.

Ohtsuki S, Schaefer O, Kawakami H, Inoue T, Liehner S, Saito A, Ishiguro N, Kishimoto W, Ludwig-Schwellinger E, and Ebner T, et al. (2012) Simultaneous absolute protein quantification of transporters, cytochromes P450, and UDP-glucuronosyltransferases as a novel approach for the characterization of individual human liver: comparison with mRNA levels and activities. *Drug Metab Dispos* **40**:83–92.

Oude Elferink RP and Paulusma CC (2007) Function and pathophysiological importance of ABCB4 (MDR3 P-glycoprotein). *Pflugers Arch* **453**:601–610.

Schaefer O, Ohtsuki S, Kawakami H, Inoue T, Liehner S, Saito A, Sakamoto A, Ishiguro N, Matsumaru T, and Terasaki T, et al. (2012) Absolute quantification and differential expression of drug transporters, cytochrome P450 enzymes, and UDP-glucuronosyltransferases in cultured primary human hepatocytes. *Drug Metab Dispos* **40**:93–103.

Shawahna R, Uchida Y, Declèves X, Ohtsuki S, Yousif S, Dauchy S, Jacob A, Chassoux F, Daumas-Duport C, and Couraud PO, et al. (2011) Transcriptomic and quantitative proteomic analysis of transporters and drug metabolizing enzymes in freshly isolated human brain microvessels. *Mol Pharm* **8**:1332–1341.

Song X, Chen Y, Valanejad L, Kaimal R, Yan B, Stoner M, and Deng R (2013) Mechanistic insights into isoform-dependent and species-specific regulation of bile salt export pump by farnesoid X receptor. *J Lipid Res* **54**:3030–3044.

Tateno C, Yoshizane Y, Saito N, Kataoka M, Utoh R, Yamasaki C, Tachibana A, Soeno Y, Asahina K, and Hino H, et al. (2004) Near completely humanized liver in mice shows human-type metabolic responses to drugs. *Am J Pathol* **165**:901–912.

Tateno C, Miya F, Wake K, Kataoka M, Ishida Y, Yamasaki C, Yanagi A, Kakuni M, Wisse E, and Verheyen F, et al. (2013) Morphological and microarray analyses of human hepatocytes from xenogeneic host livers. *Lab Invest* **93**:54–71.

Uchida Y, Ohtsuki S, Katsukura Y, Ikeda C, Suzuki T, Kamiie J, and Terasaki T (2011) Quantitative targeted absolute proteomics of human blood-brain barrier transporters and receptors. *J Neurochem* **117**:333–345.

Yamazaki H, Kuribayashi S, Inoue T, Tateno C, Nishikura Y, Oofusa K, Harada D, Naito S, Horie T, and Ohta S (2010) Approach for in vivo protein binding of 5-n-butyl-pyrazolo[1,5-a] pyrimidine bioactivated in chimeric mice with humanized liver by two-dimensional electrophoresis with accelerator mass spectrometry. *Chem Res Toxicol* **23**:152–158.

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厚生労働科学研究委託費（創薬基盤推進研究事業）

医薬品・医療機器の実用化促進のための評価技術手法の戦略的開発 （H26－創薬－一般－008）

平成26年度　委託業務成果報告書
第7/7分冊　研究分担報告書

テーマ2-4　プラスチック製医療機器の化学物質影響評価法2種の開発

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本報告書は、厚生労働省の厚生労働科学研究委託費（創薬基盤推進研究事業）による委託業務として、国立医薬品食品衛生研究所（斎藤嘉朗）が実施した平成２６年度「医薬品・医療機器の実用化促進のための評価技術手法の戦略的開発」の成果を取りまとめたものです。

目　　次

Ⅰ．委託業務成果報告（業務項目）

テーマ総括、簡易溶血性試験法の確立と血液バッグ
及び血液回路への適用 1
　蓜島 由二（国立医薬品食品衛生研究所 医療機器部）

可塑剤の炎症誘導能評価法の確立とその機序の解析
に関する研究 13
　鄭　雄一（東京大学大学院工学系研究科）

新規可塑剤を配合した溶血性試験用評価材料の開発
に関する研究 19
　柚場 俊康（川澄化学工業株式会社）

可塑剤の溶出挙動と評価結果との相関に関する研究 23
　坂口 圭介（テルモ株式会社）

Ⅱ．学会等発表実績 29

Ⅲ．研究成果の刊行物・別刷 31

Ⅰ．委託業務成果報告（業務項目）

分担研究報告書①

テーマ総括、簡易溶血性試験法の確立と血液バッグ及び
血液回路への適用

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厚生労働科学研究委託費（創薬基盤推進研究事業）
委託業務成果報告書（業務項目）

