regarding the toxicological properties of CYP2E1 in conjunction with alcohol (Caro and Cederbaum, 2004). Mitochondrial ROS species production is synergistically induced by HCV core protein and CYP2E1, resulting in a reduction of mitochondrial antioxidant capacity and sensitivity to oxidants and TNF-α (Otani et al., 2005). CYP2E1-mediated oxidative stress reportedly down-regulates glucose-regulated proteins 78 and 94 that reside in endoplasmic reticulum and protect endoplasmic reticulum from CYP2E1-dependent oxidative stress (Dey et al., 2006). Taken together, CYP2E1 down-regulation in the chronic hepatitis C-infected liver may be a protective response of liver cells against oxidative stress. The CYP3A4 mRNA titer in blood showed a tendency to decrease with the progression of viral liver disease, which is consistent with our present study (Horiike et al., 2005). Pregnane X receptor, controlling CYP3A4 expression, failed to change in accordance with fibrosis stage progression (K. Nakai, unpublished data). The biological significance of decreases in liver CYP3A4 levels and drug transporters is, at present, difficult to interpret, due to little knowledge of biological function of CYP3A4 and drug transporters and the mechanisms and pathophysiology of chronic hepatitis C.

Genome-wide analyses of gene expression in liver biopsy specimens from patients with mild or early stage fibrosis caused by chronic hepatitis C have been reported. Up-regulation in comparison with normal liver patients of IL-6 and TNF and down-regulation of CYP2E1 was observed as compared with normal liver patients (Asselah et al., 2005; Bièche et al., 2005). It was also shown that IL-6 and TNF levels in patients with liver fibrosis stages 2 to 4 were significantly higher than those in patients with stage 1 fibrosis (Asselah et al., 2005). In our study population, up-regulation of TNF-α was consistent overall with the studies by Asselah et al. (2005), but IL-6 mRNAs were below the limit of detection in many subjects. IL-6 has been shown to down-regulate CYP1A1, CYP1A2, and CYP3A4 in human hepatoma cells (Fukuda et al., 1992) and to down-regulate CYP2E1 (Hakkola et al., 2003) and CYP3A4 (Jover et al., 2002). Abdel-Razzak et al. (1993) showed that IL-6, IL-1β, and TNF-α down-regulated expression of human CYP1A1, 1A2, and 3A in adult human hepatocytes in primary culture. These results were consistent overall with our experiments using HepG2 cells and human primary hepatocytes (Fig. 5). Interferon-γ also suppressed CYP1A2 and CYP2E1 in the human primary hepatocyte system (Abdel-Razzak et al., 1993). Similarly, in human primary hepatocyte cultures, cytokines negatively affected inducible expression of CYP1A1, CYP1A2, and CYP3A4 (Muntané-Relat et al., 1995). With respect to drug transporters, murine *Ntcp* was shown to be down-regulated by IL-1β in vivo (Geier et al., 2005). Very recently, Le Vee et al. (2008) reported repression of NTCP mRNA and protein expression by IL-1β in human primary hepatocytes. IL-1β was reported to inhibit CAR-induced expression of hepatic CYP3A4 (Assenat et al., 2004). A decrease in CYP3A4 is consistent with the present results, but a decrease in CAR mRNA levels has not been related to fibrosis stage progression (K. Nakai, unpublished data). Gene promoter analysis of human OATP-C expression revealed that HNF1α stimulated OATP-C expression (Jung et al., 2001). HNF1α transcriptional activator did not strikingly correlate with fibrosis staging (K. Nakai, unpublished data). Expression of human OCT1 was activated by HNF4α, according to a study that used a luciferase reporter assay (Saborowski et al., 2006). Expression of HNF4α was also not correlated with fibrosis staging (K. Nakai, unpublished data), which suggests that HNF4α by itself does not fully explain the mechanisms involved in the decrease in OCT1 expression in chronic hepatitis C patients. TNF-α repressed CYP2E1, CYP3A4, and OCT1 expression in HepG2 cells. Our preliminary results showed IL-6 down-regulation of CYP1A2, CYP3A4, NTCP, and OCT1 expression in the human hepatocytes of both two individuals. Our present results on fibrosis stage-dependent decreases in the expression of CYP1A2, CYP2E1, CYP3A4, NTCP, OATP-C, and OCT1 suggest that these decreases were likely controlled, at least in part, by mechanisms associated with the elevated cytokine production of TNF-α. CYP1A2 mRNA levels significantly correlated with CYP2E1 ($r = 0.705$, $p < 0.0001$) and CYP3A4 ($r = 0.535$, $p < 0.0001$), but not with CYP2D6 ($r = 0.231$, $p > 0.05$). In addition, OCT1 mRNA levels significantly correlated with NTCP ($r = 0.6341$, $p < 0.0001$), but not with MDR1 ($r = 0.231$, $p > 0.05$). These results are consistent with our hypothesis that inflammatory cytokines are involved in the down-regulation of the expression of these genes. The precise molecular mechanisms of fibrosis stage-dependent decreases in the expression of the CYP1A2, CYP2E1, CYP3A4, NTCP, OATP-C, and OCT1 genes governing drug metabolism and transport should be further clarified. The data, providing information on fibrosis stage-associated changes in the gene expression related to hepatic drug metabolism and disposition, are useful for drug therapy for patients with chronic hepatitis C infection. In fact, NTCP reportedly has an important role in hepatic uptake of ursodeoxycholic acid (Mita et al., 2006). Recent report indicated INR of prothrombin time negatively correlated with antipyrine clearance, informative indices for hepatic impairment in hepatitis C patients (Mahmoud et al., 2007). The results were quite consistent with our results because antipyrine was shown to be a substrate for CYP1A2 and CYP3A4 (Rendic, 2002). In conclusion, our results indicate that in the relatively early stages of chronic hepatitis C infection without cirrhosis, factors such as cytokines are likely to affect the expression of drug metabolism enzymes and drug transporters, such as CYP1A2, CYP2E1, CYP3A4, NTCP, OATP-C, and OCT1.

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Global gene expression changes including drug metabolism and disposition induced by three-dimensional culture of HepG2 cells-Involvement of microtubules

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ABSTRACT

Constitutive upregulation and a higher degree of induction of drug metabolism and disposition-related genes were found in a three-dimensional HepG2 culture. The upregulated genes are believed to be regulated by different regulatory factors. Global gene expression analysis using the Affymetrix GeneChip indicated that altered expression of microtubule-related genes may change the expressed levels of drug metabolizing and disposition genes. Stabilization of microtubule molecules with docetaxel, a tubulin-stabilizing agent, in the two-dimensional culture showed gene expression patterns similar to those found in the three-dimensional culture, indicating that the culture environment affects drug metabolism functions in HepG2 cells.

Drug safety is currently evaluated by animal experiments during the initial stage of drug development. Species-specific differences in drug metabolism and disposition make it difficult to estimate the capacity of human drug metabolism based on experimental animal data. Human primary hepatocytes have been used to overcome this problem; however, there are remarkable inter-individual differences in drug metabolism and disposition capacities that produce other disadvantages. Deriving a cell line from the human liver that yields more reproducibly expressed drug metabolism and disposition genes than hepatocytes would make it easier to conduct drug safety evaluations. However, the expression levels of those genes are usually low when established liver cells are cultured in ordinary tissue culture plates (i.e., under two-dimensional (2-D) conditions).

