IN VIVO HEPATOTOXICITY STUDY OF RATS IN COMPARISON WITH IN VITRO HEPATOTOXICITY SCREENING SYSTEM

Rie KIKKAWA, Masaaki FUJIKAWA, Toshinori YAMAMOTO, Yoshimasa HAMADA, Hiroshi YAMADA and Ikuo HORII

Worldwide Safety Sciences, Pfizer Global Research and Development, Nagoya Laboratories, Pfizer Inc., 5-2 Taketoyo, Aichi 470-2393, Japan

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ABSTRACT — For the establishment of a high throughput screening system using primary cell cultures, investigation of elucidated toxicities to assess the correlation between in vitro and in vivo hepatotoxicity is necessary in the safety evaluation of the compound. In the previous study, we reported the usability of rat primary cultured hepatocytes for establishment of high throughput screening system. To confirm the reliability of rat primary hepatocytes culture screening system, we conducted a single-dose in vivo study with relatively high dose of hepatotoxican in rats using 4 reference compounds (acetaminophen, amiodarone, tetracycline, carbon tetrachloride), and investigated histopathological changes and expression of oxidative stress-related proteins by immunohistochemistry. We also carried out a proteomics analysis for estimating the reliable and sensitive biomarkers. Histopathologically, compound-specific hepatotoxicity was detected at 24 hr after administration in all compounds except amiodarone, which is known to induce phospholipidosis. Immunohistochemically, oxidative stress-related proteins were increased within 6 hr after administration in all treated groups. Proteomics analysis revealed several protein biomarkers related to oxidative stress and mitochondrial metabolism-regulation, which had been previously detected by proteomics analysis in in vitro screening system. Oxidative stress-related proteins were considered as useful biomarkers of hepatotoxicity; since they were detected by immunohistochemistry and proteomics analysis prior to appearance of compound-specific histopathological changes detected by light microscopy. Considering the relevance of in vitro system to in vivo system from the aspect of new biomarkers related to the toxicogenomics/toxicoproteomics, in vitro primary cell culture system would be sufficient to detect hepatotoxicity in the early stage of drug discovery.

KEY WORDS: Hepatotoxicity screening, In vitro and in vivo correlation, Oxidative stress, Biomarker

INTRODUCTION

Up to the present, in vitro hepatotoxicity predicting system using cell-line, primary cultured hepatocytes have been reported as an attractive model for elucidating the direct effects of compounds (Ekwall and Acosta, 1982; Acosta, et al., 1985; Clothier et al., 1987; Fry et al., 1990; Garle et al., 1994; Paillard et al., 1999). In in vitro system, compounds affect the cells directly and continuously until the removal of compound-containing medium. In addition, when cells are cultured using single type of cells, there are no interactions from the other interstitial cells. In in vivo system, compounds are exposed in the successive manner through absorption from the first exposed site followed by metabolism, distribution and elimination. In this context, it would consider that all the in vitro toxicity systems would not fully reflect the exposing profile and the cellular function as shown in vivo.

A numerous number of candidate compounds has been produced based on the approaches with new science and technology in drug discovery. However, high throughput toxicology, such as in vitro toxicity using small bulk amount has been required for safety evaluation in the early stage of drug discovery (Alden et al., 1999; Luber-Narod et al., 2001; Kola and Landis, 2004; Butcher, 2005). It is demanded for a hepatotoxicity screening system to use cells which well maintain
cellular functions, because the liver has numerous physiological functions and related toxicity driven by functional disorder tends to become critical (Vandenberghe, 1996; Treinen-Moslen, 2001).

We have previously reported that mitochondrial respiration was a useful parameter of in vitro hepatotoxicity screening system in the early stage of hepatotoxicity, and it was demonstrated by the proteomics analysis that mitochondrial respiration, energy or metabolism-regulatory enzymes and several oxidative stress-related proteins like acetyl-CoA acetyltransferase, argininosuccinate synthase, ATP synthase or glutathione peroxidase were up- or down-regulated after exposure to the compounds for 24 hr (Kikkawa et al., 2005).

In order to compare the correlation between in vitro and in vivo hepatotoxicity and investigate hepatotoxicity biomarkers, we conducted an in vivo hepatotoxicity study by single oral administration in rats using 4 reference compounds (acetaminophen, amiodarone hydrochloride, tetracycline hydrochloride and carbon tetrachloride). Histopathological changes and expression of oxidative stress-related proteins were examined. In addition, proteomics analysis was carried out in the case of acetaminophen.