担当研究課題　プラスチック製医療機器の化学物質影響評価法２種の開発

担当責任者　国立医薬品食品衛生研究所 医療機器部第一室長 蓜島由二

テーマ総括、簡易溶血性試験法の確立と血液バッグ及び血液回路への応用

研究要旨：溶血性試験は血液接触型医療機器に要求される安全性試験の一項目であるが、日本のガイドライン法は諸外国が採用している公定法と比較して感度的に劣ることが判明した。そこで本研究では、我々が開発を進めている新規血液バッグ用素材やその他の標準品等を評価用材料として、家兎及び青酸化合物を用いることなく、高感度に溶血性を評価する簡易試験系を構築し、その性能を代表的な公定法と比較検討した。溶血特性の異なる 8 種の標準材料を使用して検討した結果、簡易溶血性試験法は 3 種の公定法と同等以上の感度で各検体の溶血能を探知できると共に、新規血液バックの素材候補である DOTH/DINCH 配合 PVC シートの溶血特性も評価可能であった。また、当該シートの溶血抑制能と可塑剤溶出量の間には明瞭な相関性が観察された。簡易溶血性試験法は十分な基本性能を有することが確認されたことから、今後、その標準化を目指した研究を行う。

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A. 研究目的

ポリ塩化ビニル（PVC）製品の代表的な可塑剤である Di(2-ethylhexyl)phthalate (DEHP) の使用についてはリスク回避の観点から各国において規制が強化される方向にあり、日本の医療機器分野においてもリスク患者群の治療にあたっては代替機器又は代替可塑剤を利用した製品への切り替えが推奨されている。血液バッグについては、DEHP が赤血球保護作用を有することから、その使用が例外的に認められているが、近年我々は、4-cyclohexene-1,2-dicarboxylic acid bis(2-ethylhexyl) (DOTH)、1,2-cyclohexane dicarboxylic acid diisononyl ester (DINCH) 及び diisodecyl phthalate (DIDP) が DEHP と同等の赤血球保護作用を示し、中でも DOTP と DINCH が有益な代替可塑剤になり得ることを見出した[1)]。血液バッグ用代替可塑剤には、基本性能として、1)DEHP と比較して同等以上の溶血抑制能を示し、2)DEHP/PVC

製血液バッグと同等以下の溶出量、3)齧歯類に対して無毒性であることが求められる。

溶血性試験は血液接触型医療機器に要求される安全性試験の一項目であるが、世界的に標準化された評価法が存在しない。現在、当該試験の国際標準化を行うため、ISO/TC194/WG9 が国際ラウンドロビンテストを行っているが、日本のガイドライン法（MHLW 法）は諸外国が採用している公定法と比較して感度的に劣ることが判明した。そこで本研究では、我々が開発を進めている新規血液バッグ用素材やその他の標準品等を評価用材料として、家兎及び青酸化合物を用いることなく、高感度に溶血性を評価する簡易試験系を構築し、その性能を代表的な公定法と比較検討した。また、新規血液バッグ用素材の溶血抑制能と代替可塑剤溶出挙動との相関性を評価した。

現在、代替可塑剤を利用した血液バッグ及び血液回路の開発研究が世界的に進められている。これらの製品の国際市場は日本が優位性を保っている都合上、我が国も早急に新規製品を開発し、患者により安全性に優れた製品を提供する必要があることから、関連企業の要望を受けて本研究が開始された。