Three-dimensional (3-D) cell culture systems reportedly allow improved formation of glycosaminoglycan in NIH3T3 cells [1]; cell growth and differentiation of rat dorsal prostatic epithelial cells [2]; and improved albumin production in human fetal hepatocytes [3]. We previously adopted a Radial Flow Bioreactor system for the culture of HepG2 cells in a 3-D environment, and characterized gene expression during cell growth and stationary phases [4,5]. In the present study, we found elevated constitutive expression and induction of genes that encode drug metabolizing enzymes and drug transporters in the 3-D culture system, and elevated constitutive expression of microtubule- and cytoskeleton-related genes. The gene expression profile in the 3-D culture system was similar to the profile of cells in the 2-D culture system treated with docetaxel, a tubulin-stabilizing agent. This suggests that expression of cytoskeleton-related genes is an important factor supporting constitutive expression and induction of *CYP* genes and drug transporter genes.

Materials and methods

HepG2 cell line. The human hepatoblastoma cell line, HepG2, was obtained from the American Type Culture Collection (Rockville, MD, USA).

Plate (2-D) cultures. HepG2 cells were cultured in tissue culture plates (60-mm diameter; BD Falcon, Franklin Lakes, NJ, USA) at 37 °C under humidified air containing 5% CO_2.

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Radial Flow Bioreactor cell culture system (3-D cultures). A Radial Flow Bioreactor cell culture system (RFB; ABLE Corporation, Tokyo, Japan, Fig. S1) with a 5-ml bioreactor was used for the growth of high-density and 3-D cultures. The bioreactor was filled with a scaffold of hydroxyapatite beads that were 0.6–1.0 mm in diameter (Pentax Corporation, Tokyo, Japan). Culture medium was circulated between the bioreactor and the medium chamber, in which the condition of the medium was adjusted as follows: the concentration of dissolved oxygen was controlled at 4.0 ppm in the outflow of the reactor and the pH of the medium in the chamber was controlled at pH 7.6. HepG2 cell suspensions were prepared at a density of 3×10^7 cells in 200 ml of medium in the medium chamber and circulated between the medium chamber and the bioreactor at 7 ml/min until the cells were immobilized on the hydroxyapatite. After immobilization, culture medium adjusted to constant conditions was circulated between the medium chamber and the bioreactor at 7 ml/min. The volume of the culture medium in the medium chamber was maintained at 200 ml by constantly feeding fresh medium into the chamber and removing consumed medium from the chamber.

Induction of CYP3A4 by rifampicin. After cells were grown for a certain period of time (3-D culture: five days, 2-D culture: three days), they were cultured in medium containing 100 μM rifampicin for three days to induce the *CYP3A4* gene. Control cells were cultured with 0.1% of the vehicle (DMSO; Sigma, St. Louis, MO, USA). During the induction period, fresh medium, with or without rifampicin, was refed once every day.

Treatment of HepG2 cells with docetaxel. To analyze the effect of microtubule stabilization on a 2-D culture, cells were treated with docetaxel (Sigma). After cells were cultured on tissue culture plates for one day, they were cultured in medium containing various concentrations of docetaxel (0.01, 0.03, 0.1 or 0.5 μM) for three days. Control cells were cultured with 0.1% of the vehicle (DMSO). During this period, fresh medium, with or without docetaxel, was refed once every day.

RNA isolation. mRNA was extracted using the RNeasy Mini Total RNA Kit (Qiagen, Hilden, Germany).

TaqMan real-time PCR. Reverse transcription was performed with 1.0 μg of total RNA using TaqMan Reverse Transcription Reagent (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The following primer and probe sets were used for detection of each gene transcript using the Prism 7900 Real-Time PCR System (Applied Biosystems): *CYP3A4* (Hs00430021_m1), *CYP2B6* (Hs00868409_s1), *NR1I2* (*PXR*: Hs00243666_m1), *NR1I3* (*CAR*: Hs00231959_m1), and *ABCC1* (Hs00219905_m1). The expression level of each gene was normalized against *GAPDH* (*glyceraldehyde-3-phosphate dehydrogenase*) expression level. The measurement of each sample was performed in triplicate and averaged.

Expression analysis of drug metabolism-related genes by macro-array. Reverse transcription was performed with 1.0 μg of total RNA using the RT^2 PCR Array First Strand Synthesis Kit (SuperArray Bioscience Corp., Frederick, MD, USA). Simultaneous real-time PCR measurements of 84 drug metabolism-related genes were performed using RT^2 Profiler PCR Array ver. 3.0 (Drug Metabolism; SuperArray Bioscience Corp.). The dissociation curves of the PCR products were analyzed after the amplification profiles were obtained. Because the dissociation curves of the *CYP19A1* and *MTHR* gene products had two peaks, these genes were eliminated from further analysis. The expression of each gene was calculated by the ΔΔCt method using the control gene provided on the RT^2 PCR profiler Array. Each measurement was performed in triplicate and averaged. The two culture conditions were compared using a Student's *t*-test, and the level of significance was set at $p < 0.05$.

DNA microarray analysis. Total RNA was extracted from exponentially growing cells in three plates under 2-D culture for five days or from cells in three different portions of the reactor under 3-D culture for six days. The conversion of 10.0 μg total RNA into a target for the human genome U-133A Gene Chip DNA Microarray (Affymetrix, Santa Clara, CA, USA) was performed according to the manufacturer's instructions. Images of scanned microarrays were analyzed using GeneChip Expression Analysis Software (ver. 5.0) (Affymetrix). DNA microarray analysis was performed in duplicate for each sample of the 2-D and the 3-D cultures, and the signal intensities were averaged. Gene expression data were filtered according to the following criteria: (i) genes with expression differences within 1.5-fold between duplicate DNA microarray measurements, (ii) genes with a signal above 600 in either the 2-D or 3-D culture, (iii) genes with *p*-values less than 0.01 when a *t*-test was used to compare microarray data between the 2-D and 3-D cultures, (iv) genes with a $\geqslant$2.0-fold increase or a $\leqslant$0.5-fold decrease in expression in 3-D culture. The genes remaining after filtration were clustered on the basis of gene ontology using DAVID Bioinformatics Resources 2007 (Allergy and Infectious Diseases, NIH: http://david.abcc.ncifcrf.gov/) [6]. The complete dataset was submitted to the NCBI GEO (Gene Expression Omnibus) database (Accession No. GSE12939).

Results

Effect of 3-D culture on the expression of drug metabolism-related genes

To clarify the effect of 3-D culture on gene expression, an RT^2 Profiler PCR Array was used to compare the expression levels of 84 drug metabolism-related genes from cells cultured in the bioreactor for six days to those from cells cultured in plates for five days. The expression levels of 25 genes were altered ($p < 0.05$), 18 of which were upregulated in the 3-D culture (Fig. S2). The genes upregulated in 3-D culture were the followings; *ABCC1, CYP1A1, CYP2B6, ADH4, ALDH1A1, CHST1, FAAH, GAD1, GPX1, GSTA3, GSTP1, LPO, MGST1, NAT2, NOS3, PON2, SNN, ASNA1*. The genes downregulated in 3-D culture were the followings; *MT2A, CYP2J2, ALAD, FBP1, GPX3, HSD17B2, MGST2* (Table S1).

