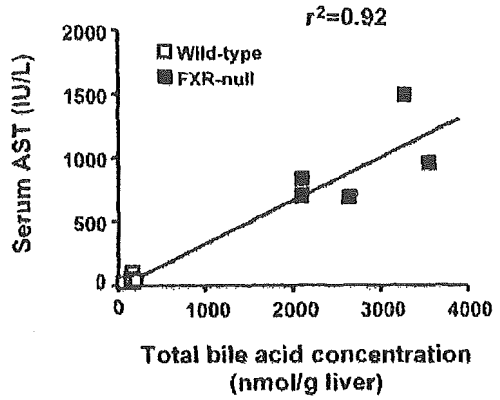

Fig. 1. Correlation between serum AST activity and hepatic total bile acid concentrations. Sera and livers were isolated from wild-type and FXR-null mice fed a 0.25% cholic acid diet for 5 days. Total bile acid concentrations and AST activities were measured by enzyme-colorimetric method.

rates were observed in FXR-null mice fed a 1% cholic acid diet, compared with FXR-null mice fed the control diet (data not shown).

Tauro-Conjugated and Unconjugated Bile Acid Levels in Liver and Bile. Biliary bile acids are conjugated with taurine before their excretion in control mice. This is consistent with reports showing no transport activity of unconjugated bile acids such as cholic acid in Bsep-expressing cells (Gerloff et al., 1998). BACS and BAT are involved in tauro-conjugation. Thus, the amount of both unconjugated and tauro-conjugated forms of hepatic bile acids in cholic acid-fed wild-type and FXR-null mice was determined by HPLC (Fig. 4A). Cholic acid, taurocholic acid, deoxycholic acid, and taurodeoxycholic acid were detected as major components in the livers of FXR-null and the wild-type mice after feeding of 0.25% cholic acid diet. Taurocholic acid was the major component in livers of FXR-null mice fed 0.25% cholic acid diet (Fig. 4B). Hepatic taurodeoxycholic acid level was also significantly higher in FXR-null mice than in wild-type mice after feeding the 0.25% cholic acid diet. Thus, hepatic tauro-conjugated bile acids accounted for more than 90% of the total bile acids detected in the livers of FXR-null mice fed a 0.25% cholic acid diet. In contrast, high levels of unconjugated cholic acid were detected in the bile of wild-type mice fed a 1% cholic acid diet (Fig. 5A). However, unconjugated deoxycholic acid levels were very low in the bile. In contrast to the control diet, the biliary ratio of unconjugated cholic acid to total cholic acid was increased to 32.9% in wild-type mice fed a 1% cholic acid diet, whereas it accounted only for 0.6 and 0.4% in the wild-type and FXR-null mice fed a 0.25% cholic acid diet, respectively (Fig. 5B).

To assess the relationship between depletion of taurine and the increased ratio of unconjugated cholic acid in liver and bile of wild-type mice fed a cholic acid diet, hepatic taurine levels were determined in both wild-type and FXR-null mice fed a cholic acid diet. Hepatic taurine level was clearly decreased upon cholic acid feeding FXR-null and the wild-type mice. The level in wild-type mice fed a 1% cholic acid diet was reduced to 10% of the control diet fed mice (Fig. 6A). It is noteworthy that ATP is necessary for the Bsep-mediated bile acid transport as well as tauro-conjugation. Since depletion of hepatic ATP affects the transport activity,

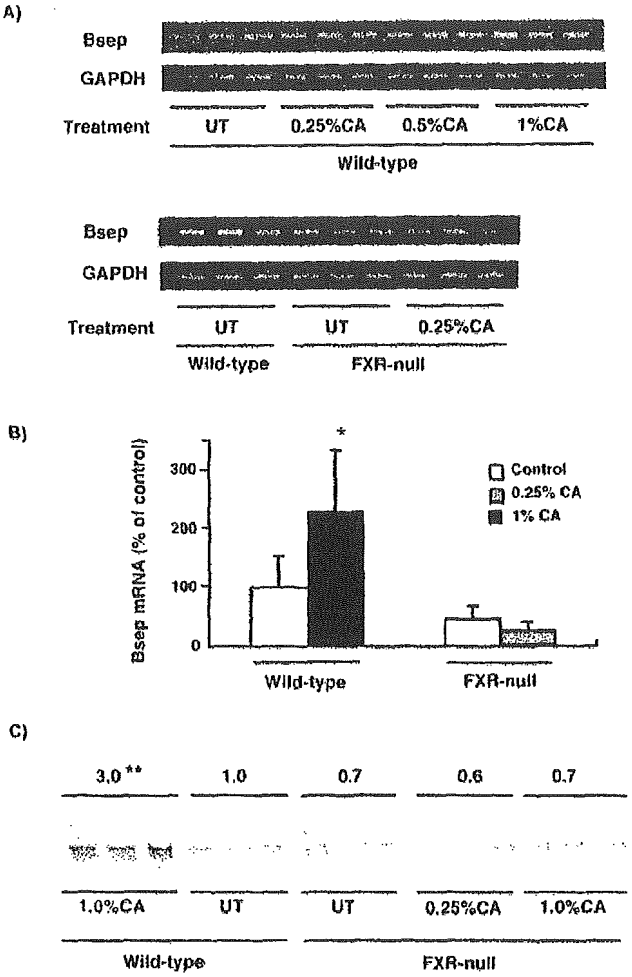

Fig. 2. Hepatic Bsep mRNA and protein levels. A, changes in hepatic Bsep mRNA levels. Hepatic mRNAs were prepared from wild-type and FXR-null mice fed a control diet (UT) or the diet containing the indicated percentage of cholic acid for 5 days. Specific primers described under *Materials and Methods* were used. B, quantification of Bsep mRNA level by real-time PCR. Data are shown as the mean ± S.D. (n = 3). *, significant difference from control group ($p < 0.05$). C, immunoblot analyses of hepatic Bsep protein. Hepatic crude liver membranes (50 μg) were analyzed by immunoblots with antibody to mouse Bsep. Values are the average obtained from three animals in each group. **, significantly different from control group ($p < 0.01$).

hepatic ATP levels were quantified in cholic acid-fed mice. Hepatic ATP levels were, however, not significantly decreased in wild-type and FXR-null mice fed the cholic acid diet, compared with the control diet (Fig. 6B).

Specific mRNA Levels of Bile Acid Transporters and Amino Acid Conjugation Enzymes. To explore the involvement of bile acid-related genes other than Bsep in protection against hepatic bile acid accumulation, changes in the hepatic mRNA levels of bile acid transporters and amino acid conjugation enzymes were analyzed by RT-PCR (Fig. 7). Hepatic Cyp7a1 mRNA was higher in FXR-null mice fed control diet than in wild-type mice. The level was decreased in cholic acid-fed wild-type and FXR-null mice. Mrp2 mRNA level was slightly increased in cholic acid-fed wild-type and FXR-null mice. On the other hand, the major bile acid uptake transporters Ntcp and Oatp1 mRNA levels were decreased upon

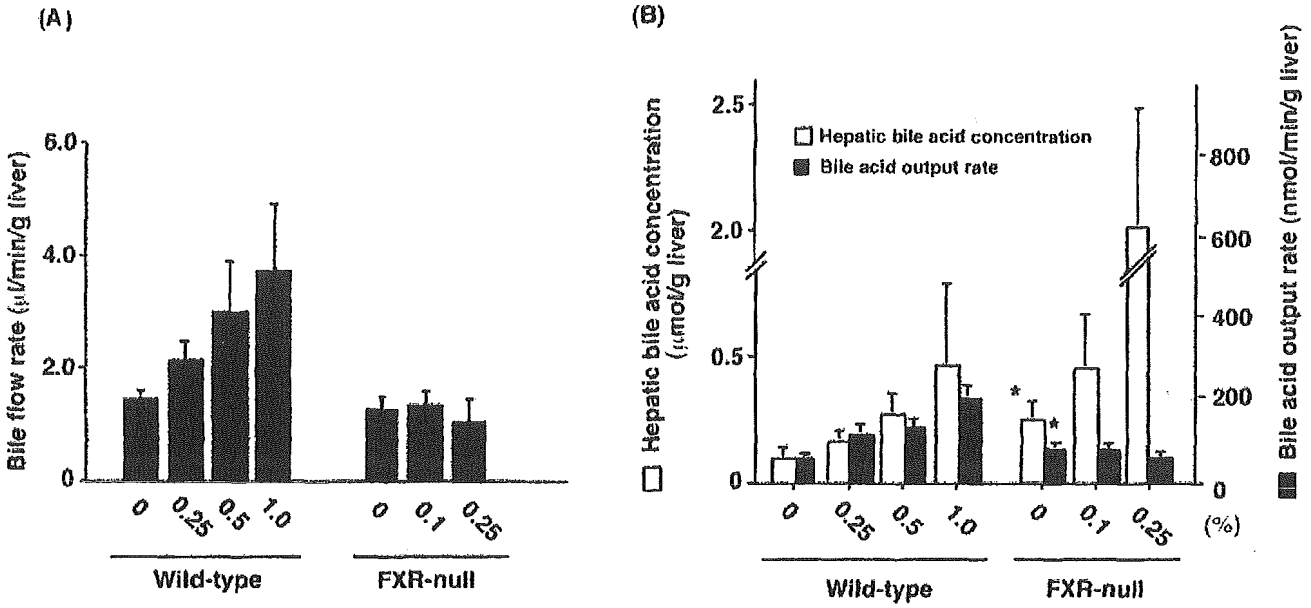

Fig. 3. Influence of cholic acid feeding on bile flow, bile output rate, and hepatic bile acid concentration. A, bile flow. B, bile acid output rate and hepatic bile acid concentrations. Bile and livers were isolated from wild-type and FXR-null mice fed a diet containing the indicated percentage of cholic acid for 5 days. Bile was collected for 30 min by bile duct cannulation. Bile flow was determined gravimetrically assuming a density of 1.0 g/ml. Hepatic bile acid concentrations were measured by enzyme-colorimetric method. Data are shown as the mean ± S.D. ($n = 5$). *, significantly different from corresponding wild-type group ($p < 0.05$).

cholic acid-feeding of wild-type mice, whereas only Oatp1 mRNA levels were decreased in cholic acid-fed FXR-null mice. Hepatic mRNA levels of BACS and BAT that mediate tauro-conjugation of bile acids were not significantly changed in FXR-null mice or after cholic acid feeding. Specific mRNA level of the taurine transporter was significantly increased in both mice fed the cholic acid diet.

Discussion

The aim of the present study was to determine the in vivo mechanism for FXR-mediated protection against extraordinarily high bile acid levels by use FXR-null and wild-type mice fed cholic acid diets. A clear correlation was found between individual hepatic bile acid concentrations and AST activities suggests that major hepatic bile acid taurocholic acid but not unconjugated cholic acid is involved in hepatotoxicity in FXR-null mice fed a cholic acid diet. Bile flow and bile acid output rates of wild-type mice fed a cholic acid diet increased with increasing hepatic bile acid concentration (from 0.1–0.5 μmol/g liver), whereas those of FXR-null mice did not increase, but decreased in spite of the marked accumulation of hepatic bile acids (2.0 μmol/g liver) after feeding a 0.25% cholic acid diet. The data on bile acid output rates and hepatic bile acid concentrations indicate that targeted disruption of FXR results in the impairment of the regulation of canalicular bile acid excretion to maintain a low level of hepatic bile acids.