MATERIALS AND METHODS

Animals
Six-week-old male Crj:CD(SD)IGS SPF rats, obtained from Charles River Japan Inc. (Kanagawa, Japan) were quarantined and acclimatized for a week before treatment. Rats were housed individually (one animal per cage) in stainless steel cages, with free access to tap water purified by reverse osmosis and certified pellet diet (CE-7, CLEA Japan Inc., Tokyo, Japan). The animal room temperature was maintained between 21 to 25°C with a relative humidity of 40 to 70%. Dark and light cycles were 12 hr each (lights on from 6:00 to 18:00) and an air cycle was 12 times per hour.

Reference compounds and preparation of administrative solution
Acetaminophen (APAP), known to induce hepatocytes death (Mitchell et al., 1973; Hart et al., 1982), was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A). Amiodarone hydrochloride (AMD), known to induce phospholipidosis (Poucell et al., 1984; Dake et al., 1985; Jain, et al., 2000; Sirajudeen et al., 2002), was purchased from ICN Biomedicals Inc. (Aurora, OH, U.S.A). Tetracycline hydrochloride (TC) and carbon tetrachloride (CTC), documented as capable of inducing fatty change (Brewer, 1965; Yeh and Shils, 1966; Breen et al., 1975; Schenker, 1976; Recknagel et al., 1989; Froment and Pessayre, 1995) were purchased from Wako Pure Chemical Co. (Osaka, Japan). All the reference compounds were dissolved in 0.5% (w/v) methylcellulose (Metolose® SM-4000, Shin-Etsu Chemical Co., Ltd., Tokyo, Japan) aqueous solution containing 0.1% Polysorbate 80 (Tween® 80, ICN Biomedicals, Inc). The concentration of each dosing solution was calculated to be 10 ml/kg.

Experimental design
Groups of 5 rats received a single dose of APAP (300 and 1,000 mg/kg), AMD (300 and 1,000 mg/kg), TC (600 and 2,000 mg/kg) or CTC (0.3 and 1 ml/kg) by oral gavage respectively. Five animals were administrated a vehicle (10 ml/kg) in the same manner and served as controls. Six or 24 hr after the dosing, blood was collected from the posterior vena cava under isoflurane inhalation anesthesia for serum chemistry and toxicokinetics analysis, and then rats were euthanized under isoflurane inhalation anesthesia. All procedures involving animals were approved by the Animal Ethics Committee of Pfizer Global Research & Development Nagoya Laboratories, and were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals (National Academy of Sciences, 1996).

Observation and measurements
1. Clinical pathology
Serum was analyzed for alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma glutamyl transferase (GGT), total bilirubin (TB), total protein (TP), albumin (ALBM), cholesterol (CHOL), triglyceride (TRIG), glucose (GLUC), lactate dehydrogenase (LDH), blood urea nitrogen (BUN), creatinine (CREA), sodium (NA), potassium (K), chloride (CL), and calcium (CA) using an H-7070 auto-analyzer (Hitachi Co., Tokyo, Japan). Albumin-globulin ratio (AG) and values of globulin (GLOB) were calculated.

2. Histopathology
The liver was removed and fixed in 4% paraformaldehyde aqueous solution, processed routinely, and embedded in paraffin. Tissue sections were stained with hematoxylin and eosin (Wako Pure Chemical Co.) and examined microscopically.
Comparison of *in vivo* and *in vitro* hepatotoxicity screening system.

3. Immunohistochemistry

For immunohistochemical analysis, sections were reacted with antibodies overnight at 4°C. The following antibodies were used: rabbit polyclonal antibody against heme oxygenase-1 (HO-1, Stressgen, Victoria BC, Canada), manganese superoxide dismutase (Mn-SOD, Stressgen) and heat shock protein 70 (HSP-70, DakoCytomation, Glostrup, Denmark), goat polyclonal antibody against selenium-dependent cellular glutathione peroxidase (GPx-1, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, U.S.A.) or mouse monoclonal antibody against 8-hydroxyguanosine (8-OHdG, NOF Corporation, Tokyo, Japan) followed by a 30-min incubation with biotinylated secondary antibody (Vector Laboratories, Burlingame, CA, USA). Antibody binding was visualized using a Vectastain Elite ABC Kit (Vector Laboratories) followed by hematoxylin staining. The dilution rate of each primary antibody was as follows: HO-1 (1:750), HSP-70 (1:750), Mn-SOD (1:500), GPx (1:250) and 8-OHdG (1:500) respectively. The result of immunohistochemical analysis was expressed using a 4-grade scale: −, ±, +, and ++.