Effect of 3-D culture on global gene expressions

The results obtained by the RT^2 Profiler PCR Array (see above, Fig. S2 and Table S1) indicated that the expression of drug metabolism-related genes tended to be upregulated in 3-D culture when compared with a 2-D culture. To elucidate the mechanism underlying this difference, expression changes in genes other than those related to drug metabolism were examined in 3-D culture using the Affymetrix human genome U-133A GeneChip. The data were filtered according to the criteria described in the Materials and methods section. The number of genes with significantly altered expression levels was 355. These genes were categorized based on ontology provided by DAVID Bioinformatics Resources 2007 [6]. Genes in the "cell cycle", "the synthesis and the metabolism of steroid and lipid", and "cytoskeleton" clusters were upregulated. A considerable number of genes related to microtubule function in the cytoskeleton cluster were upregulated (Table S2). Besides these genes, one hepatocyte marker was included among the 355 genes. The expression of *CK8*, the marker for hepatocyte cells, was significantly upregulated 2.4-fold in 3-D culture. The expression of *CK19*, which is the marker for biliary cells, was enhanced 8.0-fold in 3-D culture, though this was not a statistically significant increase.

Effect of microtubule stabilization on CYP2B6 and ABCC1 gene expression

Among the genes listed in the cytoskeleton cluster are the *tubulin-βII* genes, which reportedly act as microtubule stabilizers [7].

Several groups have found that microtubule stabilization affects the expression of a number of genes, including *CYP3A4*: Sridhar et al. reported that the expression of *CYP3A4* is upregulated after treating HepG2 cells with anti-cancer drugs such as paclitaxel and docetaxel, both of which are known microtubule stabilizing agents [8], and Zden et al. showed that the expression and induction of *CYP3A4*, *2B6*, *2C8*, and *2C9* are downregulated when microtubules are destroyed by treating human hepatocyte cells with colchicine [9].

Therefore, cells in the 2-D culture were treated with several concentrations of docetaxel to evaluate the effect of microtubule stabilization on the regulation of drug metabolism-related genes. HepG2 cells in 2-D culture were treated with docetaxel at the concentration of 0.01, 0.03, 0.1 or 0.5 μM, just around the IC_{50} (0.05 μM), and the expression levels of *CYP2B6* and *ABCC1* were measured by real-time PCR (Fig. 1). The addition of docetaxel led to upregulation of the expression of both genes, even at the concentration of 0.01 μM. Increased expression of both genes was also observed at 0.003 μM, although the magnitude of the change was less than that produced by 0.01 μM (data not shown).

Effect of docetaxel on the expression of drug metabolism-related genes in 2-D culture and comparison to gene expression in 3-D culture

The expression of drug metabolism-related genes in HepG2 cells treated with docetaxel was compared with untreated cells using the RT^2 Profiler PCR Array (Fig. S3). The expression of 72 genes was altered ($p < 0.05$) by docetaxel treatment (Table S3). Of these 72 genes, the levels of 70 were increased by docetaxel treatment. The expression profiles of genes with levels that increased more than 2-fold or decreased less than 0.5-fold in 3-D culture were compared to those from docetaxel-treated cells. Genes with increased expression in 3-D culture were also upregulated by docetaxel treatment, and genes that were downregulated in the 3-D culture were also downregulated or left unchanged by docetaxel treatment (Fig. 2). The comparison indicated that the pattern of changes induced by docetaxel treatment was similar to that created by 3-D culture.

Effect of 3-D culture and docetaxel on PXR and CAR expression

The expression levels of *CYP2B6*, *GSTA3* and *GSTP1*, which are regulated by PXR and CAR, were increased more than 2-fold in 3-D culture and by docetaxel treatment. Therefore, to examine the effects of 3-D culture or docetaxel treatment on the expression of *PXR* and *CAR*, the expression levels of *PXR* and *CAR* were measured using real-time PCR. The expression of *PXR* was significantly increased by 3-D culture (Fig. 3A) and docetaxel treatment (Fig. 3B). Although *CAR* expression also significantly increased in 3-D culture and docetaxel treatment, the degree of induction was less than that of *PXR*.

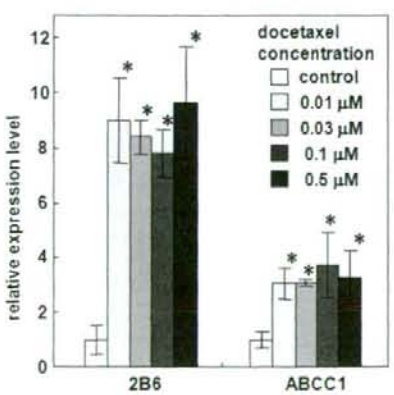

Fig. 1. Relative gene expression levels of *CYP2B6* and *ABCC1* in a 2-D culture treated with docetaxel. HepG2 cells in a 2-D culture were treated with docetaxel at the concentration of 0.01, 0.03, 0.1 or 0.5 μM, just around the IC_{50} value (0.05 μM). The expression levels of *ABCC1* and *CYP2B6* were measured by real-time PCR as described in the Materials and methods section. $^{*}p < 0.05$.

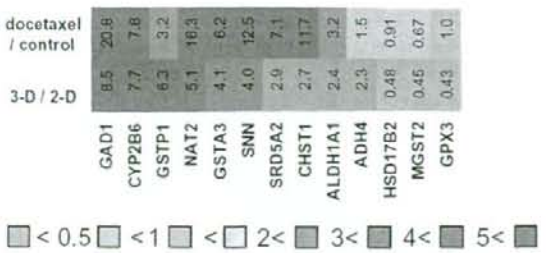

Fig. 2. Effect of docetaxel treatment on the expression of drug metabolism-related genes in 2-D culture and comparison of gene expression in 3-D culture. The changes in gene expression produced by growth in a 3-D culture and by docetaxel treatment were compared using genes with expression levels that increased more than 2.0-fold or decreased less than 0.5-fold in 3-D culture. Altered expression is indicated by the number and the pseudo-color as indicted below. The upper row shows the expression ratio of the docetaxel-treated sample and the control (docetaxel treatment/control), and the lower row shows the expression ratio of 3-D and 2-D cultures (3-D/2-D).

Effect of 3-D culture and docetaxel treatment on the induction of CYP3A4 expression

The expression of *PXR* and *CAR* was increased in 3-D culture and by docetaxel treatment. Therefore, the effect of 3-D culture and docetaxel treatment on the induction of *CYP3A4* by rifampicin, the proposed ligand for PXR, was examined by real-time PCR. Almost no induction was observed in HepG2 cells when grown in a 2-D culture. However, induction of *CYP3A4* by rifampicin was observed when the cells were grown in a 3-D culture or were treated in the 2-D culture with docetaxel (Fig. 4).

Discussion

A wide variety of drug metabolism-related genes were more highly expressed in 3-D culture than in 2-D culture (Fig. S2 and Table S1). However, no common regulatory mechanism has been reported that would provide a plausible explanation for these observations. For example, *CYP2B6* [10,11] and *ABCB1* [12] are regulated by PXR and CAR; *CYP1A1* [13] and *ALDH1A1* [14] are regulated by AHR; and *ABCC1* [15] is regulated by NRF2, indicating that 3-D culture affects not one but several transcriptional regulation pathways in a prevalent manner. We also observed the improvement of *CYP3A4* induction by rifampicin (Fig. 4A). In addition to *CYP3A4*, the inductions of *CYP3A5* and *CYP3A7* by rifampicin also improved (data not shown). Moreover, the hepatocyte marker *CK8* and the biliary cell marker *CK19* were upregulated, although the increase of *CK19* was not statistically significant (data not shown). The finding that not only genes related to drug metabolism but also the genes that are markers for hepatic function were upregulated indicates that HepG2 cells in 3-D cultures have more hepatocyte function than they do in 2-D cultures.