B. 研究方法

1. 材料

ISO/TC194/WG9 が実施している溶血性試験国際ラウンドロビンテスト用標準品であるポリエチレン（PE：陰性対照）、Latex glove（LG：弱陽性対照）、Buna rubber（BR：弱陽性対照）及び Nitrile glove（NG：陽性対照）は Nelson Laboratories 社（米国）・Michelle Lee 博士から提供された。非イオン性界面活性剤 Genapol X-080 含有 PVC シート [2] は表 1 に示した新処方に従って、川澄化学工業がコンパウンディングを行い、テルモがシーティングを担当した。T ダイ成形機により作製した DEHP 配合 PVC シート、TOTM 配合 PVC シート、DOTH 配合 PVC シート、DINCH 配合 PVC シート及び DOTH/DINCH 配合 PVC シートは川澄化学工業から提供された。DEHP、TOTM、DOTH 及び DINCH の配合量は PVC に対する重量比（PVC = 100 パーツ）として、それぞれ 55、85、55 及び 60.5 パーツとした。DOTH/DINCH の配合量は 40:16.5、25:33 及び 10:49.5 パーツとした。

2. 公定法による溶血性試験

ISO/TC194/WG9 が実施している溶血性試験国際ラウンドロビンテストプロトコル（表 2）に従い、ウサギ血液を利用して、ASTM 法/直接接触及び抽出液法（インキュベーション時間：3 時間）、NIH 法/直接接触法（1 時間）、MHLW 法/抽出液法（4 時間）により行った [3-5]。これらの試験は食品薬品安全センター及びテルモにおいて個別に実施した。

3. 簡易溶血性試験 [1,2,6]

コージンバイオ製ウサギ脱線維血 1mL を遠心分離し、得られた赤血球画分を PBS で 3 回洗浄後、同容量の PBS に再懸濁させて血液試料とした。直接接触法は、検体（6cm^2/mL）を挿入した Eppendorf チューブに PBS 1mL 及び血液 20μL を添加し、緩やかに撹拌した後、37℃でインキュベートした。抽出液法用試験液は、検体（6cm^2/mL）を挿入したスクリューキャップ付試験管に PBS 1mL を添加し、37℃下 72 時間抽出して調製した。Genapol X-080 含有 PVC シートのみは、121℃/1 時間、70℃/24 時間、50℃/72 時間及び 37℃/72 時間の条件下に抽出を行い、Eppendorf チューブに各試験液 0.5mL を採取後、血液 10μL を添加し、37℃でインキュベートし

た。インキュベート開始 1、2 及び 4 時間後に試験混液を遠心分離（700 x g, 5 分, 4℃）し、遠心上清 100µL を 96 穴プレートに採取後、SH-9000 Microplate reader を使用して 415nm における吸光度を測定した。吸光度 2.0 を越える試料は PBS を利用して適宜希釈した。陰性対照は PBS のみ、陽性対照は PBS の代わりにミリ Q 水を使用して同様の方法により調製した。溶血率は次式により算出した。

$$溶血率(\%) = (A_T \times F - A_N) / (A_P \times F - A_N) \times 100$$

A_T：試料の吸光度
A_P：陽性対照の吸光度
A_N：陰性対照の吸光度
F：希釈倍率

3. PVC シートの溶血抑制能評価

オートクレーブ処理した DEHP 配合 PVC シート、TOTM 配合 PVC シート、DOTH 配合 PVC シート、DINCH 配合 PVC シート及び DOTH/DINCH 配合 PVC シート（3.2 x 1cm, 厚さ 0.4mm）を滅菌済みスクリューキャップ付バイアル瓶に採取し、常法に従って調製したヒト MAP[1,6] 又は SAGM[6] 加濃厚赤血球液（MAP/RCC, SAGM/RCC）5mL を添加後、4℃下 49 日間インキュベーションした。インキュベーション開始後 1 週間毎に血液 50µL を Eppendorf チューブに採取し、PBS 1mL を添加して緩やかに撹拌後、遠心分離（500 x g, 2 分間, 4℃）し、その上清 100µL の吸光度を上記 2 項の方法に従って測定し、溶血率を算出した。

4. PVC シートの可塑剤溶出試験 [1,7-11]

上記 3 項において調製した血液試料 50µL を 1 週間毎にスクリューキャップ付試験管に採取し、1% NaCl 溶液（1mL）、DEHP-d_4（0.1µg）及びヘキサン（1mL）を添加後、15 分間激しく振とう抽出し、室温下で遠心分離（3,000 rpm, 10 分間）した。得られた上清に無水 Na_2SO_4 を添加して脱水し、常法に従って GC-MS/MS 分析に供した。GC-MS/ MS 測定条件は表 3 に示した。

＜倫理面への配慮＞

ヒト血液を利用した試験は、国立医薬品食品衛生研究所研究倫理審査委員会の承認を得て実施した（平成 26 年 2 月 24 日付衛研発第 114 号：受付番号 188-5）。