Consistent with previous reports (Schuetz et al., 2001; Kok et al., 2003; Lambert et al., 2003; Zollner et al., 2003), Bsep mRNA and protein levels were lower in FXR-null mice fed a control diet than that in the wild-type mice in spite of higher bile acid output rates in FXR-null mice. Bsep protein levels were not correlated with bile acid output rates between FXR-null and the wild-type mice fed control diets. These results suggest a higher efficacy for Bsep-mediated excretion in FXR-null mice. It is known that the functional activity of ABC transporters, including Bsep and Mdr1 are regulated by several posttranslational processes such as protein phosphorylation and cellular localization (Kullak-Ublick et al., 2004). Because hepatic bile acid concentrations were 2.5-fold higher in FXR-null mice fed control diets compared with wild-type mice, this discordance might reflect an adaptive response of Bsep to high levels of hepatic bile acids. In wild-type mice fed a cholic acid diet, the adaptive enhancement of canalicular bile acid excretion capacity dependent on the increase in both the Bsep protein levels and possibly the rates of Bsep-mediated excretion is likely one of the critical determinants for the suppression of hepatic bile acid accumulation.

Several factors, including hepatic bile acid levels and ATP levels, were shown to modulate canalicular bile acid excretion. Amino acid conjugation is one of the factors involved in canalicular bile acid excretion. Human BAT and BACS, involved in the conjugation of bile acids to taurine and glycine, respectively, are positively regulated by FXR (Pircher et al., 2003). As shown in Figs. 4 and 5A, nearly all of the cholic acids were detected as tauro-conjugated derivatives in bile and liver of FXR-null mice fed a 0.25% cholic acid diet. The tauro-conjugation capacity is thus unlikely to be the cause of loss of inducibility of biliary bile acid excretion in FXR-null mice fed a cholic acid diet. Tauro-conjugated bile acids accumulate in livers of FXR-null mice fed a 0.25% cholic acid diet, which is due at least in part by the loss of inducibility of bile acid output rate in FXR-null mice fed 0.25% cholic acid diet.

More than 30% of the total cholic acid content was detected as unconjugated cholic acid in the bile of wild-type mice fed a 1% cholic acid diet. The increased ratio of biliary unconjugated cholic acid to total cholic acid suggests the existence of

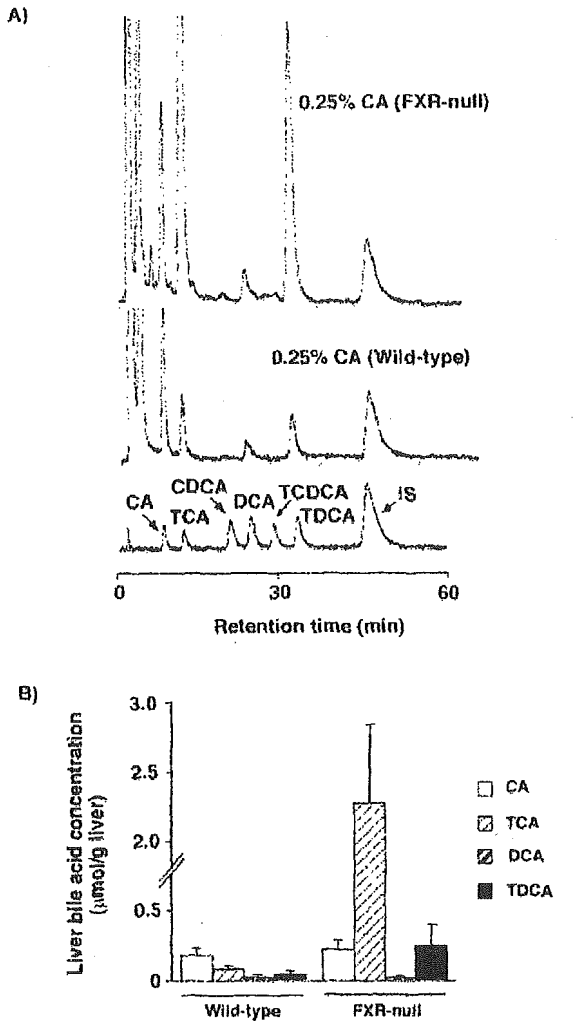

Fig. 4. Hepatic bile acid contents in FXR-null and wild-type mice fed a 0.25% cholic acid diet. A, representative HPLC chromatograms with fluorescence detection of hepatic bile acids. Equal amounts of bile acids were injected as a standard. IS, internal standard (androstandiol). B, hepatic bile acid contents. The contents of bile acids were determined by HPLC. Wild-type and FXR-null mice were fed a 0.25% cholic acid diet for 5 days. Data are shown as the mean ± S.D. (n = 5). TCA, taurocholic acid; DCA, deoxycholic acid; TDCA, taurodeoxycholic acid; CDCA, chenodeoxycholic acid; TCDCA, taurochenodeoxycholic acid.

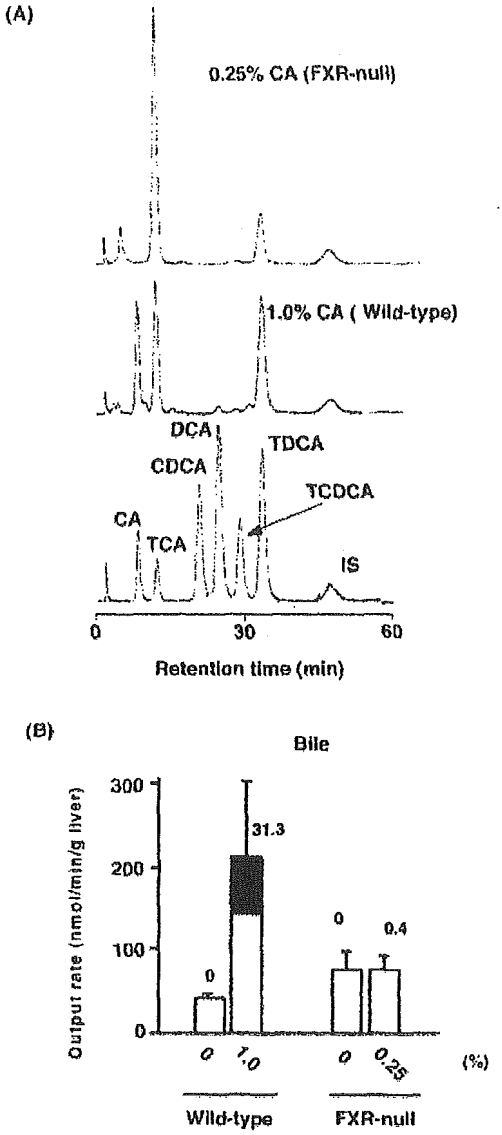

Fig. 5. Tauro-conjugated and unconjugated cholic acid levels in bile. A, representative HPLC chromatograms with fluorescence detection of biliary bile acids isolated from wild-type mice fed a 1.0% cholic acid diet and FXR-null mice fed a 0.25% cholic acid diet. Equal amounts of bile acids were injected as a standard. IS, internal standard (androstandiol). B, tauro-conjugated and unconjugated cholic acid levels in bile. Bile samples were isolated from wild-type and FXR-null mice fed a diet containing the indicated percentage of cholic acid for 5 days. Cholic acid amounts were measured by HPLC. The numbers above bar graphs indicate ratio of unconjugated cholic acid (closed bar) to total cholic acid (tauro-conjugated and unconjugated cholic acid) as percentage value. Data are shown as the mean ± S.D. (n = 5).

an unconjugated cholic acid excreting system in the wild-type mice. The excreting system may become evident as an adaptive response under the condition of hepatic taurine depletion. Amino acid conjugation is not necessarily the obligatory step for the excretion. Although the system involved in unconjugated cholic acid excretion has not been identified in the present study, it remains a possibility that there is an alternative transport system other than Bsep. Thus, it cannot be excluded that acyl glucuronides of cholic acid are formed and excreted, followed by hydrolysis in bile.

Although bile acids excreted by Bsep are the major osmotic driving force generating bile flow, no significant decrease in bile flow was found in Bsep-null mice (Wang et al., 2001). Bsep-null mice still have a capacity to excrete the bile acid into bile (30% of wild-type mice), suggesting the existence of an alternative bile acid transport system. Furthermore, bile flow and bile acid output rates of Bsep-null mice increase after feeding a 0.5% CA diet (Wang et al., 2003), a result that differs from those of FXR-null mice fed a cholic acid diet. These results suggest that a Bsep-independent bile acid excretion system was induced in Bsep-null mice fed a cholic acid diet. This excretion system is unlikely to be enhanced in FXR-null mice fed a cholic acid diet. An FXR-mediated adaptive bile acid excretion system other than Bsep might also be involved in the increase in bile acid output rate, including excretion of the unconjugated cholic acid in the wild-type

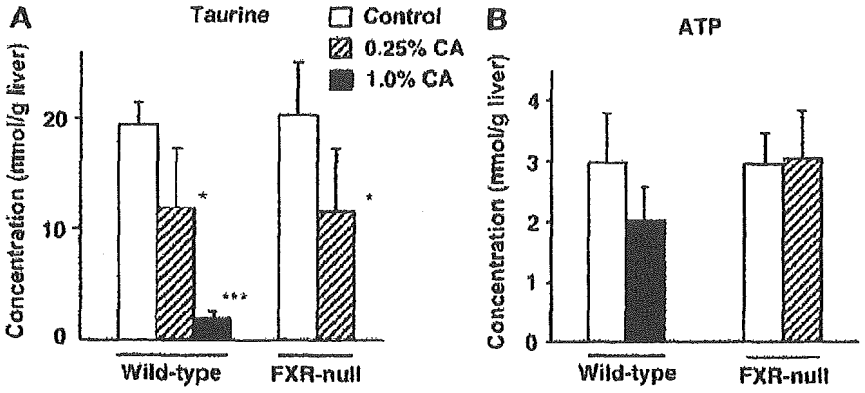

Fig. 6. Hepatic taurine and ATP level. A, taurine. B, ATP. Wild-type and FXR-null mice were fed a diet containing the indicated percentage of cholic acid for 5 days to isolate. Experimental details of the measurement of hepatic taurine and ATP contents are described under *Materials and Methods*. Data are shown as the mean ± S.D. ($n = 5$). Significantly different from corresponding control group (*, $p < 0.05$; ***, $p < 0.001$).