To explore the induction mechanism of these genes, global expression analysis was performed with an Affymetrix GeneChip. After identifying the genes whose expressions differed significantly in 3-D culture and clustering them on the basis of gene ontology, it

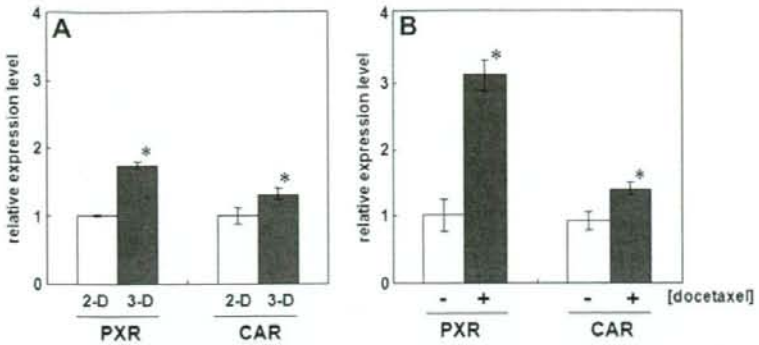

Fig. 3. Relative expression levels of *PXR* and *CAR* measured by TaqMan real-time PCR. (A) Comparison of the expression levels of 2-D and 3-D cultures. White bar: expression level in 2-D culture. Gray bar: expression level in 3-D culture. (B) Comparison of expression levels after cell treatment with or without docetaxel. White bar: gene expression of control sample. Gray bar: gene expression of 0.01 μM docetaxel-treated sample. $^{*}p < 0.05$.

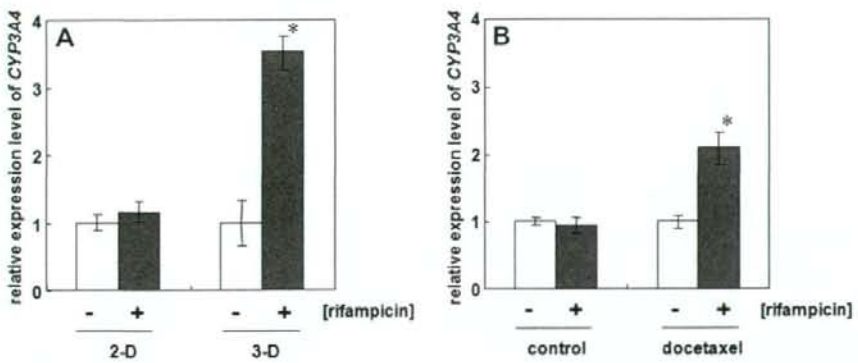

Fig. 4. Relative expression level of *CYP3A4* measured by TaqMan real-time PCR. (A) Comparison of the change in *CYP3A4* gene expression after rifampicin treatment in 2-D and 3-D cultures. (B) Comparison of changes in *CYP3A4* gene expression after rifampicin treatment when cells were or were not treated with 0.01 μM docetaxel. White bar: *CYP3A4* expression in cells treated with DMSO alone. Gray bar: *CYP3A4* gene expression in cells treated with 100 μM rifampicin for three days. $^{*}p < 0.05$.

was observed that the genes of several microtubule components were upregulated (Table S2). The *tubulin-βII* genes were included among these genes. It was reported that the increase of their products stabilizes the microtubule. When the microtubules were stabilized by adding docetaxel to the culture medium, expression of many of the drug metabolism-related genes was upregulated. Although docetaxel produced a larger magnitude of induction than 3-D culture, most of the drug metabolism-related genes altered in 3-D culture were also induced or suppressed in the same way by docetaxel treatment (Fig. 2). These results suggest that microtubules are involved in the expression and induction of drug metabolism-related genes.

Cytoskeleton gene products influence liver functions such as drug metabolism. For example, a reduction of bile flow is observed in *CK8*-knockout mice [16], and actin fibers and microtubules are necessary for the induction of *CYP2B1/2B* in hepatocytes [17]. These reports, as well as our results, indicate that the cytoskeleton plays an important role in the control of drug metabolism and other liver functions. Thus, elucidation of the mechanisms behind cytoskeleton modulation of liver functions is an important issue for further analysis. Such study will also be important for the establishment of a cell culture system that is more suitable for the testing of drug metabolism.

The results from the 3-D culture in RFB led us to examine the effects of microtubule stabilizing reagents such as docetaxel on the expression of drug metabolism-related genes and induction of the *CYP3A4* gene. A dozen genes were upregulated by docetaxel treatment (Fig. S3 and Table S3). In addition to the genes upregulated in 3-D culture, genes known to be regulated by a variety of transcription factors were included in the docetaxel-upregulated genes. For example, *BLVRB* [18] and *PON1* [19] are regulated by HNF4, *NQO1* [20] and *EPHX1* [20] are regulated by NRF2; *NQO1* [21] is regulated by AHR; *COMT* [22] is regulated by ESR1; and *APOE* [23] is regulated by LXR. As was the case with 3-D culture, docetaxel treatment of the cells in 2-D culture affected the expression of genes that are reportedly regulated by different transcriptional control mechanisms. The induction of *CYP3A4* was also enhanced by docetaxel treatment (Fig. 4B). These results suggest that docetaxel treatment in 2-D culture mimics the effects of 3-D culture on gene expression. Further study is needed to elucidate the mechanism of the induction of drug metabolism-related genes via stabilization of microtubule molecules. Our study also provides the opportunity to establish an effective and convenient analysis model for the drug metabolism.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.11.088.

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ORIGINAL ARTICLE

Validation of human skin models for skin corrosivity tests in Japan

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Abstract
As shown in OECD test guidelines 430 and 431, the human skin epidermal assay and Transcutaneous Electrical Resistance Test (TER) were validated and peer reviewed as an alternative method to corrosivity testing; however, these methods have not been used widely in Japan. The problems related to techniques and evaluation are not clear. Therefore, we performed a validation study of EPI-200 (EpiDerm™), a 3-dimensional cultured epidermal model and Vitrolife-Skin™, a 3-dimensional cultured skin model made in Japan as a catch-up validation trial of alternatives for skin corrosivity testing using 13 chemicals including a positive control: 10% potassium hydroxide solution in Japan. From the obtained data, we identified the potential of utilizing these models to evaluate the corrosivity of a chemical.

Key words: *Skin corrosivity, cultured epidermal model, cultured skin model, validation*

Introduction

Over the last decade, the European Centre for the Validation of Alternative Methods (ECVAM) has supported formal validation studies using *in vitro* tests as a replacement for the *in vivo* rabbit test for predicting skin corrosivity (Botham, et al.,1995, Barratt, et al., 1998, Fentem et al., 1998, Liebsch et al., 2000). As a result, two new test methods for skin corrosion, which incorporates a rat skin transcutaneous electrical resistance assay (TER) and two human skin epidermal assays, were included in Annex V of Directive 67/548/EEC in mid-2000, thereby making the use of *in vitro* alternatives for skin corrosivity testing of chemicals mandatory in the European Union (EC, 2000). As human epidermal model assays, two methods based on commercial human epidermal models, EPISKIN™ (EPISKIN, Chaponost, France) and

EpiDerm™ (MatTek, Ashford, MA, USA), were also endorsed.

Meanwhile, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) in the USA prepared final recommendations on these methods for their consideration and acceptance where appropriate (NIH Publication No.02-4502; ICCVAM, 2002). As a result, these assays were published as an alternative method to corrosivity testing as shown in the OECD test guidelines 430(OECD 430; 2004), and 431(OECD 431; 2004).