4. Proteomics analysis

Small pieces of rat liver were homogenized in 2D-lysis buffer containing 7 M urea and 2 M thiourea. Protein concentrations in homogenates were determined with a modified Lowry’s method (Lowry et al., 1951) using RC DC Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA), and 100 µg of proteins were applied to 2-dimensional gel electrophoresis (2DGE). The 2D-gels images were acquired by means of a Typhoon 9200 image scanner (Amersham Biosciences) after staining with SyproRuby protein gel stain (Molecular Probes, Eugene, OR, USA). The protein expression profiles were compared in triplicate between control and treated groups employing the Progenesis Workstation Analysis System (Nonlinear dynamics, Cuthbert House, UK). The protein spots whose expressions displayed greater than two-fold alteration were nominated as differential expressed spots, and then they were excised from gels for further protein identification. Following reduction and alkylation, the gel pieces were dehydrated in acetonitrile and the dried gel pieces were rehydrated in TPCK-modified trypsin (Promega, Madison, WI, USA). In-gel tryptic digestion was carried out at 37°C overnight and then the digested peptides were recovered into 5% aqueous formic acid. The product ion spectra derived from the tryptic peptide were acquired using a liquid chromatograph-mass spectrometer (CapLC and Q-Tof Ultima API, Micromass, Manchester, UK). The collected datasets were searched against the SWISS-PROT (GeneBio, Geneva, Switzerland) database using Mascot Protein Identification Systems (Matrix Science, London, UK).

5. Toxicokinetics measurement

To confirm exposure to the compounds, the serum concentration of the compounds was measured using liquid chromatography-tandem-mass spectrometry (API 3000, Applied Biosystems, Foster City, CA, U.S.A.). Serum was collected from the rats administered APAP, AMD and TC via vena cava under isoflurane inhalation anesthesia immediately prior to necropsy.

Statistical analysis

Statistical analyses of clinicopathological findings were performed separately for each compound. Each parameter was compared with the control group mean. Dunnett’s multiple comparison procedure was used if a preliminary Bartlett’s test for variance homogeneity was not significant at the p = 0.05 level. Statistical significance of the comparisons was indicated at both the p<0.05 and 0.01 levels. Tests were two-tailed (Dunnett, 1955, 1964; Bartlett, 1968; Yoshimura, 2003).

RESULTS

Clinicopathological and histopathological observations

The clinicopathological and histopathological findings are summarized in Tables 1 and 2, respectively. As for clinicopathological data, APAP induced increase of ALT, AST and GGT in 1,000 mg/kg at 24 hr after administration, while there were no noteworthy changes at 6 hr after administration. AMD induced increase of ALT in 1,000 mg/kg at 6 hr after administration, and increases of CHOL in 300 and 1,000 mg/kg and ALP in 1,000 mg/kg at 24 hr after administration. CTC induced increases of ALT and AST at 6 hr after administration, and ALT, AST and GGT in 1 ml/kg at 24 hr after administration. There were no noteworthy changes after administration in 300 mg/kg of APAP and TC.

Regarding histopathological findings in the liver, eosinophilic changes, necrosis of centrilobular hepatocytes which characterized as coagulation and shrinkage of hepatocytes in being lost their nuclei, and
Table 1. Changes in clinicopathological parameters in rats at 6 or 24 hr after administration in APAP, AMD, TC and CTC.