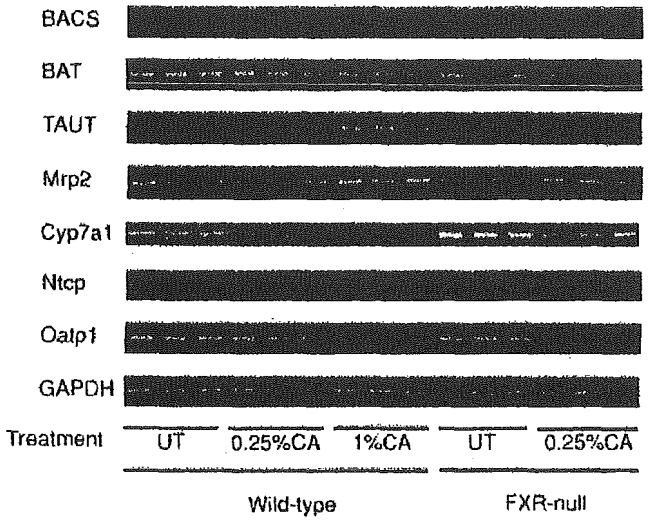

Fig. 7. Hepatic mRNA levels of bile acid-related genes. Hepatic mRNA levels of bile acid-related genes were measured by RT-PCR. Hepatic mRNAs were prepared from wild-type and FXR-null mice fed a control diet (UT) or the diet containing the indicated percentage of cholic acid for 5 days. Specific primers described under *Materials and Methods* were used.

mice fed a cholic acid diet, although the increase in Bsep protein levels likely explains at least in part the increase in bile acid output rate in wild-type mice fed a cholic acid diet.

Disruption of FXR enhances the sensitivity to cholic acid-induced toxicity. The enhancement of bile acid output rate mediated by FXR is likely a critical determinant for protection against cholic acid-induced liver toxicity. In contrast to cholic acid-induced toxicity, induction of toxicity by the secondary bile acid lithocholic acid is ameliorated by hydroxysteroid sulfotransferase St2a induced through PXR (Sonoda et al., 2002; Kitada et al., 2003) (our unpublished observation). This fact supports the idea that PXR is involved in the protection against lithocholic acid-induced liver toxicity (Staudinger et al., 2001; Xie et al., 2001). Nuclear receptor-mediated protective mechanisms of cholic acid-induced toxicity might be different from that of lithocholic acid-induced toxicity. Recently, it was reported that constitutive androstane receptor is also involved in protection against cholic acid-induced toxicity by using PXR-FXR double null mice (Guo et al., 2003). Role of the nuclear receptor interaction among FXR, PXR, and constitutive androstane receptor in the protection against bile acid-induced toxicity remains to be fully clarified.

The present study demonstrated that the adaptive enhancement of canalicular bile acid excretion is one of the critical protective mechanisms for cholic acid-induced toxicity. Furthermore, the transport system of unconjugated cholic acid is at least in part involved in protection against hepatic cholic acid accumulation under the condition of hepatic taurine depletion. Ileal mRNA levels of apical sodium-dependent bile acid transporter was 3.0-fold higher in FXR-null mice fed a cholic acid diet, compared with that of the wild-type mice fed the same diet (Maeda et al., 2004). It was also reported that the absolute amounts of bile acids reabsorbed from the intestine is enhanced by 2-fold in FXR-null mice (Kok et al., 2003). Although the analysis of the reabsorption process in intestine is necessary to explore the precise mechanism for protection against hepatic bile acid accumulation, FXR-null mice fed a cholic acid diet might be useful model to evaluate the possible involvement of Bsep in cholestasis.

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References

Ananthanarayanan M, Balasubramanian N, Makishima M, Mangelsdorf DJ, and Suchy FJ (2001) Human bile salt export pump promoter is transactivated by the farnesoid X receptor/bile acid receptor. *J Biol Chem* **276**:28857–28865.

Bjorkhem I (1985) mechanism of bile acid biosynthesis in mammalian liver, in *Steroids and Bile acids* (Danielsson H and Sjovall J eds) pp 231–260, Elsevier Science, New York.

Byrne JA, Strautnieks SS, Mieli-Vergani G, Higgins CF, Linton KJ, and Thompson RJ (2002) The human bile salt export pump: characterization of substrate specificity and identification of inhibitors. *Gastroenterology* **123**:1649–1658.

Chiang JY, Kimmel R, Weinberger C, and Stroup D (2000) Farnesoid X receptor responds to bile acids and represses cholesterol 7alpha-hydroxylase gene (CYP7A1) transcription. *J Biol Chem* **275**:10918–10924.

Czuba B and Vessey DA (1981) Purification and characterization of cholyl-CoA: taurine N-acetyltransferase from the liver of domestic fowl (*Gallus gallus*). *Biochem J* **195**:263–266.

Denson LA, Sturm E, Echevarria W, Zimmerman TL, Makishima M, Mangelsdorf DJ, and Karpen SJ (2001) The orphan nuclear receptor, shp, mediates bile acid-induced inhibition of the rat bile acid transporter, ntcp. *Gastroenterology* **121**:140–147.

Falany CN, Fortinberry H, Leiter EH, and Barnes S (1997) Cloning, expression and chromosomal localization of mouse liver bile acid CoA:amino acid N-acyltransferase. *J Lipid Res* **38**:1139–1148.

Gerloff T, Stieger B, Hagenbuch B, Madon J, Landmann L, Roth J, Hofmann AF, and Meier PJ (1998) The sister of P-glycoprotein represents the canalicular bile salt export pump of mammalian liver. *J Biol Chem* **273**:10046–10050.

Goodwin B, Jones SA, Price RR, Watson MA, McKee DD, Moore LB, Galardi C, Wilson JG, Lewis MC, Roth ME, et al. (2000) A regulatory cascade of the nuclear receptors FXR, SHP-1 and LRH-1 represses bile acid biosynthesis. *Mol Cell* **6**:517–526.

Guo GL, Lambert G, Negishi M, Ward JM, Brewer HB Jr, Kliewer SA, Gonzalez FJ, and Sinal CJ (2003) Complementary roles of farnesoid X receptor, pregnane X receptor and constitutive androstane receptor in protection against bile acid toxicity. *J Biol Chem* **278**:45062–45071.

Honma W, Shimada M, Sasano H, Ozawa S, Miyata M, Nagata K, Ikeda T, and

Yamazoe Y (2002) Phenol sulfotransferase, ST1A3, as the main enzyme catalyzing sulfation of troglitazone in human liver. *Drug Metab Dispos* **30**:944–949.
Kitada H, Miyata M, Nakamura T, Tozawa A, Honma W, Shimada M, Nagata K, Sinal CJ, Guo GL, Gonzalez FJ, et al. (2003) Protective role of hydroxysteroid sulfotransferase in lithocholic acid-induced liver toxicity. *J Biol Chem* **278**:17838–17844.
Kok T, Hulzebos CV, Wolters H, Havinga R, Agellon LB, Stellaard F, Shan B, Schwarz M, and Kuipers F (2003) Enterohepatic circulation of bile salts in farnesoid X receptor-deficient mice: efficient intestinal bile salt absorption in the absence of ileal bile acid-binding protein. *J Biol Chem* **278**:41930–41937.
Kullak-Ublick GA, Stieger B, and Meier PJ (2004) Enterohepatic bile salt transporters in normal physiology and liver disease. *Gastroenterology* **126**:322–342.
Lambert G, Amar MJ, Guo G, Brewer HB Jr, Gonzalez FJ, and Sinal CJ (2003) The farnesoid X-receptor is an essential regulator of cholesterol homeostasis. *J Biol Chem* **278**:2563–2570.
Lee CH, Bradley G, Zhang JT, and Ling V (1993) Differential expression of P-glycoprotein genes in primary rat hepatocyte culture. *J Cell Physiol* **157**:392–402.
Lu TT, Makishima M, Repa JJ, Schoonjans K, Kerr TA, Auwerx J, and Mangelsdorf DJ (2000) Molecular basis for feedback regulation of bile acid synthesis by nuclear receptors. *Mol Cell* **6**:507–515.
Lundin A and Thore A (1975) Analytical information obtainable by evaluation of the time course of firefly bioluminescence in the assay of ATP. *Anal Biochem* **66**:47–63.
Maeda T, Miyata M, Yotsumoto T, Kobayashi D, Nozawa T, Toyama K, Gonzalez FJ, Yamazoe Y, and Tamai I (2004) Regulation of drug transporters by the farnesoid X receptor in mice. *Mol Pharmaceutics* **1**:281–289.
Makishima M, Lu TT, Xie W, Whitfield GK, Domoto H, Evans RM, Haussler MR, and Mangelsdorf DJ (2002) Vitamin D receptor as an intestinal bile acid sensor. *Science (Wash DC)* **296**:1313–1316.
Makishima M, Okamoto AY, Repa JJ, Tu H, Learned RM, Luk A, Hull MV, Lustig KD, Mangelsdorf DJ, and Shan B (1999) Identification of a nuclear receptor for bile acids. *Science (Wash DC)* **284**:1362–1365.
Noe J, Stieger B, and Meier PJ (2002) Functional expression of the canalicular bile salt export pump of human liver. *Gastroenterology* **123**:1659–1666.
Parks DJ, Blanchard SG, Bledsoe RK, Chandra G, Consler TG, Kliewer SA, Stimmel JB, Willson TM, Zavacki AM, Moore DD, et al. (1999) Bile acids: natural ligands for an orphan nuclear receptor. *Science (Wash DC)* **284**:1365–1368.
Pircher PC, Kitto JL, Petrowski ML, Tangirala RK, Bischoff ED, Schulman IG, and Westin SK (2003) Farnesoid X receptor regulates bile acid-amino acid conjugation. *J Biol Chem* **278**:27703–27711.
Repa JJ, Turley SD, Lobaccaro JA, Medina J, Li L, Lustig K, Shan B, Heyman RA, Dietschy JM, and Mangelsdorf DJ (2000) Regulation of absorption and ABC1-mediated efflux of cholesterol by RXR heterodimers. *Science (Wash DC)* **289**:1524–1529.
Schersten T, Bjorntorp P, Ekdahl PH, and Bjorkerud S (1967) The synthesis of taurocholic and glycocholic acids by preparations of human liver. II. An analysis of the stimulating effect of the L fraction. *Biochim Biophys Acta* **141**:155–163.
Schuetz EG, Strom S, Yasuda K, Lecureur V, Assem M, Brimer C, Lamba J, Kim RB, Ramachandran V, Komoroski BJ, et al. (2001) Disrupted bile acid homeostasis reveals an unexpected interaction among nuclear hormone receptors, transporters and cytochrome P450. *J Biol Chem* **276**:39411–39418.
Sinal CJ, Tohkin M, Miyata M, Ward JM, Lambert G, and Gonzalez FJ (2000) Targeted disruption of the nuclear receptor FXR/BAR impairs bile acid and lipid homeostasis. *Cell* **102**:731–744.
Solaas K, Ulvestad A, Soreide O, and Kase BF (2000) Subcellular organization of bile acid amidation in human liver: a key issue in regulating the biosynthesis of bile salts. *J Lipid Res* **41**:1154–1162.
Sonoda J, Xie W, Rosenfeld JM, Barwick JL, Guzelian PS, and Evans RM (2002) Regulation of a xenobiotic sulfonation cascade by nuclear pregnane X receptor (PXR). *Proc Natl Acad Sci USA* **99**:13801–13806.
Staudinger JL, Goodwin B, Jones SA, Hawkins-Brown D, MacKenzie KI, LaTour A, Liu Y, Klaassen CD, Brown KK, Reinhard J, et al. (2001) The nuclear receptor PXR is a lithocholic acid sensor that protects against liver toxicity. *Proc Natl Acad Sci USA* **98**:3369–3374.
Stieger B, Fattinger K, Madon J, Kullak-Ublick GA, and Meier PJ (2000) Drug- and estrogen-induced cholestasis through inhibition of the hepatocellular bile salt export pump (Bsep) of rat liver. *Gastroenterology* **118**:422–430.
Wang H, Chen J, Hollister K, Sowers LC, and Forman BM (1999) Endogenous bile acids are ligands for the nuclear receptor FXR/BAR. *Mol Cell* **3**:543–553.
Wang R, Lam P, Liu L, Forrest D, Yousef IM, Mignault D, Phillips MJ, and Ling V (2003) Severe cholestasis induced by cholic acid feeding in knockout mice of sister of P-glycoprotein. *Hepatology* **38**:1489–1499.
Wang R, Salem M, Yousef IM, Tuchweber B, Lam P, Childs SJ, Helgason CD, Ackerley C, Phillips MJ, and Ling V (2001) Targeted inactivation of sister of P-glycoprotein gene (spgp) in mice results in nonprogressive but persistent intrahepatic cholestasis. *Proc Natl Acad Sci USA* **98**:2011–2016.
Waterfield CJ (1994) Determination of taurine in biological samples and isolated hepatocytes by high-performance liquid chromatography with fluorimetric detection. *J Chromatogr B Biomed Appl* **657**:37–45.
Xie W, Radominska-Pandya A, Shi Y, Simon CM, Nelson MC, Ong ES, Waxman DJ, and Evans RM (2001) An essential role for nuclear receptors SXR/PXR in detoxification of cholestatic bile acids. *Proc Natl Acad Sci USA* **98**:3375–3380.
Zollner G, Fickert P, Fuchsbichler A, Silbert D, Wagner M, Arbeiter S, Gonzalez FJ, Marschall HU, Zatloukal K, Denk H, et al. (2003) Role of nuclear bile acid receptor, FXR, in adaptive ABC transporter regulation by cholic and ursodeoxycholic acid in mouse liver, kidney and intestine. *J Hepatol* **39**:480–488.