In Japan, these methods have not been widely used. The problems related to techniques and evaluation are not clear. In the present study, therefore we performed a catch-up validation trial to evaluate skin corrosivity using the human epidermal and skin models, that is, evaluations were made based on the ECVAM experimental protocol.

We performed a validation study of EPI-200 (EpiDerm™), a 3-dimensional cultured epidermal model and Vitrolife-Skin™, a 3-dimensional cultured skin model as validation trials of alternative for skin corrosivity testing in Japan. From the obtained data, we investigated the possibility of utilizing these models to evaluate the corrosivity of a chemical. We may suggest using these models to the ad hoc. committee of toxicology at MHLW in Japan.

Materials and Methods

Study management and organization

The study was performed according to the Japanese Society for the Alternative to Animal Testing Experiments (JSAAE) validation scheme as shown in Fig.1. The chairman was Dr. I Yoshimura at the Fac. Eng. Tokyo Univ. Science, who is head of the validation committee in JSAAE. Dr. Ohno at the National Institute of Health Sciences (NIHS) prepared the protocol and supported this validation with a grant from MHLW. Six Laboratories joined the study as shown in Table 1, and a blind trial with 13 chemicals including a positive control (10% potassium hydroxide solution) was performed using the protocol. In addition, Dr. Y. Ohno, the chemical distributor, coded and distributed the test chemicals to be used in the blind trial. After submission of all coded data to biostaticians, an independent biostatistical analysis of the blind trial was performed at the Fac. Med. Kyoto Univ. and Fac. Eng. Tokyo Univ. Science. The study director at each laboratory, a chemical distributor, biostaticians and kit suppliers were organized into study management teams in this validation assay as shown in Fig. 1. Finally, the chairman reported the outcome of this validation and forwarded this report to JSAAE.

Technical transfer and preliminary tests

The management team performed the technical transfer by kit suppliers at NIHS, Tokyo on January 28, 2004. After that, technicians performed the preliminary test using 10% potassium hydroxide solution and benzalkonium chloride 10 % solution. A qualified technician from each laboratory participated in the technical transfer and the preliminary

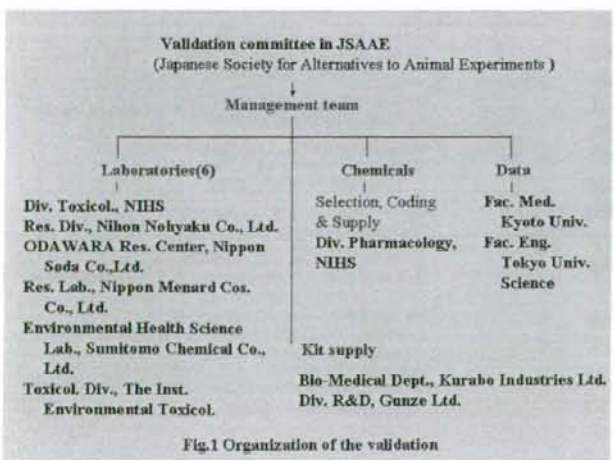

Fig.1 Organization of the validation

400μm 100μm

Fig.2 EpiDerm™ **Fig.3** Vitrolife-Skin™

test. All technicians obtained good results in this test.

Cultured epidermal and skin models

EpiDerm™ (EPI-200) models were purchased from KURABO Corporation (Osaka, Japan) as kits containing 24 models as shown in Fig.2, with sufficient amounts of Dulbecco's modified Eagle's medium (DMEM)-based assay medium, and phosphate-buffered saline (PBS) solution. These kits are made by MatTek Corporation (Ashland, MA, USA). The human epidermal model consisting of an epidermis with cornified layers was prepared as previously described (Liebsch et al., 2000).

Vitrolife-Skin™ models were supplied from Gunze Corporation Ltd. (Kyoto, Japan) as kits containing 24 models, collagen sponges without cells and sufficient amounts of DMEM-based assay medium, as shown in Fig.3. The human skin model consisting of a dermis and epidermis with cornified layers was prepared as previously described (Morikawa et al., 2002; Morota et al., 1998; Morota et al., 1999).

Materials

A total of 13 test chemicals including a positive control (10 % potassium hydroxide solution) were selected from the chemicals tested in the ECVAM skin corrosive validation study (Fentem et al., 1998, Liebsch et al., 2000). The chemical distributors selected test chemicals considering a balanced representation of the chemical classes, rate of corrosion or non-corrosion, solubility etc. from the total 60 chemicals tested in the ECVAM validation study. Test chemicals included six of which are known to be corrosive *in vivo*, six which are non-corrosive, six liquids, four solids and two powders, excluding the positive control. Each laboratory was sent the rotated 11 chemicals, including the positive control, in 13 test chemicals as shown in Table 2. Therefore, five data items from each laboratory for each chemical were obtained. All blinded test chemicals were treated as powerful drugs or poisons in each laboratory. The management team considered the minimum appropriate number of chemicals for catch up validation.

All test chemicals used were from the same batch and were purchased from Sigma Aldrich(Milwaukee, USA) and Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and were supplied to each laboratory by the chemical distributors. Phosphate-buffered saline (PBS) and isopropanol were obtained from Wako Pure Chemical Industries,

Table 1 List of members in skin corrosivity validation assay

Japanese Society for Alternative to Animal Experiments Validation Executive Committee

	Organization	Name
Chairman	Tokyo University of Science, Faculty of Engineering, Dept. Management Science	Isao Yoshimura
	National Institute of Health Science, Biological Safety Research Center, Division of Pharmacology	Yasuo Ohno

Study Director

	Organization	Name
	National Institute of Health Sciences, Biological Safety Research Center, Division of Toxicology	Tomoko Ando
	Nihon Nohyaku Co., Ltd., Research Division, Toxicological & Pharmaceutical Research Center	Katsuhiro Inagaki
	Nippon Soda Co., Ltd., Odawara Research Center, Toxicological Research Department	Mami Kuboki
	Nippon Menard Cosmetic Co., Ltd., Research Laboratories	Hajime Kojima
	Sumitomo Chemical Co., Ltd., Environmental Health Science Laboratory, Biochemistry Group	Yosuke Nakamura
	The Institute of Environmental Toxicology, Toxicology Division II, Laboratory of Immunotoxicology	Tadashi Kosaka

Kit supplier

	Organization	Name
	Kurabo Industries Ltd., Bio-medical Department	Hisashi Torishima
	Kurabo Industries Ltd., Biomedical Department	Michiru Genno
	Gunze Limited, Division of Research & Development	Noriyuki Morikawa

Coordinator

	Organization	Name
	Sumitomo Chemical Co., Ltd., Environmental Health Science Laboratory, Biochemistry Group	Naohiko Isobe
	Nippon Soda Co., Ltd., Agro Product Division, Regulatory Affairs Group	Yukihiro Kanaguchi
	National Institute of Health Sciences, Biological Safety Research Center, Division of Toxicology	Jun Kanno
	The Institute of Environmental Toxicology, Toxicology Division II	Takanori Harada
	Nihon Nohyaku Co., Ltd., Research Division, Toxicological & Pharmaceutical Research Center	Masaru Nogata
	Nippon Soda Co., Ltd., Agro Product Division, Regulatory Affairs Group	Mitsuo Hattori
	Nippon Soda Co., Ltd., Odawara Research Center, Toxicological Research Department	Yoshinobu Fujii
	The Institute of Environmental Toxicology, Toxicology Division II, Laboratory of Neurotoxicology	Sayaka Ishimine
	Sumitomo Chemical Co., Ltd., Environmental Health Science Laboratory, Biochemistry Group	Takashi Morimoto

Ltd. and 3-(4,5-dimethylthiazol-2-yl)- 2,5- diphenyltetrazolium bromide (MTT) and MTT formazan were obtained from Sigma Aldrich. They were supplied by the management team.