| Parameter | Control | 0 hr | 30 min | 60 min | 2 hr | 4 hr | 6 hr | 8 hr | 10 hr | 12 hr | 14 hr | 16 hr | 18 hr | 20 hr | 22 hr | 24 hr |
|-----------|---------|------|--------|--------|------|------|------|------|------|------|------|------|------|------|------|------|------|
| ALT (U/l) | 41.0 ± 6.6 | 31.6 ± 4.3* | 47.6 ± 6.5 | 51.2 ± 12.5 | 65.6 ± 17.6* | 44.8 ± 6.2 | 51.8 ± 11.6 | 39.4 ± 4.3 | 64.3 ± 11.3* |
| AST (U/l) | 75.6 ± 14.1 | 65.8 ± 7.8 | 76.2 ± 6.1 | 67.8 ± 6.2 | 74.2 ± 6.1 | 79.0 ± 15.0 | 73.2 ± 5.2 | 79.8 ± 10.2 | 118.8 ± 14.6** |
| ALP (U/l) | 872.4 ± 128.3 | 688.8 ± 115.6* | 855.2 ± 89.2 | 900.4 ± 118.1 | 830.9 ± 84.7 | 906.0 ± 146.6 | 673.0 ± 44.5* | 781.6 ± 114.3 | 833.3 ± 154.9 |
| GGT (U/l) | 0.0 ± 0.0 | 0.2 ± 0.5 | 0.2 ± 0.5 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.1 ± 0.5 | 0.0 ± 0.0 | 0.5 ± 0.5 | 1.0 ± 0.0 | 1.1 ± 0.0 | 1.0 ± 0.0 | 0.8 ± 0.3 | 0.8 ± 0.3 | 0.8 ± 0.3 |
| TB (mg/dl) | 0.10 ± 0.00 | 0.10 ± 0.00 | 0.10 ± 0.00 | 0.10 ± 0.00 | 0.10 ± 0.00 | 0.10 ± 0.00 | 0.10 ± 0.00 | 0.10 ± 0.00 | 0.10 ± 0.00 | 0.10 ± 0.00 | 0.10 ± 0.00 | 0.10 ± 0.00 | 0.10 ± 0.00 |
| TP (g/dl) | 5.68 ± 0.19 | 5.56 ± 0.15 | 5.68 ± 0.19 | 5.78 ± 0.18 | 5.76 ± 0.17 | 5.73 ± 0.17 | 5.76 ± 0.17 | 5.73 ± 0.17 | 5.76 ± 0.17 | 5.73 ± 0.17 | 5.76 ± 0.17 | 5.73 ± 0.17 | 5.76 ± 0.17 |
| ALB (g/dl) | 2.72 ± 0.11 | 2.7 ± 0.07 | 2.84 ± 0.17 | 2.80 ± 0.14 | 2.68 ± 0.11 | 2.73 ± 0.10 | 2.76 ± 0.11 | 2.73 ± 0.10 | 2.76 ± 0.11 | 2.73 ± 0.10 | 2.76 ± 0.11 | 2.73 ± 0.10 | 2.76 ± 0.11 |
| GLOB (g/dl) | 2.94 ± 0.15 | 3.02 ± 0.18 | 3.24 ± 0.20* | 3.02 ± 0.08 | 2.88 ± 0.13 | 2.95 ± 0.13 | 3.02 ± 0.08 | 3.02 ± 0.08 | 3.02 ± 0.08 | 3.02 ± 0.08 | 3.02 ± 0.08 | 3.02 ± 0.08 | 3.02 ± 0.08 |
| AG | 0.93 ± 0.04 | 0.90 ± 0.05 | 0.88 ± 0.06 | 0.93 ± 0.06 | 0.93 ± 0.06 | 0.93 ± 0.04 | 0.91 ± 0.04 | 0.91 ± 0.04 | 0.91 ± 0.04 | 0.91 ± 0.04 | 0.91 ± 0.04 | 0.91 ± 0.04 | 0.91 ± 0.04 |
| CHOL (mg/dl) | 73.2 ± 14.4 | 84.2 ± 11.4 | 88.6 ± 10.8 | 81.0 ± 13.6 | 82.8 ± 11.8 | 63.0 ± 4.2 | 70.4 ± 9.8 | 72.4 ± 11.0 | 66.8 ± 5.7 |
| TRIG (mg/dl) | 76.4 ± 27.1 | 65.8 ± 16.9 | 79.2 ± 14.0 | 46.0 ± 15.5 | 35.4 ± 5.4* | 100.8 ± 31.0 | 100.0 ± 48.3 | 94.2 ± 15.1 | 63.3 ± 14.8 |
| GLUC (mg/dl) | 267.2 ± 51.3 | 214.4 ± 32.0 | 270.6 ± 39.0 | 265.4 ± 53.3 | 242.4 ± 30.5 | 219.5 ± 12.2 | 216.6 ± 22.6 | 218.6 ± 17.4 | 199.8 ± 20.1* |
| LDH (U/l) | 196.2 ± 71.7 | 133.6 ± 12.7 | 166.6 ± 28.5 | 119.6 ± 11.2 | 170.2 ± 15.5 | 238.5 ± 154.7 | 124.8 ± 23.8 | 247.4 ± 135.1 | 413.5 ± 142.9* |
| BUN (mg/dl) | 110.0 ± 1.4 | 106.5 ± 1.5 | 132.0 ± 5.5* | 10.0 ± 1.6 | 11.6 ± 1.5 | 13.5 ± 1.9 | 15.6 ± 2.7** | 11.2 ± 1.5 | 13.0 ± 0.8 |
| CREA (mg/dl) | 0.22 ± 0.05 | 0.20 ± 0.00 | 0.20 ± 0.00 | 0.20 ± 0.00 | 0.20 ± 0.00 | 0.20 ± 0.00 | 0.20 ± 0.00 | 0.20 ± 0.00 | 0.20 ± 0.00 |
| NA (mmol/l) | 146.4 ± 1.3 | 145.6 ± 0.6 | 145.0 ± 1.6 | 146.8 ± 1.6 | 144.0 ± 0.7* | 146.5 ± 2.4 | 145.6 ± 1.1 | 145.6 ± 1.3 | 145.5 ± 1.3 |
| K (mmol/l) | 6.83 ± 0.72 | 8.14 ± 0.60* | 7.54 ± 1.02 | 6.92 ± 0.98 | 8.08 ± 0.73 | 7.10 ± 0.74 | 7.18 ± 1.30 | 7.36 ± 0.68 | 6.70 ± 0.81 |
| CL (mmol/l) | 108.4 ± 1.8 | 101.8 ± 1.5 | 99.2 ± 1.9 | 101.4 ± 1.5 | 101.2 ± 1.6 | 103.0 ± 0.8** | 102.4 ± 1.3* | 100.8 ± 1.3 | 100.8 ± 1.5 |
| CA (mg/dl) | 13.28 ± 0.97 | 12.90 ± 0.52 | 13.48 ± 0.61 | 13.12 ± 0.90 | 12.38 ± 0.28 | 12.75 ± 0.30 | 13.02 ± 0.34 | 13.32 ± 0.18 | 12.43 ± 0.59 |