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In silico assessment of chemical mutagenesis in comparison with results of Salmonella microsome assay on 909 chemicals

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Abstract

Genotoxicity is one of the important endpoints for risk assessment of environmental chemicals. Many short-term assays to evaluate genotoxicity have been developed and some of them are being used routinely. Although these assays can generally be completed within a short period, their throughput is not sufficient to assess the huge number of chemicals, which exist in our living environment without information on their safety. We have evaluated three commercially available in silico systems, i.e., DEREK, MultiCASE, and ADMEWorks, to assess chemical genotoxicity. We applied these systems to the 703 chemicals that had been evaluated by the Salmonella/microsome assay from CGX database published by Kirkland et al. [1]. We also applied these systems to the 206 existing chemicals in Japan that were recently evaluated using the Salmonella/microsome assay under GLP compliance (ECJ database). Sensitivity (the proportion of the positive in Salmonella/microsome assay correctly identified by the in silico system), specificity (the proportion of the negative in Salmonella/microsome assay correctly identified) and concordance (the proportion of correct identifications of the positive and the negative in Salmonella/microsome assay) were increased when we combined the three in silico systems to make a final decision in mutagenicity, and accordingly we concluded that in silico evaluation could be optimized by combining the evaluations from different systems. We also investigated whether there was any correlation between the Salmonella/microsome assay result and the molecular weight of the chemicals: high molecular weight (>3000) chemicals tended to give negative results. We propose a decision tree to assess chemical genotoxicity using a combination of the three in silico systems after pre-selection according to their molecular weight.

Keywords: In silico; (Quantitative) structure-activity relationship; (Q)SAR; Chemical genotoxicity; Decision tree

1. Introduction

It is said that more than 20,000 chemicals are in use in Japan. Among them, only approximately 10% are thought to have been assessed for human hazard based on data from in vitro and in vivo bioassays. According to the "Law Concerning the Evaluation of Chemical Substances and Regulation of Their Manufacture, etc." [2], the Salmonella/microsome (Ames) assay, in vitro chromosomal aberration assay (or alternatively mouse lymphoma TK assay), and 28-day repeat dose toxicity test in rodents are obligatory to notify new chemicals for production/import at a level of more than 10 t per year.

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To screen the remaining 18,000 chemicals for human hazard by application of this three-test battery is not realistic from the time and economical point of view. We need a much higher-throughput system to assess these chemicals, at least for prioritization of those chemicals that should be submitted to biological testing. To assess human hazard for regulatory purposes, in silico systems are now beginning to be used [3]. Here, we evaluated three commercially available in silico (quantitative) structure-activity relationship ((Q)SAR) systems and tried to construct a decision tree for prioritization of which chemicals need in vitro and/or in vivo testing. Also, within the drug discovery process, integrated computational analysis has been proposed to be incorporated as a toxicity prediction tool [4].

Kirkland et al. [1] published a database (CGX database, see http://www.lhasalimited.org/cgx) for nearly 1000 carcinogens and non-carcinogens with results of representative in vitro genotoxicity assays, i.e., Salmonella/microsome assay (Ames), mouse lymphoma TK assay using L5178Y cells (MLA), and in vitro chromosomal aberration assay or in vitro micronucleus assay (CA/MN). We used 703 chemicals that had been assessed in the Ames assay for evaluation of the three in silico systems, i.e., DEREK, MultiCASE (MCase), and ADMEWorks (AWorks). We also used a database (the ECJ database) that we constructed from chemicals existing in Japan that had recently been assessed in the Ames assay, in vitro chromosomal aberration assay, and 28 day repeat dose rodent toxicity test and/or reproductive and developmental toxicity test for their safety evaluation under GLP compliance. The ECJ database consisted of 206 chemicals but only 26 chemicals were positive by the Ames assay. Initially we evaluated both sensitivity and specificity of these three systems using the ECJ database of 206 chemicals [5].

We selected these three in silico systems because of their different modes of analysis. DEREK is a rule-based system [6], MCase [7] is a database/substructure based system, and AWorks is a QSAR. We applied these systems individually to assess gene-mutation induction on the 703 and 206 chemical sets described above and evaluated their sensitivity, specificity, concordance, and applicability (how many chemicals could be assessed), independently.

It is known that high molecular weight polymers tend not to induce gene mutation and chromosomal aberrations mainly because they cannot enter the target cells to react with DNA, or other bio-molecules necessary for genetic stability. We analyzed 194 Ames positive chemicals (confidential source) for the effect of molecular weight.

2. Materials and methods

2.1. Data sources for chemicals assessed

Of about 1000 chemicals, 703 that had been assessed in the Ames test were chosen from the CGX database published by Kirkland et al. [1]. All chemical structures were re-drawn using Chemdraw Ultra (Cambridge Soft Corporation, USA) and converted to MOL files before application to each system. We also used the database of 206 chemicals evaluated in the MHLW project "Safety Examination of Existing Chemicals and Safety Programmes in Japan" (ECJ database). The test summary for each of these chemicals can be seen at http://wwwdb.mhlw.go.jp/ginc/html/db1.html. In addition, we collected 194 Ames positive chemicals from a confidential source and investigated the relationship between gene mutation induction and molecular weight, with identification of any active side chain that might have contributed to the positive result in the Ames assay.

2.2. In silico systems used and definition of positive and negative responses

We used DEREK (Lhasa Ltd., UK) version 8.0.1.When the system gave an evaluation as "certain", "probable" or "plausible" we considered this as "positive", and when the system gave "equivocal", "doubted", "improbable", "impossible", or "no alert" we considered this as "negative". We used MCase (Multicase Co. Ltd.) version mc4pc. When the system gave "active" or "marginal" we considered this as "positive", and when the system gave "Inactive" we considered this as "negative". In the case of AWorks (Fujitsu Kitakyushu, Co. Ltd., version 2.0), we considered as "positive" when system evaluation was "positive", and considered as "negative" when the system evaluation was "negative". We excluded chemicals from further analysis when DEREK or AWorks gave no answer, or the evaluation was "inconclusive" by MCase.

2.3. Definition of sensitivity, specificity, concordance, and applicability

We calculated sensitivity, specificity, concordance, and applicability as follows:

$$\text{sensitivity}=\frac{N_{A+S+}}{N_{A+}}\times 100, \qquad \text{specificity}=\frac{N_{A-S-}}{N_{A-}}\times 100,$$

$$\text{concordance}=\frac{N_{A+S+}+N_{A-S-}}{N_{\text{eval}}}\times 100,$$

$$\text{applicability}=\frac{N_{\text{eval}}}{N_{\text{all}}}\times 100$$

where N_{A+} is number of chemicals revealing positive in Ames assay; N_{A-} is number of chemicals negative in Ames assay; N_{A+S+} is number of chemicals revealing positive by both Ames assay and in silico evaluation; N_{A-S-} is number of chemicals negative in both Ames assay and in silico evaluation; N_{eval} is

Table 1
Performance of in silico systems

	Ames result	+	–	Total	Sensitivity (%)	Specificity (%)	Concordance (%)	Applicability (%)
CGX database								
DEREK	+	288	64	352				
	–	69	267	336	81.8	79.5	80.7	97.9
	Total	357	331	688				
MCase	+	235	32	267				
	–	6	249	255	88.0	97.6	92.7	74.3
	Total	241	281	522				
AWorks	+	267	89	356				
	–	149	187	336	75.0	55.7	65.6	98.4
	Total	416	276	692				
ECJ database								
DEREK	+	19	7	26				
	–	21	159	180	73.1	88.3	86.4	100.0
	Total	40	166	206				
MCase	+	13	7	20				
	–	13	133	146	65.0	91.1	88.0	80.6
	Total	26	140	166				
AWorks	+	19	7	26				
	–	54	124	178	73.1	69.7	70.1	99.0
	Total	73	131	204				

MCase: MultiCASE; AWorks: ADMEWorks.

number of chemicals evaluated; and N_{all} is total number of chemicals subjected.

3. Results

Among the set of 703 CGX chemicals with published Ames data, 358 were positive and 345 were negative. The results of the in silico evaluation are summarized in Table 1. The highest sensitivity, specificity, and concordance with Ames assay results was provided by MCase, then followed by DEREK. However, the systems that showed the best applicability were AWorks and (almost the same) DEREK, then followed by MCase. For the database of 206 ECJ chemicals, 26 were positive and 180 were negative. The outcomes of the in silico analyses are summarized in Table 1. The pattern of performance was very similar to that with the 703 chemicals in the CGX database.