Methods

Chemical application procedure according to the ECVAM validation study.

The experimental steps of the method were performed according to the protocol used in phase III of the EpiDerm™ skin corrosivity test (Liebsch et al., 2000) with slight modifications. The EpiDerm™ models were equilibrated at 37°C and 5% CO_2 within one hour after receiving a kit and placed in 1 mL of DMEM-based assay medium in 6-well plates before use. If kept for a few days, it was preserved in a refrigerator. The Vitrolife-Skin™ models were placed in 250 µL of DMEM-based assay medium in 24-well plates and equilibrated for several hours' incubation (37°C, 5% CO_2) within a few days after receiving a kit. One hour before dosing, the models were transferred in 1 mL of DMEM-based assay medium to 6-well plates. Test chemicals were applied directly to the stratum corneum of two replicate models per chemical. Liquids (50 µL) were applied using a positive displacement pipette. Solids were crushed to a powder, if necessary, and 25 mg was applied using a spatula with the addition of 25µL of distilled water to ensure good contact with the surface. Two models were dosed with 100 µL distilled water as a negative control. After exposure for three or 60 min. at room temperature (15-25°C), two replicate models for each exposure time were rinsed thoroughly with PBS to remove the test chemical from the surface.

Table 2 Test chemicals

No.	Name	C/NC	Comments
1	Potassium hydroxide(10%aq)	C	Positive control
2	Sulfuric acid(10% wt)	C	
3	Octanoic (Caprylic) acid	C	
4	Sodium hydroxide(4.88%)	C	
5	Phenol	C	
6	Chromium trioxide	C	
7	Phosphoric acid	C	
8	Sodium perborate	NC	
9	Tetrachloroethylene	NC	
10	Potassium hydroxide(5% aq)	NC	
11	4-Amino-1,2,4-triazole	NC	
12	L-Lactic acid	NC	
13	Isopropanol (2-propanol)	NC	

Calculation of cell viability

The effects of the test chemicals on cell viability were determined using an MTT reduction assay. After blotting, the models were incubated in 0.3 mL (EpiDerm™) or 1 mL (Vitrolife-Skin™) of each DMEM-based assay medium containing 0.5 mg of MTT for an additional three hours at 37°C and 5% CO_2. Living cells were dyed dark-violet by the MTT reagents. After the models were washed with PBS, biopsies of Vitrolife-Skin™ models were taken using a biopsy punch (6 mm diameter), although this operation is not used in EpiDerm™ models. The biopsies were separated from the models using forceps, and placed into acidified isopropanol (2.0mL: EpiDerm™,1.0 mL: Vitrolife-Skin™), after removing excess water by placing the samples on absorbent paper. Precipitated formazan was extracted overnight at room temperature with protection from light. The absorbance of the extracts was measured at 570 nm using a UV-VIS spectrophotometer. Adequate absorbance of spectrophotometers was checked using 0.1mg/mL solution of MTT formazan prior to the validation study. Cell viability of EpiDerm™ models determined by the MTT reduction assay method was expressed as follows:

$$\text{Cell viability} = \frac{A_t}{A_c} \times 100\,(\%), \qquad (1)$$

where *At* and *Ac* are the absorbancies of the extracts when test chemicals and a negative control, respectively, are applied to the cultured skin model.

In case of Vitrolife-Skin™, additional tests using collagen sponges without cells were performed, with the potential to interfere with the MTT assay, and thus cell viability was expressed as follows:

$$\text{Cell viability} = \left(\frac{A_t - A_{bt}}{A_c - A_{bc}} \right) \times 100\,(\%), \qquad (2)$$

where *At* and *Ac* are absorbancies of the extracts

when test chemicals and a negative control, respectively, are applied to the viable Vitrolife-Skin™ model, and A_{bt} and A_{bc} are the values obtained for a blank test using a test chemical and the negative control, respectively, with a collagen sponge without cells.

Prediction models

Predictions of *in vitro* corrosiveness/non- corrosiveness were made according to the refined final prediction model (PM2) used in phase III of the EpiDerm™ skin corrosivity test (Liebsch *et al.*, 2000). Hence, chemicals that reduced cell viability to less than 50% upon exposure to the Vitrolife-Skin™ model for three min. were predicted to be 'corrosive' *in vivo*. If 3 min. exposure produced cell viability of ≥ 50%, the chemical was classified as 'non-corrosive' after a 3 min. exposure, but the same chemical was still be classified as 'corrosive' if viability after a 60 min. exposure was below 15%. The results obtained using the EpiDerm™ and Vitrolife-Skin™ models in this study were compared with the results of ECVAM validation studies using EpiDerm™ (Liebsch *et al.*, 2000) and EPISKIN ™ (Fentem *et al.*, 1998) for skin corrosivity testing.

This test was repeated twice. If different results from the two tests were obtained, a third test was performed at each laboratory and used for final judgment.

Table 3 Data from each laboratory

Chemical	Potassium hydroxide (10%). Corrosive						Sulfuric acid (10%). Corrosive					Tetrachloroethylene. Non-Corro				
Lab.	NIHS	NN	NS	NM	SC	IET	NIHS	NN	NS	NM	SC	NIHS	NN	NS	NM	IET
Blind No.	1	2	3	4	5	6	13	14	15	16	17	18	19	20	21	22
EpiDerm -test 1-	C	C	C	C	C	C	NC	NC	NC	C	C	NC	NC	NC	NC	NC
EpiDerm -test 2-	C	C	C	C	C	C	NC	C	C	C	C	NC	NC	NC	NC	NC
EpiDerm re-trial	C	C	C	C	C			C	C		C					
Judge	C	C	C	C	C	C	NC	C	C	C	C	NC	NC	NC	NC	NC
Vitrolife-Skin -	C	C	C	C	C	C	C	C	C	C	C	NC	NC	NC	NC	NC
Vitrolife-Skin -	C	C	C	C	C	C	C	C	NC	C	C	NC	NC	NC	NC	NC
Vitrolife-Skin re-	C	C	C						C							
Judge	C	C	C	C	C	C	C	C	C	C	C	NC	NC	NC	NC	NC

Chemical	Octanoic acid. Corrosive					Potassium hydroxide(5%). Non-					Sodium hydroxide(4.88%). Corrosive				
Lab.	NIHS	NN	NS	NM	IET	NIHS	NN	NS	SC	IET	NIHS	NN	NS	SC	IET
Blind No.	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37
EpiDerm -test 1-	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
EpiDerm -test 2-	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
EpiDerm re-trial		C	C				C	C							
Judge	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
Vitrolife-Skin -	C	C	NC	C	C	C	C	C	C	C	C	NC	C	C	C
Vitrolife-Skin -	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
Vitrolife-Skin re-			C				C	C				C			
Judge	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C