All data points shown represent the mean ± SD (n=5).
* significantly different from control (p<0.05).
** significantly different from control (p<0.01).
Comparison of *in vivo* and *in vitro* hepatotoxicity screening system.

Inflammatory cells infiltrations were observed in 1,000 mg/kg of APAP at 24 hr after administration (Photo 1-f). Slight inflammatory cell infiltration was observed in the liver in 1,000 mg/kg of AMD at 24 hr after administration (Photo 1-g). Fatty changes and focal inflammatory cell infiltration were observed in the liver in 2,000 mg/kg of TC (Photo 1-h) at 24 hr after administration. Centrilobular fatty changes with foamy structure, swelling of hepatocytes and centrilobular necrosis in 0.3 and 1 ml/kg of CTC were observed after 6 and 24hr. At 24 hr after administration, slight infiltration of inflammatory cells was seen at 0.3 ml/kg of CTC (Photo 1-e, i).

**Immunohistochemistry**

The results of the immunohistochemical analysis are summarized in Table 3, and those of immunohistochemical staining using anti-Mn-SOD of control and AMD administered liver are shown in Photo 2. In *in vivo* immunohistochemistry, increased expressions of oxidative stress-related proteins were demonstrated in all treated animals administered hepatotoxicity compounds. Expression of each oxidative stress-related protein was as follows:

**1. HO-1**

As previously reported (Chiu *et al*., 2002), HO-1-positive Kupffer cells were observed in all the rats including the control at 6 and 24 hr after administration; at 24 hr after administration, a slight increase in positive Kupffer cells were observed in 300 and 1,000 mg/kg of APAP and in 1 ml/kg of CTC. At 6 hr after administration, HO-1-positive hepatocytes were observed, especially in the centrilobular area in 1,000 mg/kg of APAP, 1,000 mg/kg of AMD, and 0.3 and 1 ml/kg of CTC, but not in the control animals. At 24 hr after administration, HO-1-positive hepatocytes were observed in the centrilobular cells, particularly degenerative and necrotic ones, seen in the liver treated in 1,000 mg/kg of APAP and 1 ml/kg of CTC.

**2. HSP-70**

At 6 hr after administration, HSP-70-positive hepatocytes were observed mainly in the centrilobular area in 300 and 1,000 mg/kg of AMD, 2,000 mg/kg of TC and 0.3 and 1 ml/kg of CTC. At 24 hr after administration, HSP-70-positive hepatocytes were seen in the liver administered 300 mg/kg of APAP (periportal area), 1,000 mg/kg of APAP (centrilobular area), 300 mg/kg of AMD (centrilobular area), 600 and 2,000 mg/kg of TC (centrilobular area), 0.3 ml/kg of CTC (centrilobular area) and 1 ml/kg of CTC (diffuse).

**3. Mn-SOD**

At 6 hr after administration, Mn-SOD-positive hepatocytes were observed in the centrilobular area in 300 and 1,000 mg/kg of AMD, 600 and 2,000 mg/kg of TC, 0.3 ml/kg of CTC and in the periportal area and 1 ml/kg of CTC. Mn-SOD-positive Kupffer cells are also observed in 1 ml/kg of CTC. Mn-SOD-positive Kupffer cells in 0.3 and 1 ml/kg of CTC and in Kupffer cells in 0.3 and 1 ml/kg of CTC.