動物血液の採取については、所属機関が規定する動物実験指針に従い、動物に対する苦痛が最小となるよう務めた（承認番号：食品薬品安全センター1120331A、テルモ 120163）。

C. 研究結果

1. Genapol X-080 含有 PVC シートの溶血特性

改良型溶血性試験用対照材料 Genapol X-080 含有 PVC シート（陰性対照：Y-1, 弱溶血性材料：Y-2・Y-3, 強溶血性材料：Y-4）の溶血挙動を簡易溶血性試験法及び 3 種の公定法により評価した。図 1a に示したとおり、Y-4 は直接接触法による簡易溶血性試験において強溶血性を示し、Y-2 は 4 時間インキュベーション時、Y-3 は 1 時間インキュベーション時にそれぞれ弱溶血性（溶血率：25.1%, 21.1%）を呈することが確認された。Y-1 の溶血性は陰性対照（PBS）と同等であった。これらの材料の溶血特性を抽出液法による簡易溶血性試験により評価した結果、全ての抽出条件において、Y-4 は強溶血性を示した（例：図 1b）。Y-2 及び Y-3 は、121℃/1 時間及び 70℃/24 時間抽出では溶血活性を示さず、37℃/72 時間抽出では強溶血性を示した（図 1b, 1c, 1e）。50℃/72 時間抽出により、Y-3 は強溶血性を呈したが、Y-2 は 2 時間又は 4

時間インキュベーション時に弱溶血性（溶血率：20.4%, 60.6%）を示すことが確認された（図1d）。

Genapol X-080含有PVCシートの溶血特性を公定法により評価した結果を図2に示した。なお、抽出液法用試験液は37℃/72時間抽出により調製した。Y-4は、いずれの試験法においても強溶血性を示した。Y-2はASTM法/直接接触法及びMHLW法/抽出液法において弱又は中溶血性（溶血率：8.7%, 49.9%）を示したが、ASTM法/抽出液法では強溶血性（86.8%）を呈した。Y-3はNIH法/直接接触法において弱溶血性（溶血率：8.0%）を示したが、その他の試験法では全て強溶血性を呈した。

2.ラウンドロビンテスト用標準品の溶血特性

ISO/TC194/WG9が実施している溶血性試験国際ラウンドテスト用標準品であるPE、LG、BR及びNGの溶血特性を公定法により評価した。図3に示したとおり、陰性対照であるPEは全ての試験法において溶血活性を示さなかった。主に直接接触法における弱陽性対照材料であるLG及びBRはASTM法/直接接触法（溶血率：5.0%, 8.0%）及びNIH法/直接接触法（溶血率：11.0%, 4.6%）により弱溶血性を呈したが、ASTM法/抽出液法及びMHLW法/抽出液法では溶血活性が検出されなかった。陽性対照であるNGはASTM法/直接接触法、ASTM法/抽出液法及びNIH法/直接接触法において強溶血性を示したが、MHLW法/抽出液法ではNGの溶血活性が殆ど探知できなかった（溶血率：4.0%）。

これらの材料の溶血特性を簡易溶血性試験により評価した結果（図4a, 4b）、公定法同様、PEは簡易溶血性試験法においても顕著な溶血活性を示さなかった。LG及びBRの弱溶血性は直接接触法（溶血率：11.8%/4時間, 17.5%/4時間）により検出されたほか、公定法と異なり、抽出液法（溶血率：16.7%/4時間, 24.0%/4時間）によっても探知可能であった。NGは直接接触法及び抽出液法ともに強溶血性を示した。

3.PVCシートの溶血抑制能評価

MAP/RCC及びSAGM/RCCに対する各PVCシートの溶血抑制能を簡易溶血性試験法により評価した。図5に示したとおり、TOTM含有PVCシートはMAP/RCCに対する溶血抑制能を示さず、49日間インキュベーション後の溶血率は4.5%に達した（陰性対照：4.9%）。DEHP配合PVCシートはMAP/RCCに対して有意な溶血抑制能を示し、49日間インキュベーション時の溶血率は1.4%であった。DOTH配合PVCシート、組成比40:16.5及び25:33のDOTH/DINCH配合PVCシートはDEHP配合PVCシートと同等の溶血抑制能を示した（49日後溶血率：1.5%, 1.3%, 1.4%）。一方、DINCH配合PVCシート及び組成比10:49.5のDOTH/ DINCH配合PVCシートの溶血抑制能はDEHP配合PVCシート比較して若干低く、49日間インキュベーション後の溶血率は、それぞれ2.1%及び2.3%であった。