Chemical	4-Amino-1,2,4-triazole. Non-Corro.					Phosphoric acid. Corrosive					L-Lactic acid. Non-Corro.				
Lab.	NIHS	NN	NM	SC	IET	NN	NS	NM	SC	IET	NIHS	NS	NM	SC	IET
Blind No.	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52
EpiDerm -test 1-	NC	NC	NC	NC	NC	C	C	C	C	C	NC	C	C	NC	C
EpiDerm -test 2-	NC	NC	NC	NC	NC	C	C	C	C	C	C	C	C	C	C
EpiDerm re-trial											C	C	C	C	
Judge	NC	NC	NC	NC	NC	C	C	C	C	C	C	C	C	C	C
Vitrolife-Skin -	NC	NC	NC	NC	NC	C	C	C	C	C	C	C	C	C	C
Vitrolife-Skin -	NC	NC	NC	NC	NC	C	C	C	C	C	C	C	C	C	C
Vitrolife-Skin re-											C	C	C		
Judge	NC	NC	NC	NC	NC	C	C	C	C	C	C	C	C	C	C

Chemical	Isopropanol. Non-Corro.					Phenol. Corrosive					Sodium perborate. Non-Corro.					Chromium trioxide. Corrosive				
Lab.	NN	NS	NM	SC	IET	NIHS	NN	NM	SC	IET	NIHS	NN	NS	NM	SC	NIHS	NS	NM	SC	IET
Blind No.	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72
EpiDerm -test 1-	NC	NC	NC	NC	NC	C	C	C	C	C	NC	NC	NC	NC	NC	C	C	C	C	C
EpiDerm -test 2-	NC	NC	NC	NC	NC	C	C	C	C	C	NC	NC	NC	NC	NC	C	C	C	C	C
EpiDerm re-trial							C													
Judge	NC	NC	NC	NC	NC	C	C	C	C	C	NC	NC	NC	NC	NC	C	C	C	C	C
Vitrolife-Skin -	NC	NC	NC	NC	NC	C	C	C	C	C	NC	NC	NC	NC	NC	C	C	C	C	C
Vitrolife-Skin -	NC	NC	NC	NC	NC	C	C	C	C	C	NC	NC	NC	NC	NC	C	C	C	C	C
Vitrolife-Skin re-	NC											NC								
Judge	NC	NC	NC	NC	NC	C	C	C	C	C	NC	NC	NC	NC	NC	C	C	C	C	C

Laboratory:
NIHS: Div. Toxicol., NIHS
NS: ODAWARA Res. Center, Nippon Soda Co., Ltd.
SC: Environmental Health Science Lab., Sumitomo Chemical Co., Ltd.
NN: Res. Div., Nihon Nohyaku Co., Ltd.
NM: Res. Lab., Nippon Menard Cosmetic Co., Ltd.
IET: Toxicol. Div., The Inst.Environmental Toxicol.

C: Corrosive, NC: Non-Corrosive

Results

This validation study was not performed under GLP. However, all data obtained in each laboratory followed GLP compliance and spirit. Their records (data and detailed documents) could be checked after the assays, and raw data was sent to Tokyo Univ. of Science for analysis by biostatisticians. All documents were checked by the chairperson, biostatisticians and chemical distributors, and are stored in the NIHS.

Predictivity

Using cell viability after exposure to test chemicals for three or 60 min., the chemical classifications according to the EpiDerm™ prediction model are shown in Table 3. Data for positive controls in the two models were evaluated correctly at all laboratories. The EpiDerm™ data summarized in Table 4 excluded the positive control data. Of 30 classifications of six chemicals in the corrosive class, 29 classifications of EpiDerm™ were correctly predicted to be corrosive, and sensitivity was 96.7%. All six chemicals in the corrosive class were correctly predicted excluding one laboratory. Lab.1 gave a negative classification of sulfuric acid from two data sets, but this chemical is corrosive. Cell viability values after expose to sulfuric acid for 60 min. were 18.54% and 38.80%, and these values

Table 4 Contigency table for EpiDerm™ predictions

Vitro / *Vivo*	Corrosive	Non-Corrosive
Corrosive	29	1
Non-Corrosive	10 (5% KOH, Lactic Acid)	20

Table 5 Contingency table for Vitrolife-Skin™ predictions

Vitro / *Vivo*	Corrosive	Non-Corrosive
Corrosive	30	0
Non-Corrosive	10 (5% KOH, Lactic Acid)	20

Table 6 Key statistical parameters for the four tests

	EpiDerm™	Vitrolife-Skin™	EpiDerm™ (ECVAM)	EPISKIN™ (ECVAM)
No. of Chemicals	12	12	24	60
Sensitivity	100% (12/12)	100% (12/12)	92%	82%
Specificity	66.7% (4/6)	66.7% (4/6)	83%	84%
Accuracy	83.3% (10/12)	83.3% (10/12)	92%	83%
False positive rate	16.7% (2/12)	16.7% (2/12)	17%	19%
False negative rate	0% (0/12)	0% (0/12)	8%	14%

were slightly high compared to 15%, which is the border line. On the other hand, of 30 classification of six chemicals in the non-corrosive class, 20 classifications of EpiDerm™ were correctly predicted to be non-corrosive, and specificity was 66.7%, but two were false positives. There were 5% potassium hydroxide and lactic acid. All the laboratories gave them positive classifications from two data sets, which is a non-corrosive chemical. Positive predictivity was 74.4% (29 true corrosive classifications / 39 corrosive classifications in this assay). Negative predictivity was 95.2% (20 true non-corrosive classifications / 21 non-corrosive classifications in this assay). The total consistency rate was 81.7% (49 true classifications / 60 classifications in this assay).

The Vitrolife-Skin™ data are summarized in Table 5, excluding the positive control data. Of 30 classifications of six chemicals in the corrosive class, 30 of Vitrolife-Skin™ were correctly predicted to be corrosive, and sensitivity was 100%. All six chemicals in the corrosive class were correctly predicted.

On the other hand, of 30 classification of six chemicals in the non-corrosive class, 20 of Vitrolife-Skin™ were correctly predicted to be non-corrosive, and specificity was 66.7%, but two were false positives. They were 5% potassium hydroxide and lactic acid, which all laboratories gave a positive classification from two data sets. This chemical is non-corrosive. Positive predictivity was 75% (30 true corrosive classifications / 40 corrosive classifications in this assay). Negative predictivity was 100% (20 true non-corrosive classifications / 20 non-corrosive classifications in this assay). The total consistency rate was 83.8% (50 true classifications / 60 classifications in this assay).

Predictability of these two models was similar to the results obtained by the ECVAM validation study.

Intralaboratory variation

Most chemicals did not show any great differences in scores on tests repeated at each laboratory. Different classifications of EpiDerm™ accounted for 6.66% (4/60). These data are not shown in the Tables. Cell viabilities of sulfuric acid after exposure for 60 min. in Lab. 2 were 17.26%, 9.46% and 12.02%, and those in Lab.3 were 15.72%, 10.58% and 9.01%, respectively. On the other hand, cell viabilities of lactic acid after exposure for 60 min. in Lab. 1 were 16.55%, 13.39% and 7.19%, while those in Lab.5 were 15.85%, 12.01% and 15.89%, respectively. These cell viabilities were around 15% after exposure for 60 min. (the success criteria).

Different classifications of Vitrolife-Skin™ accounted for 5.0% (3/60). Cell viabilities of sulfuric acid after exposure for 60 min. in Lab. 3 were 5.90%, 16.09% and 6.34%, while after exposure to octanoic acid for 60 min in Lab.3 were 21.37%, 11.77% and 10.71%. These cell viabilities were around 15% after exposure for 60 min (the success criteria). Meanwhile, cell viabilities of sodium hydroxide (4.88%) after exposure for 3 min. in Lab. 2 were also 55.12%, 15.41% and 17.51%. These cell viabilities were around 50% after exposure for 3 min. (the success criteria).