Fig. 1 shows the cumulative percent of Ames positive chemicals against molecular weight. It can be seen that 87.1% of those positive chemicals had molecular weights less than 1000, and 96.4% had molecular weights less than 3000; in other words, only 3.6% of the chemicals with a molecular weight >3000 gave a positive response in the Ames assay. Seven of 194 Ames positive chemicals had a molecular weight >3000 and four of these seven polymers had epoxy groups.

When we combined the in silico systems, the performance was different from that when assessed individually (Table 2). If we considered the in silico mutagenicity as positive (or negative) when two or more systems gave positive (or negative) evaluations, 87.8

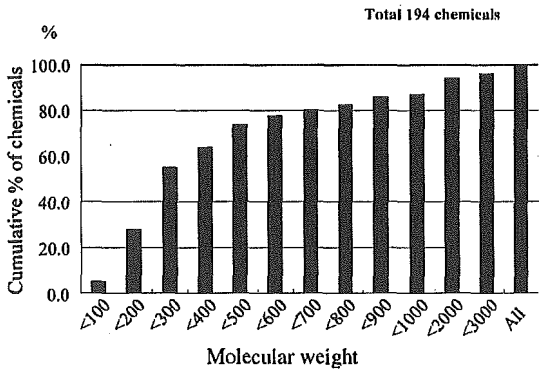

Fig. 1. Cumulative percentage of chemicals based on their molecular weight. 194 Ames positive chemicals were analyzed. 7/194 chemicals were more than 3000 molecular weight and Ames positive and 4/7 contained epoxy groups.

Table 2
Performance of in silico systems after combined

CGX database

In silico / Ames	+ + or + + +	- - or - - -	Total	Sensitivity (%)	Specificity (%)	Concordance (%)	Applicability (%)
+	279	40	319				
-	42	249	291	**87.8**	**85.6**	**86.7**	**86.8**
Total	321	289	610				
	+ + +	- - -					
+	166	1	167				
-	3	127	130	**99.4**	**97.7**	**98.7**	**42.2**
Total	168	129	297				

ECJ database

In silico / Ames	+ + or + + +	- - or - - -	Total	Sensitivity (%)	Specificity (%)	Concordance (%)	Applicability (%)
+	19	7	26				
-	23	147	170	**73.1**	**86.5**	**84.7**	**95.1**
Total	42	154	196				
	+ + +	- - -					
+	13	2	15				
-	5	94	99	**86.7**	**94.9**	**93.9**	**55.3**
Total	18	96	114				

Table 3
Performances of DEREK and MCase in several published papers.

Target compounds	In silico system	Sensitivity (%)	Specificity (%)	Concordance (%)	Applicability (%)	Reference
394 Drugs	DEREK	52	75	74	94[a]	[11]
	MCase	48	93	90	91[a]	
217 Non-drugs	DEREK	86	50	81	100[a]	[10]
	MCase	91	62	83	100[a]	
520 Drug candidates	DEREK	28	80	72	100	[13]
	MCase	50	86	81	41	
	DEREK + MCase	29	95	88	29	
	DEREK + Mcase + TOPKAT	75	96	95	15	
123 Drug candidates	DEREK	8[b]	31[c]	61	100[d]	[4]
	MCase (A2H)	13[b]	15[c]	72	97[d]	
	Topcat (Ames Mut)	18[b]	15[c]	67	98[d]	
	DEREK + MCase	6[b]	19[c]	75	97[d]	
	DEREK + Mcase + TOPKAT	5[b]	9[c]	86	46[d]	
94 Non-drugs	DEREK	63	81	76	100	[13]
	MCase	40	90	76	75	
	DEREK + MCase	47	100	85	56	
	DEREK + Mcase + TOPKAT	55	100	86	37	
516 Non-drugs	DEREK	6[b]	24[c]	70	100[d]	[4]
	MCase (A2H)	7[b]	12[c]	81	98[d]	
	Topcat (Ames Mut)	25[b]	19[c]	56	97[d]	
	DEREK + MCase	2[b]	16[c]	82	98[d]	
	DEREK + Mcase + TOPKAT	7[b]	10[c]	83	43[d]	

[a] Calculated by us
[b] % False negative.
[c] % False positive.
[d] (1-Indeterminate).

and 73.1% sensitivity, 85.6 and 86.5% specificity, 86.7 and 84.7% concordance, and 86.8 and 95.1% applicability were obtained for the CGX and ECJ databases, respectively. If we considered the in silico mutagenicity as positive (or negative) only when all three systems gave positive (or negative) evaluations, all performance measures (sensitivity, specificity, etc.) increased up to 98.7 and 93.9%. However, applicability decreased to 42.2 and 55.3%, which meant only about half of the chemicals in the CGX and ECJ databases could be evaluated. One chemical, *o*-phenylphenol [90-43-7], was positive in the Ames test but negative by all three in silico systems and three chemicals, carboxymethylnitrosourea [60391-92-6], methidathion [950-37-8], 1-nitroso-3,5-dimethyl-4-benzoylpiperazine [61034-40-0], were negative in the Ames test although all three in silico system gave positive evaluation for mutagenicity in the CGX database. When we used the ECJ database, 2-amino-1-naphthalenesulfonic acid [81-16-3] and 2-vinylpyridine [100-69-6] were positive in the Ames test but negative by all three in silico systems and there was no chemical that was negative in the Ames assay and all positive in in silico system. These exceptional chemicals are listed in Table 3 together with such chemicals taken from literatures.

4. Discussion

It is important to construct a strategy for efficient evaluation of the toxicity of a large number of existing chemicals. Even so-called short-term assays, e.g., Ames assay and in vitro chromosomal aberration assay, can practically assess only 100 chemicals per year according to our experiences in Japan. In this case, it will take 180 years to assess the outstanding 18,000 existing chemicals for genotoxicity, and it will take even longer when repeat dose toxicity tests are also performed, as these are not short-term assays. We therefore need higher-throughput systems to assess chemical safety, or at least to set priorities for those chemicals that should be tested in in vitro and/or in vivo tests. In silico systems have the capability for high throughput but have not yet been well validated for assessment of human hazard, although some regulatory bodies have started to use these methods.

Correlation between the Ames assay result and molecular weight could be explained by the lack of membrane permeability of high molecular weight chemicals, making it more difficult for them to reach target molecules such as DNA and proteins that contribute to the fidelity of cell division. Therefore, only a few chemicals with molecular weight >3000 gave positive responses in the Ames assay. This phenomenon is also true for induction of chromosomal aberrations in vitro (data not shown). The other important issue is the contribution of epoxy group in the polymer. Although of molecular weight >3000, some polymers with an epoxy group gave positive results in both the Ames and chromosomal aberration assays. Epoxy embedding reagents employed in electron microscopy (e.g., epon and araldite) have been reported as mutagenic in the Ames assay [8]. According to these findings, we should include a step to evaluate molecular weight and existence of any epoxy groups in the molecule.

In the present study, we used the CGX database recently published by Kirkland et al. [1] for microbial mutagenicity data on 358 carcinogens and 345 non-carcinogens for validation of three commercially available in silico (Q)SAR systems. When applied individually, MCase gave high sensitivity, specificity, and concordance compared to other two systems. One of the reasons may be because the CGX database contained many results from the U.S. National Toxicology Program (NTP), and the learning dataset of MCase would have used many of the same results. Therefore, some of them were evaluated by direct matching. Moreover, the applicability of MCase was relatively low compared with the other systems in this study (Table 1). MCase judged 119 chemicals as inconclusive and one chemical as marginal, and could not evaluate 67 chemicals. Such selectivity in MCase may contribute to the high concordance. On the other hand, the other systems were not influenced directly by the NTP data. We applied the in silico systems to another dataset, the ECJ database, that does not contain the NTP data and we obtained similar patterns of sensitivity, specificity, etc.

Each in silico system showed different outcomes on some chemicals complimentary by some extent. These different evaluation patterns were mainly due to the different evaluation rules. The DEREK is a rule-based system, AWorks is a discriminant-based system mainly depending on physicochemical descriptors, and MCase is a hybrid system based on a database. Therefore, we concluded that in silico evaluation could be optimized by combining the evaluations from the three systems. Sensitivity, specificity and concordance were increased when we combined the three in silico systems to make a final conclusion of mutagenicity (Table 1). Concordance was much higher after combining but the applicability became poor (42.2%). When two of the in silico systems gave the same evaluations, the applicability (86.8%) was good but the concordance was lower (86.7%) than when all three were combined (98.7).

Recently, several in silico studies for prediction of mutagenicity have been conducted on drugs or non-

Table 4
Exceptional chemicals that showed Ames test gave positive but all three in silico systems (DEREK, MCase, TOPKAT/AWorks) gave negative and Ames test gave negative but all three systems gave positive

Compound	CAS	Ames test	DEREK	MCase	TOPKAT/Aworks	Source[a]
Bupropion	34911-55-2	+	–	–	–	1
Citalopram	59729-33-8	+	–	–	–	1
Naloxone	465-65-6	+	–	–	–	1
Oxcarbazepime	28721-07-5	+	–	–	–	1
Quetiapine	111976-69-7	+	–	–	–	1
Rabeprazole	117976-89-3	+	–	–	–	1
Zolmitriptan	139264-17-8	+	–	–	–	1
2-(2-Methylpropyl) thiazole	18640-74-9	+	–	–	–	2
2-Chloropyridine	109-09-1	+	–	–	–	2
Pyrogallol	87-66-1	+	–	–	–	2
o-Phenylphenol	90-43-7	+	–	–	–	3
2-Amino-1-naphthalenesulfonic acid	81-16-3	+	–	–	–	3
2-Vinylpyridine	100-69-6	+	–	–	–	3
Fosfomycin	23155-02-4	–	+	+	+	1
Toremifene	89778-26-7	–	+	+	+	1
Poly (2-hydroxypropyl methacrylate)	25703-79-1	–	+	+	+	2
Carboxymethylnitrosourea	60391-92-6	–	+	+	+	3
Methidathion	950-37-8	–	+	+	+	3
1-Nitroso-3,5-dimethyl-4-benzoylpiperazine	–	+	+	+	3	

[a] 1: Synder et al. [11] (with TOPKAT), 2: White et al. [13] (with TOPKAT), 3: this study (with AWorks).

drug chemicals with commercially available programs, e.g., DEREK, MCase or TOPKAT, or newly developed computational approaches [4,9–12]. The performances of DEREK and MCase in several of these studies are summarized in Table 4. Generally, similar performance in sensitivity, specificity, concordance, and applicability were shown between DEREK and MCase but with some exceptions, e.g., sensitivity in 520 drug candidates [13], specificity in 516 non-drugs [4], and applicability in 520 pharmaceutical drug candidates and 94 non-drugs [13]. These differences might be due to the chemical class of target compounds in each database. However, there was no remarkable difference in performance whether the chemical was intended for use as a pharmaceutical, agricultural, or industrial agent. Our results on performance of in silico systems showed similarity with the published analyses. With respect to the combination of in silico prediction systems, White et al. [13] reported that combination improved the overall accuracy and specificity, but sensitivity was barely above the 50% level (Table 4). On the other hand, their analysis showed quite low applicability in the combination of three prediction systems, DEREK, MCase and TOPKAT. Our analysis of the combination of DEREK, MCase and AWorks showed good improvements in sensitivity, specificity and concordance, but applicability was low, especially in the 3-system combination.