**Table 2. Histopathological findings at 6 or 24 hr after administration with APAP, AMD, TC and CTC.**

<table>
<thead>
<tr>
<th>Histopathological findings</th>
<th>APAP (mg/kg)</th>
<th>AMD (mg/kg)</th>
<th>TC (mg/kg)</th>
<th>CTC (ml/kg)</th>
</tr>
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<tbody>
<tr>
<td><strong>6 hr</strong></td>
<td></td>
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<td></td>
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<tr>
<td>Necrosis of hepatocytes</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Eosinophilic changes of hepatocytes</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Fatty changes of hepatocytes</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Inflammatory cell infiltration</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
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<tr>
<td><strong>24 hr</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Necrosis of hepatocytes</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>±</td>
</tr>
<tr>
<td>Eosinophilic changes of hepatocytes</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>±</td>
</tr>
<tr>
<td>Fatty changes of hepatocytes</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Inflammatory cell infiltration</td>
<td>−</td>
<td>−</td>
<td>±±</td>
<td>±±±±</td>
</tr>
</tbody>
</table>

Grade of changes: −; NAD, ±; Slight, +; Mild, ++; Severe.
4. GPx
At 6 hr after administration, GPx-positive hepatocytes were increased in 300 and 1,000 mg/kg of APAP, 300 and 1,000 mg/kg of AMD and 600 and 2,000 mg/kg of TC and 0.3 ml/kg of CTC, while they decreased in 1 ml/kg of CTC compared with the control. At 24 hr after administration, GPx-positive cells were increased in 1,000 mg/kg of APAP, 300 and 1,000 mg/kg of AMD and 600 and 2,000 mg/kg of TC.

5. 8-OHdG
At 6 hr after administration, 8-OHdG-positive nuclei in the hepatocytes were observed in 600 mg/kg of TC, 0.3 ml/kg of CTC. At 24 hr after administration, they were seen in 1,000 mg/kg of APAP, 600 mg/kg of TC and 0.3 ml/kg of CTC. It is reported that NAPQI, an active metabolite of APAP, affects the nuclei in mice hepatocytes administered APAP (Rogers, et al., 1997), and it coincides with the result obtained in rats administered APAP in this study. But 8-OHdG was not sensitive enough for the other compounds.

The antibodies used were oxidative stress-related proteins: HO-1, HSP-70, Mn-SOD, GPx and 8-OHdG. Their antibody-characters are as follows: HO-1, which is known as heat shock protein-32 and exerts an antioxidative function by catalyzing the degradation of heme into biliverdin, iron, and carbon monoxide (Schwartz, 2001; Chiu et al., 2002), HSP-70 which is a family of molecular chaperones not abundant in normal physiological situations and strongly induced under oxidative stress (Gathering and Sambrook, 1992; Georgopoulos and Welch, 1993; Liu et al., 1996; Callahan et al., 2002), Mn-SOD which abounds in the mitochondria matrix and content depends on mitochondrial oxidant production (Fridovich, 1975; Kanbagli et al., 2002), GPx which is a major peroxide scavenging enzyme found in cytoplasmic and mitochondrial fractions of cells (Kanbagli et al., 2002), and

Photo 1. Light microscopic photographs of rat livers after single oral administration of the reference compounds for 24 hr.
Doses that were treated to each of the rats were 300 mg/kg (1-b) or 1,000 mg/kg (1-f) of APAP, 300 mg/kg (1-c) or 1,000 mg/kg (1-g) of AMD, 600 mg/kg (1-d) or 2,000 mg/kg (1-h) of TC, or 0.3 ml/kg (1-e) or 1 ml/kg (1-i) of CTC, respectively. The compound-related hepatotoxicity was described as eosinophilic changes, centrilobular necrosis of hepatocytes, and inflammatory cells infiltrations that were treated with 1,000 mg/kg of APAP (1-f), fatty changes of the hepatocytes with 2,000 mg/kg of TC (1-h), centrilobular fatty changes with foamy structure and centrilobular necrosis at 0.3 and 1 ml/kg of CTC (1-e, i), and infiltration of inflammatory cells and eosinophilic changes in 1 ml/kg of CTC (1-i). H.E.
Comparison of in vivo and in vitro hepatotoxicity screening system.

8-OHdG which is a DNA-base modified product generated by reactive oxygen species (ROS) (Boiteux and Radicella, 1999; Kitada et al., 2001). Although the functions and locations of oxidative stress-related protein were different in the cells, they were commonly increased by treatment with hepatotoxic compounds.

**Proteomics analysis**

In the proteomics analysis, 71 candidate proteins were identified among a total of 74 proteins that displayed altered expression spots 24 hr after the administration of APAP (Table 4). Details of the analysis are as follows: there were 21 down-regulated proteins (7 unique at control), and 53 up-regulated proteins (6 unique at higher dose), indicating that they may serve as specific biomarkers of hepatotoxicity. The main altered proteins were related to the functions of respiration and metabolism-related enzymes in the mitochondria, oxidative stress-related protein and proteasomes, as previously observed by in vitro proteomics analysis (Kikkawa et al., 2005).

**Toxicokinetics measurement**

Serum concentration of APAP, AMD and TC prior to necropsy confirmed that the animals had been exposed to the corresponding compound (Table 5).