各PVCシートはSAGM/RCCに対しても類似した特性を示した（図6）。組成比25:33のDOTH/DINCH配合PVCシートの溶血抑制能はDEHP配合PVCシートと同等であり、49日間インキュベーション時の溶血率は、それぞれ2.1%及び2.3%であった。一方、DINCH配合PVCシートの溶血抑制能はDEHP配合PVCシート比較して若干低く、49日間インキュベーション後の溶血率4.4%であった。

4.PVCシートからの可塑剤溶出挙動

表4に示したとおり、各PVCシートをMAP/RCCに浸漬した際に溶出する可塑剤量は時間経過

と共に増大し、DEHP配合PVCシートからのDEHP溶出量はインキュベーション開始後 49 日目で 50.5μg/mL に達した。49 日目における DOTH 配合 PVC シート及び DINCH 配合 PVC シートからの可塑剤溶出量は、それぞれ 114 及び 25.6μg/mL であり、DEHP と比較して、DOTH は易溶出性、DINCH は難溶出性であることが確認された[1)]。DOTH/DINCH配合PVCシートから溶出する総可塑剤量は DOTH 含量の増加に伴って上昇し、組成比 25:33 の DOTH/DINCH 配合シート（49 日目可塑剤総溶出量：51.1μg/mL = DOTH 37.1μg/mL + DINCH 14.0μg/mL）が DEHP 配合 PVC シートとほぼ同等の溶出挙動を示すことが判明した。表 5 に示したとおり、DOTH/DINCH 配合 PVC シートからの DOTH/DINCH 溶出比は時間経過に伴って低下したことから、DOTH の溶出速度は DINCH と比較して早いことが明らかとなった。なお、TOTM 配合 PVC シートからの TOTM 溶出量は非常に低いことも確認された（0.047μg/mL）。

DINCH 配合 PVC シート及び組成比 25:33 の DOTH/DINCH 配合シートは SAGM/RCC 系においても同様の可塑剤溶出特性を示すことが明らかとなった（表 6）。

D. 考察

本研究では、溶血特性の異なる 8 種の標準材料を使用して、簡易溶血性試験法の性能を 3 種の公定法と比較検討した。その結果、簡易溶血性試験法は MHLW 法/抽出液法と異なり、陽性対照材料である NG の溶血特性を探知することが可能であった。また、抽出液法でも LG 及び BR の溶血特性を感度良く評価できる等、3 種の公定法と比較して同等以上の感度で各検体の溶血能を探知できることも明らかとなった。また、簡易溶血性試験法は新規血液バックの素材候補である DOTH/DINCH 配合 PVC シートの溶血特性も評価可能であった。Genapol X-080 は曇点である 64℃を堺として溶解性が変化する。Y-2 及び Y-3 の簡易溶血性試験法／抽出液法において観察された抽出温度の低下に伴う溶血能の上昇は当該性質に由来する現象である。

表 2 に示したとおり、簡易溶血性試験法は、採血のために家兎を飼育する必要がないと共に、青酸化合物を含む Drabkin 試薬を利用しない利点を有している。また、ヘモグロビン型の定性能力には劣るが、ヘモグロビンの第一吸収極大であるソーレー帯（415nm）で測定するため、感度的に優れており、試験スケールを 1/10 に縮小しても良好な成績が得られる。公定法では血液を直接試験に供するが、簡易溶血性試験法ではバックグランドを低下させることを目的として、赤血球を PBS により洗浄する。この洗浄操作に伴い血漿成分が除去されるため、赤血球自体は不安定になるが、溶血剤に対する感度は逆に上昇すると思われる。また、簡易溶血性試験法は ASTM 法及び NIH 法と異なり、使用する血液のヘモグロビン濃度や吸光度調整が不要である利点もある。このように、公定法と比較して簡易溶血性試験法は操作性に優れていると共に、医用材料の溶血特性を十分探知できる基本性能を有することが確認されたことから、今後、その標準化を目指した研究を行う。