Exceptional chemicals that gave positive Ames results but were negative in all three in silico systems (DEREK, MCase, TOPKAT/AWorks), and those that were negative in the Ames test but gave positive evaluations in all three systems, are summarized in Table 4. This table, which includes data from Synder et al. [11] and White et al. [13] shows there are 19 exceptional chemicals from both drug and non-drug families. Although it would be unrealistic to expect zero exceptions using this approach, further improvement of the prediction systems is needed. We do not have good reasons to explain the discordance, therefore we will verify the results from both sides, i.e., in silico system and Ames test.

Considering these outcomes, we propose a decision tree (Fig. 2), in order to evaluate chemical induction of gene mutation. We may use the decision tree to prioritize chemicals to be assayed by in vitro and/or in vivo tests. A final goal being that eventually, chemical mutagenicity will be evaluated by in silico systems alone for regulatory use. The decision tree consists of three steps; namely to assess the molecular weight, the existence of epoxy groups, and the in silico evaluation for genotoxicity. Based on the purpose of the in silico evaluation, the tree might be altered by the different final call of the in silico evaluation, i.e., regarding as positive (negative) all three systems show positive (negative). The choice of definition for final call applying to the decision tree should be based on the balance between accuracy of eval-

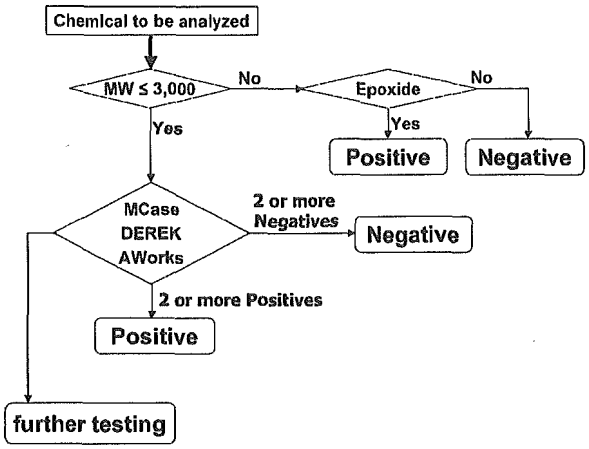

Fig. 2. Decision tree. In in silico evaluation, when two or more give positive then the final call is "positive" and two or more negative then call "negative".

uation and applicability, which are especially important for regulatory purpose. The decision should be made on a case-by-case basis depending upon the purpose of the decisions to be made.

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References

[1] D. Kirkland, M. Aardema, L. Henderson, L. Müller, Evaluation of the ability of a battery of three in vitro genotoxicity tests to discriminate rodent carcinogens and non-carcinogens. I. Sensitivity, specificity and relative predictivity, Mutat. Res. 584 (2005) 1–256.

[2] Law Concerning the Evaluation of Chemical Substances and Regulation of Their Manufacture, etc., Law No. 117, 16 October 1973 as last amended by Law No.49, 28 May 2003.

[3] M.T.D. Cronin, J.S. Jaworska, J.D. Walker, M.H.I. Comber, C.D. Watts, A.P. Worth, Use of QSARs in international decision-making frameworks to predict health effects of chemical substances, Environ. Health Perspect. 111 (2003) 1391–1401.

[4] G.M. Pearl, S. Livingstone-Carr, S.K. Durham, Integration of computational analysis as sentinel tool in toxicological assessments, Curr. Topoics Med. Chem. 1 (2001) 247–255.

[5] A. Hirose, M. Takahashi, M. Kamata, M. Ema, M. Hayashi, Development of genotoxicity predicting QSAR system for registered and existing industrial chemicals in Japan, Toxicol. Appl. Pharmacol. 197 (2004) 358.

[6] N. Greene, P.N. Judson, J.J. Langowski, C.A. Marchant, Knowledge-based expert systems for toxicity and metabolism prediction: DEREK, StAR and METEOR, SAR QSAR Environ. Res. 10 (1999) 299–314.

[7] H.S. Rosenkranz, A.R. Cunningham, Y.P. Zhang, H.G. Claycamp, O.T. Macina, N.B. Sussman, S.G. Grant, G. Klopman, Development, characterization and application of predictive-toxicology models, SAR QSAR Environ. Res. 10 (1999) 277–298.

[8] M.P. Murray, J.E. Cummins, Mutagenic activity of epoxy embedding reagents employed in electron microscopy, Environ. Mutagen. 1 (1979) 307–313.

[9] N.F. Cariello, J.D. Wilson, B.H. Britt, D.J. Wedd, B. Burlinson, V. Gombar, Comparison of the computer programs DEREK and TOPKAT to predict bacterial mutagenicity. Deductive estimate of risk from existing knowledge. Toxicity prediction by computer assisted technology, Mutagenesis 17 (4) (2002) 321–329.

[10] J.R. Votano, M. Parham, L.H. Hall, L.B. Kier, S. Oloff, A. Tropsha, Q. Xie, W. Tong, Three new consensus QSAR models for the prediction of Ames genotoxicity, Mutagenesis 19 (5) (2004) 365–377.

[11] R.D. Snyder, D.E. Ewing, L.B. Hendry, Evaluation of DNA intercalation potential of pharmaceuticals and other chemicals by cell-based and three-dimensional computational approaches, Environ. Mol. Mutagen. 44 (2) (2004) 163–173.

[12] R.D. Snyder, G.S. Pearl, G. Mandakas, W.N. Choy, F. Goodsaid, I.Y. Rosenblum, Assessment of the sensitivity of the computational programs DEREK, TOPKAT, and MCASE in the prediction of the genotoxicity of pharmaceutical molecules, Environ. Mol. Mutagen. 43 (3) (2004) 143–158.

[13] A.C. White, R.A. Mueller, R.H. Gallavan, S. Aaron, A.G. Wilson, A multiple in silico program approach for the prediction of mutagenicity from chemical structure, Mutat. Res. 539 (1–2) (2003) 77–89.

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SEMI-QUANTITATIVE IMMUNOHISTOCHEMICAL ANALYSIS OF MALE RAT-SPECIFIC α_{2u}-GLOBULIN ACCUMULATION FOR CHEMICAL TOXICITY EVALUATION

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ABSTRACT — We purified male rat urinary α_{2u}-globulin, prepared the antibody in rabbits, and improved an immunohistochemical detection method using this antibody for male rat-specific α_{2u}-globulin accumulation appearing as hyaline droplets in the kidneys. Our prepared antibody reacted specifically with α_{2u}-globulin in both immunohistochemical and Western blotting analyses, furthermore, and the graded immuno-reactivities on the slide were well associated with computational image analyzing results. Using this method, we retrospectively analyzed the renal sections from the toxicity studies of 12 nephrotoxic chemicals, which had already been conducted under the Japanese Existing Chemicals Survey Program. We demonstrated that the hyaline droplets induced by treatment with 10 chemicals (1,4-dibromobenzene, dicyclopentadiene, 3,4-dimethylaniline, 1,4-dicyanobenzene, tetrahydrothiophene-1,1-dioxide, 1,3-dicyanobenzene, acenaphthene, 3,4-dichloro-1-butene, 3a,4,7,7a-tetrahydro-1H-indene and 3,5,5-trimethylhexan-1-ol) were directly associated with α_{2u}-globulin accumulation. This immunohistochemical method is convenient for applying, even retrospectively, paraffin sections from general toxicity studies and could be useful for qualifying male rat-specific hyaline droplets consisting of α_{2u}-globulin and renal risk in humans.

KEY WORDS: α_{2u}-globulin, Immunohistochemistry, Hyaline droplet, Nephrotoxicity

INTRODUCTION

For risk assessment of chemicals, the most critical data are derived from animal toxicity studies because of a general lack of information on humans. Although all available results from animal studies have been applied to human risk assessment, in principle, exclusion of some specific toxicities, which might not occur in humans, should be taken into account. Among laboratory animals, the rat has been commonly used for toxicity studies, especially sub-acute, long-term or carcinogenicity studies. Nephropathy with hyaline droplets and renal tubular neoplasia caused by chemicals inducing α_{2u}-globulin accumulation (CIGA) are considered to be a male rat-specific toxicity, not occurring in female rats or other animals, including primates. Although low molecular proteins homologous to α_{2u}-globulin can be detected in other species, including mice and humans, none of these proteins have been confirmed to bind to CIGA, followed by accumulation of the protein-CIGA complex as in the case of α_{2u}-globulin. It is therefore believed that renal toxicity induced by CIGA in male rats is unlikely to occur in humans (Hard *et al.*, 1993).

α_{2u}-Globulin was first identified in male rat urine (Roy and Neuhaus, 1966), and had been reported to be a male rat-specific protein with a molecular weight of 18 to 20 kDa. The major source of urinary α_{2u}-globulin

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is the liver, where α_{2u}-globulin mRNA constitutes approximately 1% of the total hepatic mRNA (Sippel *et al.*, 1976; Kurtz and Feigelson, 1977). Neither α_{2u}-globulin nor its mRNA is detectable in the female liver (Sippel *et al.*, 1975, 1976; MacInnes *et al.*, 1986). The blood α_{2u}-globulin secreted from the liver is freely filtered through the glomerulus, and in mature rats, about two-thirds of the filtered protein is reabsorbed by tubules and the remainder is excreted through the urine (Neuhaus *et al.*, 1981). CIGA binds noncovalently to α_{2u}-globulin, and the resulting complex shows less degradability with proteolytic enzymes, causing an accumulation of the complex that is detectable as hyaline droplets with a light microscope. Various chemicals have been suspected of being CIGA based on detection of the evidence for exacerbation of hyaline droplets in renal proximal tubules in male rats, though not in females. Direct evidence for increasing α_{2u}-globulin levels has been demonstrated for only a few of these chemicals, however, including 2,2,4-trimethylpentane (Stonard *et al.*, 1986; Charbonneau *et al.*, 1987; Lock *et al.*, 1987), decalin (Kanerva *et al.*, 1987), d-limonene (Lehman-McKeeman *et al.*, 1989; Webb *et al.*, 1989), 1,4-dichlorobenzene (Charbonneau *et al.*, 1989), isophorone (Strasser *et al.*, 1988), lindane (Dietrich and Swenberg, 1990), tri- or per-chloroethylene and pentachoroethane (Goldsworthy *et al.*, 1888).