**DISCUSSION**

In a previous study, we reported that the hepatotoxicity at early stage changes could be detected by measuring mitochondrial respiration and compound-specific morphological changes using in vitro primary cell cultures. Comparing the differences between in vitro and in vivo hepatotoxicity, changes of in vivo and in vitro hepatotoxicities were well correlated in the case of APAP, TC and CTC. The clinicopathological and histopathological findings indicated compound-related hepatotoxicity in the rats administered 1,000 mg/kg of APAP, 2,000 mg/kg of TC for 24 hr, and 0.3 and 1 ml/kg of CTC at 6 and 24 hr after administration. As for AMD, CHOL were slightly increased, however, no histopathological change except for slight inflammatory cells infiltration was observed at 24 hr after administration in 1,000 mg/kg of AMD. The result showed the consistency that AMD is known to induce phospholipidosis and it takes time to detect AMD-induced phospholipidosis (Zimmerman and Ishak, 1994; Jain et al., 2000). In the primary cell culture system, release of LDH, a kind of leakage enzyme and a parameter of cytotoxicity, can be detected only after the failure of the cell membrane. In vivo, the serum LDH was increased only in some rats administered 1,000 mg/kg of APAP and 0.3 and 1 ml/kg of CTC, even after histopathological changes had occurred. This result that a clinicopathological parameter is not always sensitive as histopathological observation can be applied to other enzymes, such as ALT and AST, which increased only when administered 1,000 mg/kg of APAP and 1 ml/kg of CTC, which showed severe...
morphological changes in the liver. In the AMD-treated animals, compound-specific hepatotoxicity, such as phospholipidosis, was not observed. In the primary culture system, however, compound specific morphological changes could be detected within 24 hr with low bulk. These results indicated the significance of the in vitro screening system for the safety evaluation of hepatotoxicants.

Oxidative stress-related protein plays a major role of erasing ROS. ROS are produced constantly during vital activities and many degenerative changes followed by cell death have been reported to occur when the rate of ROS generation exceeds the detoxification abilities of the cell (Hogg, 1998; Hollan, 1995; Gate et al., 1999; Andreyev et al., 2005). Since oxidative stress-related proteins firstly react with these ROS instead of cellular components like nuclear acids (Richter et al., 1988; Dizdaroglu, 1991), proteins (Dubois et al., 1991; Pinto et al., 1991; Stadtman, 1993; Hogg, 1998), and lipids (Ernster, 1993) to eliminate ROS and prevent tissue injury at physiological situation, oxidative stress-related proteins are thought to be useful biomarkers for the establishment of a high throughput hepatotoxicity screening.

Photo 2. Mn-SOD expression changes after single oral administration of AMD for 6 or 24 hr. Doses that were administered to each rat were 300 mg/kg (2-b, -c) or 1,000 mg/kg (2-d, 2-e) of AMD, respectively. Mn-SOD positive hepatocytes are recognized from 6 hr after AMD treatment.
Comparison of *in vivo* and *in vitro* hepatotoxicity screening system.

**Table 4.** Proteins identified utilizing peptide sequence tag method from a total of 74 proteins demonstrating altered expressions of rat liver at 24 hr after administrated 1,000 mg/kg of APAP.