溶血とは、物理的、化学的又は生物学的要因により赤血球の細胞膜が破壊され、ヘモグロビンを含む赤血球内容物が細胞外に放出される現象である。溶血は非常に単純な機序に基づく現象であり、適用する試験法により得られる結果が大きく相違することは考え難いが、MHLW 法/抽出液法は ASTM 法/抽出液法と比較して、NG の溶血性評価に関して感度的に劣ることが本研究においても確認された。両試験法は使用する抽出液と抗凝固処理法が異なる。ASTM 法は抽

出溶媒としてPBSを利用するが、MHLW法は生理食塩液により抽出を行う。pH変化を伴う材料の場合、緩衝能力を有していない生理食塩液の方が溶血性に与える影響は大きいため、理論的に考えて、ASTM法とMHLW法の感度の相違を抽出溶媒の違いから説明することは難しい。ASTM法において利用する血液はNIH法及び簡易溶血性試験法と同様、クエン酸Naにより抗凝固処理を施しているが、MHLW法に使用する血液は抗凝固剤を含まない脱繊維血である。表7に示したとおり、ASTM法、NIH法及び簡易溶血性試験法に使用する血液は時間経過と共に415nmにおける吸光度が増加するが、MHLW法に利用する血液は4時間インキュベーション後でも大きな吸光度変化を生じない。これは、その他の試験法と比較して、MHLW法に利用する血液（赤血球）の安定性が高いことを意味している。今後、MHLW法が感度的に劣る要因を特定するため、赤血球膜の蛋白質組成や血液中に存在する凝固因子量を解析する予定である。

新規血液バッグの基本的な設計コンセプトとして、既存製品の可塑剤暴露量を超えることなく、同等の溶血抑制能を示すことが要求される。DOTH/DINCH配合PVCシートの溶血抑制能と可塑剤溶出量を評価した結果、両者間には明瞭な相関性が観察された。組成比 10:49.5 の DOTH/DINCH配合PVCシートはDEHP配合PVCシートと比較して、可塑剤総溶出量は低いが、十分な溶血抑制能を示さないことが確認された。一方、組成比 40:16.5 の DOTH/DINCH 配合 PVC シートは DEHP 配合 PVC シートと同等の溶血抑制能を示すが、可塑剤総溶出量が高いことが判明したことから、新規血液バッグ素材としては DEHP 配合PVCシートと同等の溶血抑制能及び可塑剤溶出挙動を持つ組成比 25:33 の DOTH/DINCH 配合シートが最適であると思われる。

受託研究費において、日本発の新規可塑剤 DL9TH のラット亜慢性毒性試験を実施した結果、当該可塑剤はラットに対する毒性が非常に低いことが判明した。DOTH及びDINCHと比較して、DL9TH は耐寒性に優れており、血液バッグ用素材として DOTH/DINCH 配合 PVC シートを越える性能を発揮する可能性が高いため、次年度、DOTH/DL9TH配合PVCシートを作製し、溶血抑制能と可塑剤溶出挙動の相関性について検討する。

E. 結論

簡易溶血性試験法は各公定法と同等以上の感度で各検体の溶血能を探知できると共に、新規血液バックの素材候補である DOTH/DINCH 配合 PVC シートの溶血特性も評価可能であった。また、当該シートの溶血抑制能と可塑剤溶出量の間には明瞭な相関性が観察されたことから、簡易溶血性試験法は十分な基本性能を有することが確認された。

F. 健康危機情報

特になし

G. 研究発表等

論文発表等

1) Haishima Y, Kawakami T, Fukui C, Tanoue A, Yuba T, Ozono S, Kumada H, Inoue K, Morikawa T, Takahashi M, Fujisawa A, Yamasaki K, Nomura Y, Isama K, Chung U, Ogawa K, Niimi S, Yoshida M.: Characterization of alternative plasticizers in polyvinyl chloride sheets for blood containers. *J. Vinyl Add. Technol.*, 2015, in press.