A number of initial safety assessments has so far been conducted for industrial chemicals, including both new and existing chemicals by the Japanese government or the OECD high production volume chemicals programs. Certain chemicals among these industrial chemicals have been suspected of being CIGA. In some cases, however, renal changes in male rats have been assessed as the endpoint for extrapolation to human health risk owing to a lack of direct evidence caused by α_{2u}-globulin accumulation, because no antibody against α_{2u}-globulin is commercially available for general toxicity studies. Some immunohistochemical α_{2u}-globulin analysis methods had already been developed (Burnett *et al.*, 1989; Hashimoto and Takaya, 1992; Caldwell *et al.*, 1999). As these methods required glycolmethacrylate embedding or specific computational analysis, they would be inappropriate for confirming α_{2u}-globulin accumulation in routinely conducted guideline-based toxicity studies. We therefore improved an immunohistochemical α_{2u}-globulin detection system using paraffin sections, which are generally used for standard toxicity studies. We evaluated the several chemicals suspected of being CIGA, moreover, and indicated the direct evidence caused by α_{2u}-globulin accumulation.

MATERIALS AND METHODS

Preparation of anti α_{2u}-globulin antibody

α_{2u}-globulin as an antigen was obtained from the urine collected from aged male rats, pooled, and used to immunize rabbits. The immunization procedures, including the amount of antigen and immunizing intervals, were determined from the results of a preliminary test referring to the methods of Kurtz *et al.* (1976). The antigen was injected under the skin at a dose of 1 mg/animal (1st injection) or 0.5 mg/animal (2nd and subsequent injections) once at two weeks. Blood sampling was conducted periodically and the antibody titer measured. When the antibody titer level reached a plateau, whole blood was collected and antiserum was obtained from the blood. The antiserum was used for immunohistochemistry and immuno-electron microscopy. For measurement of the α_{2u}-globulin content in the urine and tissues, the antibody was purified from the antiserum using a DEAE ionic exchange column after ammonium sulfate precipitation. The singularity of the antibody was confirmed as a single diffuse band of approximately 19 kDa by Western blotting analysis. This study and the following study were carried out in accordance with the Law for the Humane Treatment and Management of Animals and the Standards Relating to the Care and Management, etc. of Experimental Animals in Japan.

Experiment 1 Confirmation of specific reactivity of the antibody to α_{2u}-globulin

1. Preparation of α_{2u}-globulin nephropathy rats

To confirm the specific reactivity of the anti-α_{2u}-globulin antibody, we prepared α_{2u}-globulin nephropathy rats as follows. Male and female Crj:CD(SD)IGS rats were obtained from Charles River Japan Inc. and used at the age of 11 weeks. d-Limonene (Nacalai Tesque Inc.), a well-known α_{2u}-globulin nephropathy inducer, was administered to the rats, consisting of 4 males and 4 females each, for 10 days at doses of 0, 150 and 300 mg/kg/day by gavage using corn oil as a vehicle. The rats were housed individually in stainless steel wire cages in an animal room with a controlled temperature of 24±2°C, humidity of 55±10% and a 12-hr light/dark cycle (lighting from 7:00 to 19:00) and allowed access to food and water ad libitum.

Pooled urine was collected for 24 hr on the day before the start of administration and on Day 9 of administration. After the 10-day administration period,

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Table 1. Chemical name and effect dose derived from the general toxicity studies.

Chemical	Test type	Original study doses (mg/kg/day)	Effect doses (mg/kg/day) [a]			Original reported NOEL (mg/kg/day) [a]	The selected doses for analyzing (contr./low/high) (mg/kg/day)
			Histopathological findings		Non histopathological observations		
			AN	Other			
1,4-Dibromobenzene	RD	0/ 4/ 20/100/500	20≤ / –	100≤	100≤ / 20≤	4	0/ 20/500
Dicyclopentadiene	RT	0/ 4/ 20/100	4≤ / –	20≤ / 100	20≤ / 100	<4 / 20	0/ 4/100
3,4-Dimethylaniline	RD	0/10/ 50/250	50≤ / –	250	250 / 50≤	10	0/ 50/250
1,4-Dicyanobenzene	RD	0/ 1.25/ 5/ 20/ 80	5≤ / –	20≤ / –	20≤	1.25 / 5	0/ 5/ 80
Tetrahydrothiophene-1,1-dioxide	RD	0/60/ 200/700	200≤ / –	–	700	60 / 200	0/200/700
1,3-Dicyanobenzene	RD	0/ 8/ 40/200	8≤ / –	40≤ / 200	40≤	<8 / 8	0/ 8/200
Acenaphthene	RD	0/12/ 60/300	60≤ / –	300	300 / 60≤	12	0/ 60/300
3,4-Dichloro-1-butene	RT	0/ 0.4/ 2/ 10/ 50	10≤ / –	50	10≤ / 50	2 / 10	0/ 10/ 50
3a,4,7,7a-Tetrahydro-1*H*-indene	RT	0/ 67/200/600	67≤ / –	600	67≤ / 200≤	<67 / 67	0/ 67/600
3,5,5-Trimethylhexan-1-ol	RT	0/ 12/ 60/300	12≤ / –	60≤	60≤	12	0/ 12/300
2,4-di-*tert*-butylphenol	RD	0/ 5/ 20/ 75/300	– / –	300	300 / 75≤	75 / 20	0/ – /300
4-aminophenol	RD	0/ 4/ 20/100/500	– / –	100≤	100≤	20	0/100/500

[a] The data were described in a pattern of male/female when the data were different between the male and female.
RD, 28-day Repeat Dose Toxicity Test; RT, Combined Repeat Dose and Reproductive/Developmental Toxicity Test.
AN, α_{2u}-globulin nephropathy including hyaline droplets and subsequent tubular alteration.

α_{2u}-globulin antiserum by the above-mentioned protocol. HE-stained sections were used to examine the degree of hyaline droplets and to determine whether or not other findings were present. The degree of occurrence of hyaline droplets was divided into five grades, including none (−), minimal (±, barely detectable minimal appearance), slight (+, multifocal but not dispersed appearance), moderate (++, dispersed appearance over the cortex) and severe (+++, diffused appearance over the whole cortex). The staining sections with PAS, Azan-Mallory and anti-α_{2u}-globulin reaction were also graded similarly for positive-stained droplets. In addition, computational image analysis was carried out to verify the above-mentioned grading criteria using three typical immuno-stained samples for each grade. Images including almost all the renal superficial cortex were captured using a light microscope (Olympus BHS) and a digital camera (Olympus DP12). The captured images were measured for positive area using an image analyzing system (C-Imaging System, Compix Inc.), and the positive area (%) was then calculated from the data.

RESULTS

Experiment 1 Specific reactivity of the antibody to α_{2u}-globulin

On the HE-stained sections of the kidneys, hyaline droplets with round to irregular shapes were observed in the renal proximal tubular epithelium only in males administered d-limonene (Photo. 1a). The hyaline droplets were negative for PAS reaction (Photo 1b) but stained positively with Azan-Mallory staining (Photo 1c). With immuno-staining with the anti-α_{2u}-globulin antibody, the hyaline droplets were more clearly stained and more distinguishable than with Azan-Mallory staining (Photo 1d). The hyaline droplets showed a dose-dependent increase on the HE-stained sections (Photo 2, a-c) and positive reactions for hyaline droplets showed a correlational increase with immuno-staining (Photo 2, d-f). Very fine positive granules were also detected on the immuno-stained sections for all the males as background, but no positive reactions were observed in other tissue components. This background was observed generally in male kidneys and was, therefore, excluded from the grading in experiment 2. In the liver, all the males showed a positive reaction for the antibody in centrilobular hepatocytes. The degree of intensity was weaker than in the kidneys, and there was no clear intensification by d-limonene. No positive reaction for the anti-α_{2u}-globulin antibody was detected in the liver or kidneys in any females.

With electron microscopy, electron-dense and irregular-shaped inclusions surrounded by a single membrane were observed as changes corresponding to the hyaline droplets in the renal proximal tubular epithelium, and positive reactions were observed for the antibody with post-embedding method in the inclusions (Photo 3). A similar positive reaction was observed in the lysosomes of the renal tubule epithelium, but no positive reaction was detected in the hepatocytes.

The α_{2u}-globulin content in the kidneys of the males was increased dose-dependently by administration with d-limonene (Fig. 1). A dose-dependent but mild increase in α_{2u}-globulin content was also observed in the liver of the males. While no dose-dependent increase in the urine was noticeable, a lower molecular type of α_{2u}-globulin appeared in the males in the highest dose group, with the α_{2u}-globulin type reported as an early marker for α_{2u}-globulin nephropathy (Saito *et al.* 1991).

Experiment 2 α_{2u}-globulin analysis for industrial chemicals

Table 2 indicates the grades of all the samples with respect to hyaline droplets, positive droplets and immunological positive droplets analyzed with HE, Azan-Mallory and anti-α_{2u}-globulin antibody staining, respectively. In the controls there was a minimal to moderate amount of hyaline droplets in some animals and consequent variation for Azan-Mallory and anti-α_{2u} -globulin reaction. This variation was due to the arbitrary sampling of specimens, or probably related to the lot of the animals or to the difference of food used in each study. Dose-dependent increases of hyaline droplets in the renal proximal tubular epithelium were, however, confirmed for HE-staining of 10 chemicals suspected of being CIGA (1,4-dibromobenzene, dicyclopentadiene, 3,4-dimethylaniline, 1,4-dicyanobenzene, tetrahydrothiophene-1,1-dioxide, 1,3-dicyanobenzene, acenaphthene, 3,4-dichloro-1-butene, 3a,4,7,7a-tetrahydro-1H-indene, 3,5,5-trimethylhexan-1-ol). This was described in the original reports (Toxicity Testing Reports of Industrial Chemicals), although the occurrence of hyaline droplets varied in shape, size and number/cell with chemicals and showed no clear common features. In the highest dose groups of these chemicals, basophilic tubules, granular casts in the tubules and/or tubular dilatation were intensified or occurred as in the original reports. These changes

showed similar features in spite of the various severity and incidence with the chemicals. In serial sections prepared simultaneously, Azan-Mallory-positive reactions for hyaline droplets were detected dose-dependently in these 10 chemicals. No PAS-positive reaction was detected in any chemical. These staining behaviors of the hyaline droplets were the same as those in the case of d-limonen described above. Immunohistochemical staining using the anti-α_{2u}-globulin antibody revealed thoroughly dose-dependent positive reactions for hyaline droplets in all these chemicals. The resulting grades from three types of analysis were the same, demonstrating that a highly positive correlation exists among the three staining methods. As for the remainder not suspected of being CIGA (2,4-di-tert-butylphenol, 4-aminophenol), there was no increase of hyaline droplets or positive immunohistochemical reactions in any dose groups, as well as no stain in either PAS or Azan-Mallory staining. In addition, computational image analysis using three typical immuno-stained sections for each grade (Photo 4) showed a close correlation between the quantitative analysis and semi-quantitative grading (Fig. 2).