<table>
<thead>
<tr>
<th>Type of Change</th>
<th>Rate</th>
<th>Protein Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Up-regulated</td>
<td>&gt; 3-fold</td>
<td>NSFL1 cofactor p47, Argininosuccinate synthase, Senesence marker protein-30 (Regucalcin), Proteasome subunit alpha type 3, Catalase, C-1-tetrahydrofolate synthase, cytoplasmic</td>
</tr>
<tr>
<td></td>
<td>&gt; 2-fold</td>
<td>Glutamate dehydrogenase 1, mitochondrial precursor, Argininosuccinate synthase, Hydroxymethylglutaryl-CoA synthase, mitochondrial precursor, Actin, cytoplasmic 1, Fructose-bisphosphate aldolase B, Uroporphyrinogen decarboxylase, Insulin-degrading enzyme, Nucleic acid binding factor pRM10, Keratin, type II cytoskeletal, Senesence marker protein-30 (Regucalcin), Pyruvate carboxylase, mitochondrial precursor (PCB), Electron transfer flavoprotein-ubiquinone oxidoreductase, mitochondrial precursor, Alpha-1-antiproteinase precursor, Glutathione S-transferase Ya-2, Heat shock 70kDa protein 1A/1B, Glutathione S-transferase 8, Tubulin alpha-6 chain, Glutathione S-transferase Ya-2, Hydroxymethylglutaryl-CoA synthase, mitochondrial precursor, ATP synthase gamma chain, mitochondrial, Phenylalanine-4-hydroxylase (PAH), 60 kDa heat shock protein, mitochondrial precursor (Hsp60), Calpain small subunit 1 (CSS1), Rho, GDP dissociation inhibitor (GDI) beta (predicted), Pyruvate carboxylase, mitochondrial precursor (PCB), Glutathione S-transferase Ya-1, Similar to nicotinamide N-methyltransferase, Acyl-CoA dehydrogenase, long-chain specific, mitochondrial precursor, Arylamine N-acetylimidazole transferase 1 (NAT-1), Arginase 1, Affiloxin B1 aldehyde reductase member 1, Alcohol dehydrogenase [NADP+], Protein disulfide-isomerase A3 precursor (ERp60), Glycerol kinase, Aldehyde dehydrogenase family 7 member A1, 26S protease regulatory subunit 7, 26S protease regulatory subunit 8, Alpha enolase</td>
</tr>
<tr>
<td></td>
<td>Unique on high dose</td>
<td>L-lactate dehydrogenase A chain (LDH-A), Carbonic anhydrase III, Triosephosphate isomerase, Adenylate kinase isoenzyme 4, mitochondrial precursor, NADP-dependent malic enzyme, 2-oxoisovalerate dehydrogenase alpha subunit, mitochondrial precursor</td>
</tr>
<tr>
<td></td>
<td>&gt; 5-fold</td>
<td>Estrogen sulfotransferase, isoform 1</td>
</tr>
<tr>
<td></td>
<td>&gt; 3-fold</td>
<td>Transitional endoplasmic reticulum ATPase, Isopentenyl-diphosphate delta-isomerase 1, NDRG1 related protein NDRG2b1</td>
</tr>
<tr>
<td>Down-regulated</td>
<td>&gt; 2-fold</td>
<td>14-3-3 protein zeta/delta (Protein kinase C inhibitor protein-1), Carbonic anhydrase III, Fatty acid-binding protein, epidermal (E-FABP), similar to microsomal triglyceride transfer protein, GrpE protein homolog 1, mitochondrial precursor (Mt-GrpE#1), Fructose-bisphosphate aldolase B, Serotransferrin precursor</td>
</tr>
<tr>
<td></td>
<td>Unique on control</td>
<td>N-hydroxyarylamine sulfotransferase (HAST-1), Catalase, Triosephosphate isomerase, Acetyl-CoA dehydrogenase, long chain specific, mitochondrial precursor, Long-chain -fatty-acid-CoA ligase 1, Peptide methionine sulfoxide reductase, Superoxide dismutase [Mn], mitochondrial precursor</td>
</tr>
</tbody>
</table>
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Proteomics analysis enables us to identify toxicologically responsible proteins, and plays a very important role in uncovering new biomarkers (Gorg et al., 2000; Bandara and Kennedy, 2002; Wetmore and Merrick, 2004). The biomarkers obtained by in vitro proteomics analysis suggest oxidative stress-related proteins can serve as biomarkers of early stage hepatotoxicity (Yamamoto et al., 2005). In order to confirm whether the protein alteration detected by in vitro proteomics analysis reflected an in vivo protein alteration, an immunohistochemical examination of the liver of rats administered hepatotoxic compounds was carried out to detect oxidative stress-related proteins. As a result, rats in all treated groups including those administered AMD which showed no compound-specific morphological changes expressed oxidative stress-related proteins including Mn-SOD. Mn-SOD expression is an interesting evidence because Mn-SOD is an oxidative stress-related protein located in mitochondria. This result supports the findings of our previous report in the sense that measurement of mitochondrial respiration is a useful in vitro parameter of early stage hepatotoxicity. As significant biomarkers of hepatotoxicity, we could detect the changes of oxidative stress-related proteins before histopathological changes occur even if the toxicity is caused not only by acute but by chronic exposure. Although the mitochondrial respiration- or metabolism-related protein and oxidative stress-related proteins showing an altered expression by in vitro proteomics analysis were also found to be altered in vitro proteomics analysis, the in vivo changes tended to be smaller compared with those observed in the in vitro proteomics analysis. This phenomenon in vivo liver tissue would be dependent on the protective hepatocyte homeostasis with the effects of other components such as Kupffer cells. These results showed that an in vitro system is appropriate to detect the direct effect of early stage hepatotoxicity for high throughput screening with low bulk and high speed.

On the whole, we could demonstrate a fairly good correlation between in vitro and in vivo test systems for hepatotoxicity evaluation from the aspect of toxicologically responsible biomarkers. In this context, it was shown that oxidative stress-related proteins were usefully significant biomarkers of hepatotoxicity.

In conclusion, in vitro primary cell culture system would be sufficient to detect hepatotoxicity in the early stage of drug discovery.

REFERENCES


Comparison of in vivo and in vitro hepatotoxicity screening system.