学会発表等

1) 蓜島由二，福井千恵，山﨑佳世，野村祐介，小園 知，熊田秀文，藤澤彩乃，井上 薫，森川朋美，市村亮平，前田 潤，高橋美和，河上強志，伊佐間和郎，柚場俊康，鄭 雄一，小川久美子，新見伸吾，吉田 緑：新規血液バッグ用代替可塑剤 DOTH のラット亜慢性毒性試験．第 36 回日本バイオマテリアル学会大会(2014.11，船堀)．
2) 蓜島由二，河上強志，福井千恵，田上昭人，柚場俊康，向井智和，野村祐介，伊佐間和郎，新見伸吾：新規血液バッグ素材 DOTH/DINCH 配合 PVC シートの性能評価．第 36 回日本バイオマテリアル学会大会(2014.11，船堀)．
3) 野村祐介，福井千恵，柚場俊康，新藤智子，坂口圭介，谷川隆洋，杉山知子，竹ノ内美香，新見伸吾，蓜島由二：簡易溶血性試験法の性能評価と公定法との比較検証．第 36 回日本バイオマテリアル学会大会(2014.11，船堀)．
4) 蓜島由二，福井千恵，野村祐介，藤澤彩乃，山﨑佳世，熊田秀文，井上 薫，森川朋美，高橋美和，河上強志、伊佐間和郎，柚場俊康，宮崎謙一，鄭 雄一，小川久美子，新見伸吾，吉田 緑：PVC 製血液バッグに適用可能な新規可塑剤 NJC-NP の毒性評価．日本薬学会第 135 年会(2015.3，神戸)．

報道発表等

特になし

H. 知的財産権の出願・登録状況

1) 蓜島由二，福井千恵，河上強志，迫田秀行，野村祐介，伊佐間和郎，新見伸吾，柚場俊康，向井智和，清麻里子．「医療用バッグ」．出願番号：特願 2015-17514，出願日：2015 年 1 月 30 日．

参考文献

1) Haishima Y, Kawakami T, Hasegawa C, Tanoue A, Yuba T, Isama K, Matsuoka A, and Niimi S. Screening study on hemolysis suppression effect of an alternative plasticizer for the development of a novel blood container made of polyvinyl chloride. *J. Biomed. Mater. Res. Part B*, 102:721-728 (2014).
2) Haishima Y, Hasegawa C, Nonura Y, Kawakami T, Yuba T, Shindo T, Sakaguchi K, Tanigawa T, Inukai K, Takenouchi M, Isama K, Matsuoka A, and Niimi S. Development and performance evaluation of a positive reference material for hemolysis testing. *J. Biomed. Mater. Res. Part B*, 102:1809-1816 (2014).
3) ASTM Standard F756-08 (2008) Practice for assessment of hemolytic properties of materials, ASTM International, West Conshohocken, PA.
4) National Institutes of Health (1977) Evaluation of hemodialyzers and dialysis membranes, <Hemolysis-Rabbit Blood> DHEW Publication 77-1294, Bethesda, MD.
5) ISO/TC194 国内委員会：第 8 部血液適合性試験．医療機器の製造販売承認申請等に必要な生物学的安全性評価の基本的考え方について（編集: 松岡厚子）．薬事日報社（東京）2013, pp. 189-203.
6) Haishima Y, Kawakami T, Fukui C, Tanoue A, Yuba T, Ozono S, Kumada H, Inoue K, Morikawa T, Takahashi M, Fujisawa A, Yamasaki K, Nomura Y, Isama K, Chung U, Ogawa K, Niimi S, Yoshida M.: Characte- rization of alternative plasticizers in polyvinyl chloride sheets for blood containers. *J. Vinyl Add. Technol.*, 2014, in press.
7) Ito R, Seshimo F, Haishima Y, Hasegawa C, Isama K, Yagami T, Nakahashi K, Yamazaki H, Inoue K, Yoshimura Y, Saito K, Tsuchiya Y, Nakazawa H.: Novel approach for reducing migration of di-2-ethylhexyl phthalate from polyvinyl chloride medical device. *Int. J. Pharm.*, 303:104-112 (2005).
8) Haishima Y, Seshimo F, Higuchi T, Yamazaki H, Hasegawa C, Izumi S, Makino T, Nakahashi K, Ito R, Inoue K, Yoshimura Y, Saito K, Yagami T, Tsuchiya T, Nakazawa H.: Development of a simple method for predicting the levels of di(2-ethylhexyl)phthalate migrated from PVC medical devices into pharmaceutical solutions. *Int. J. Pharm.*, 298: 126-142 (2005).
9) Haishima Y, Matsuda R, Hayashi Y, Hasegawa C, Yagami T, Tsuchiya T.: Risk assessment of di(2-ethylhexyl) phthalate eluted from PVC blood cir-