DISCUSSION

Many toxicity studies using laboratory animals have been conducted on environmental and industrial chemicals to ensure their safety or toxicity levels concerning human health. On extrapolating the results to humans, toxic mechanisms that are unlikely to occur in humans should be taken into account. A typical example of such toxicities is α_{2u}-globulin-related nephropathy and the consequent renal tumorigenesis in repeated

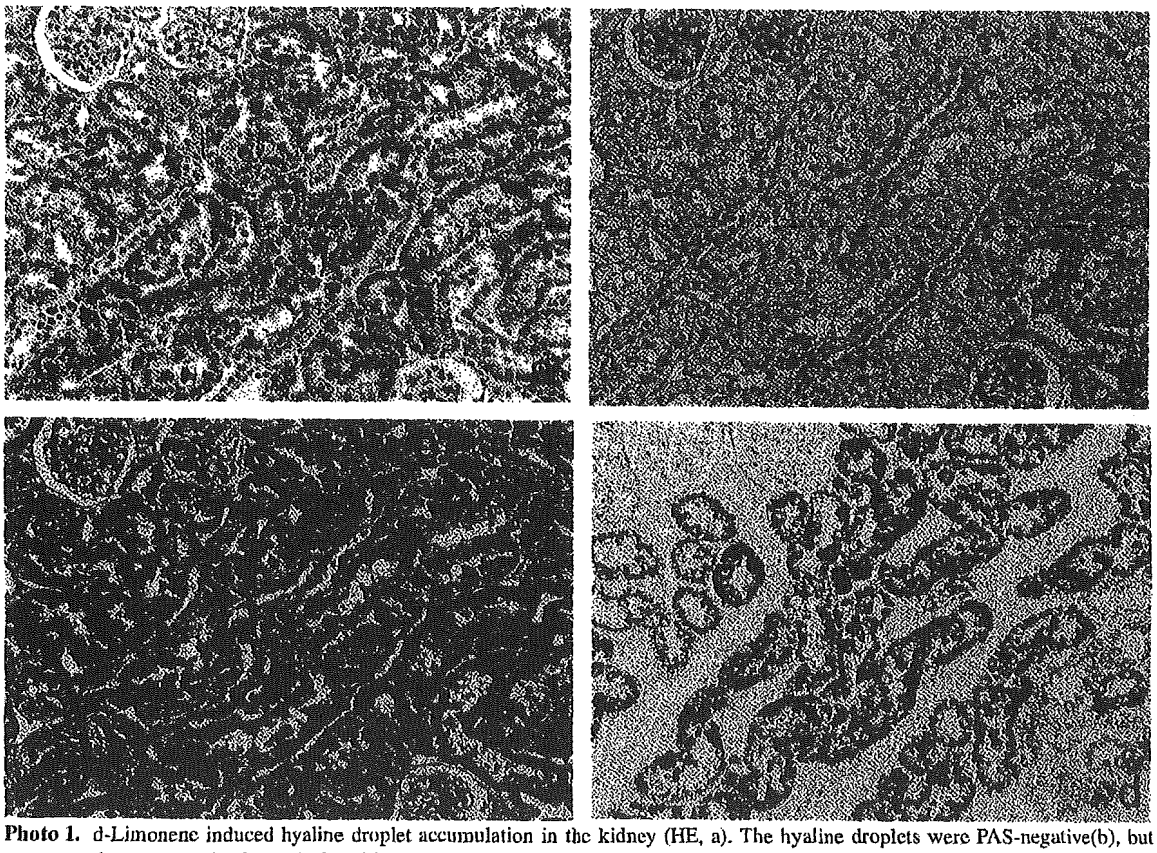

Photo 1. d-Limonene induced hyaline droplet accumulation in the kidney (HE, a). The hyaline droplets were PAS-negative(b), but they were stained positively with Azan-Mallory staining (c). Immunohistochemistry using the anti-α_{2u}-globulin antibody showed a clear positive reaction consistent with the hyaline droplets (d). Original magnification, ×66.

dose toxicity studies using male rats. This male rat-specific nephrotoxicity is not considered to occur in humans (Hard *et al.*, 1993). To exclude this male rat-specific toxicity from chemical risk assessment, it is necessary to demonstrate properly that such renal toxicity results from α_{2u}-globulin-CIGA complex accumulation. Detection analysis of α_{2u}-globulin in the nephrotoxicity has not been conducted in most conventional toxicity studies, however, especially in sub-acute toxicity screening studies for industrial chemicals. As

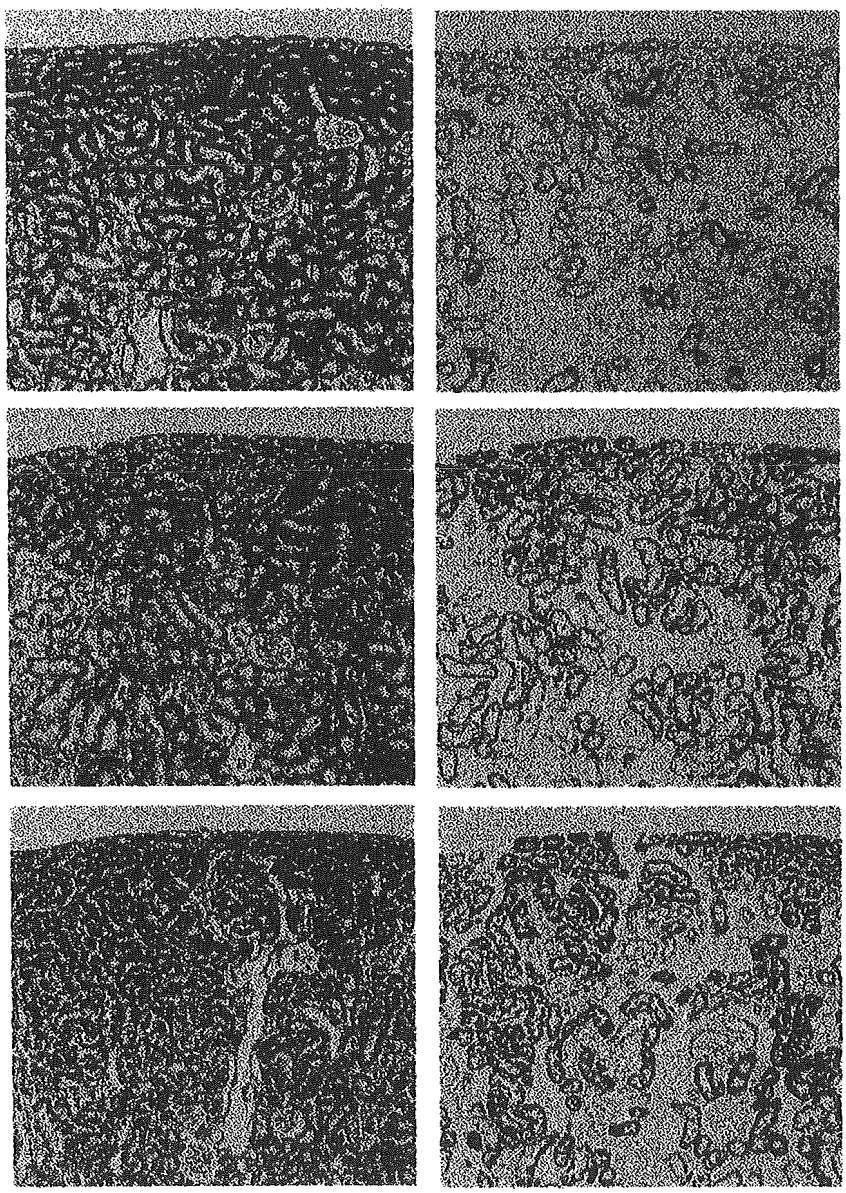

Photo 2. An increase of hyaline droplets in the kidney in correlation to the doses of *d*-limonene(HE, a - c). Positive reaction for the anti-α_{2u}-globulin antibody also increased with similar dose dependency (d - f). Original magnification, ×33.

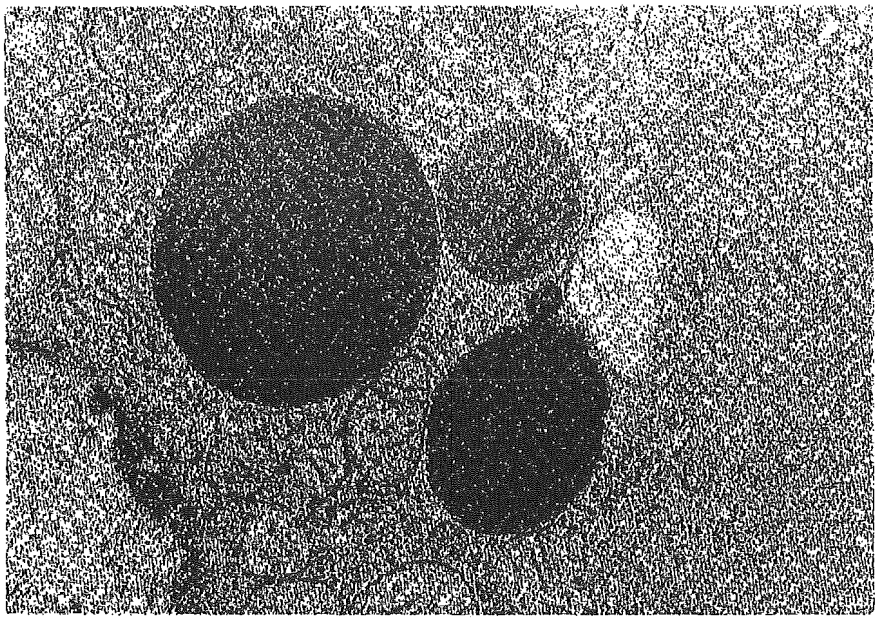

Photo 3. Immuno-electron micrograph of cytoplasmic inclusions, corresponding to the *d*-limonene induced hyaline droplets, in the epithelial cell of the renal proximal tubule. Colloidal gold particles are dispersed in the inclusions. Original magnification, ×10,000.

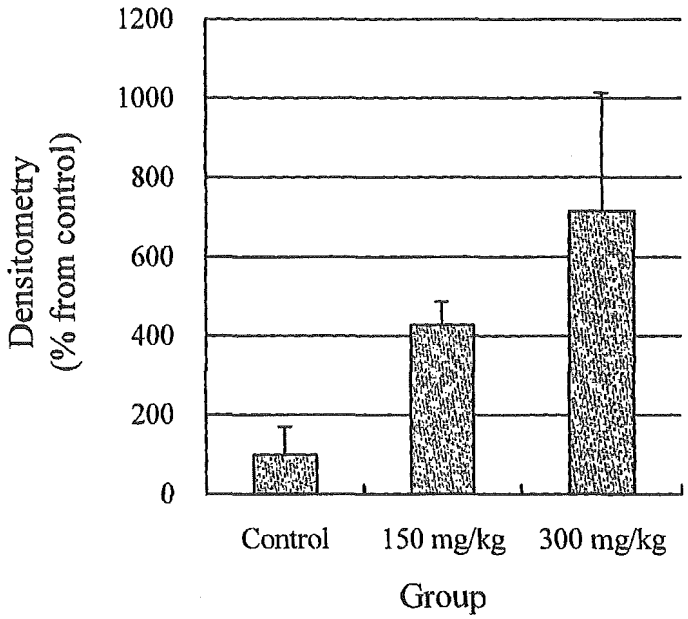

Fig. 1. Western blot analysis of α_{2u}-globulin in kidney from male rats treated with *d*-limonene.
Results are expressed as mean ± SD (n=4).