These cell viabilities were in an extremely narrow range despite the different classifications. Therefore, intralaboratory variation between the two models is presumed to be small.

Interlaboratory variation

In EpiDerm™, inter-laboratory variation was significant for only sulfuric acid. The classification of sulfuric acid in Lab. 1 was different from the data in the other four laboratories. In the data of Lab.1, not shown in the Tables, cell viabilities after exposure for 60 min. were 18.54% and 38.80%, and these values were almost the same as the positive classification. For Vitrolife-Skin™, inter- laboratory variation was not significant. From these results, the feasibility of using EpiDerm™ and Vitrolife-Skin™ was suggested by the experiment.

Discussion

From the obtained data, we confirmed the potential of using EpiDerm™ and Vitrolife-Skin™ as methods to evaluate the corrosivity of a chemical. We consider the data form these models has high predictivity, and low intra- and inter-laboratory variation.

With Vitrolife-Skin™, however, it is necessary to use limited blank data using collagen sponges without cells.

Modified points of Vitrolife-Skin™ from the ECVAM skin corrosivity validation study

Application volume

Although the surface of the Vitrolife-skin™ model (0.5 cm^2) is similar to that of EpiDerm™ (0.63cm^2), 50 μL of Liquid chemical was often insufficient for the surface. In this study, therefore,

the application volume of liquids was increased from 50 μL, the volume used in the phase III protocol in the EpiDerm™ skin corrosivity test, to 100 μL. For the same reason, 50 mg of solid chemical was applied and 50 μL of water was added to ensure good contact with the surface (in contrast to the Phase III protocol, in which 25mg of solid and an additional 25 μL of water were applied. Additional tests using collagen sponges without cells, the Vitrolife-Skin™ model uses a collagen sponge without cells to construct the dermal layer, and this allows test chemicals to be easily absorbed and bound, compared with epidermal models consisting of only an epidermal layer and supporting material. In a previous study, tests using collagen sponges without cells, instead of non-viable Vitrolife-Skin™ models, were performed for several test chemicals with the potential to interfere with the MTT assay (Mirokawa, 2006). For 3- methoxypropylamine and n-heptylamine, these experiments suggested about 50-60% and 80% "viability", respectively, due to a chemical reaction with the MTT medium. Hence, the 70-80% viability obtained for 3-methoxypropylamine with the Vitrolife-Skin™ model should be corrected to about 20%. In the same way, the 120% viability obtained for n-heptylamine should be decreased to about 40%. Therefore, these two chemicals, which were incorrectly classified as negatives by testing without using blank collagen sponges, should correctly be classified as corrosive by adding blank collagen sponges, in agreement with the results from the EpiDerm™ model. The additional test for the other six chemicals gave results of around 15% "viability", such that the Vitrolife-Skin™ *in vitro* prediction of corrosivity was not changed.

Therefore, we obtained blank data using collagen sponges without cells in the validation of Vitrolife-Skin™. In this validation study, we detected solibilization, swelling and color change after exposure to chemicals, and the need to use blank collagen sponges without cells.

Comparison of skin models

As shown in Table 6, there was no difference in sensitivity, specificity, accuracy, false positive rate or false negative rate between EpiDerm™ and Vitrolife-Skin™ in this validation study. The result in this validation study may be due to no difference in structure between a two-layer skin model consisting of a dermis and epidermis (Vitrolife-Skin™) and epidermal models (EpiDerm™). The barrier function of cornified layers of the cultured epidermal and skin model is less effective compared with human skin tissue (Kojima *et al.*, 2000). In addition, as chemical exposure times become longer, stronger cytotoxicity occurs due to the accumulation of chemicals which permeate the cornified layer of the skin model. However, it is considered the barrier function of these model is similar.

The sensitivity was 92% in phase III of the EpiDerm™ study and 82% in EPISKIN™ study, and the present values (100%) were higher than data of these previous validation assays. The specificity, however, was 83% in phase III of the EpiDerm™ study and 84% in the EPISKIN™ study, and the present ones (66.7%) were lower than those. We consider these accuracy and false positive rates to be no different between the present validation and previous validation study. On the other hand, none of the false negative rates in present validation study were lower than data from previous validation studys. This issue must be handled carefully, because this assay is a catch-up validation trial, and the number of chemicals and classes is small.

Though peer review of these models is in progress, the ad hoc. committee of toxicology at MHLW in Japan has approved the utilization of these models to evaluate the corrosivity of a chemical.

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マイクロドーズ臨床試験ガイダンスおよび早期探索的臨床試験について

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1—序

医薬品開発のなるべく早期にヒト試験を行う早期探索的臨床試験は、薬物動態、薬理作用、安全性の面から最適化合物を早期に選択し、従来の第一相から第三相の臨床試験段階での成功率を上げることに役立つと考えられる。また、第一相試験以後の開発経費の浪費を削減するとともに、開発段階に応じたリスクを小さくし、医薬品開発をスムースにし、有用な医薬品を一日も早く患者に届けるために極めて重要である。欧米では医薬品開発を促進するためマイクロドーズ臨床試験（MD試験）を初めとする早期探索的臨床試験を導入するなど、国レベルで積極的な施策を採ってきた(EMEA 2003.1, FDA 2006.1)。我が国においても、「早期臨床試験による医薬品開発促進に関する意見書」が日本薬物動態学会から提出され(2006.3.1)、また、総合科学技術会議・基本政策推進専門調査会が、臨床研究を効率的に進めるための手法として、マイクロドージング等の新しい技術の導入に向けて検討すべきであると指摘された(2006.7.26)。その結果、2008年6月3日に我が国においてもMD試験が導入された(薬食審査発第0603001号)。本項では、探索的臨床試験全体の意義を紹介するとともに、MD臨床試験のガイドラインを紹介する。なお、臨床試験の一般指針に記述された探索的臨床試験と区別するため、本原稿では開発候補物質の選択のために従来の第一相試験のなかで行われる探索的臨床試験を早期探索的臨床試験とした。

2—早期探索的臨床試験の定義とその目的

早期探索的臨床試験はその目的と投与量に基づき、概略、①極めて低用量を用いて薬物動態を検討するMD試験（Ⅰ型）、②MD試験よりは高いが、薬効用量よりは低い用量で薬物動態と薬効につながる作用を評価する準薬効用量早期探索的臨床試験（Ⅱ型）、及び③薬効は現れるが毒性は現れないと思われる用量を用い、薬物動態や薬物相互作用、及び薬効を評価する薬効用量早期探索的臨床試験（Ⅲ型）に分けられる。これらを単回あるいは反復投与の回数に応じて分けることもある（**表**）。従来の第一相試験は推定薬効用量を超え、被験者が耐えられる最高用量（最大耐量）まで投与するが、早期探索的臨床試験では、用量や投与回数を限定することにより、また、試験の目的を限定することにより、試験実施に必要な非臨床試験の範囲を狭め、少量の被験物質での評価を可能とし、また、GMP上の負荷を軽くし、従来よりも臨床試験に入りやすくなるように企図したものである。

1）MD試験（Ⅰ型）：6月3日に通知されたガイダンスではMD試験とは、「ヒトにおいて薬理作用を発現すると推定される投与量（以下「薬効発現量」という）の1/100を超えない用量又は100 μgのいずれか少ない用量の被験物質を、健康な被験者に単回投与することにより行われる臨床試験をいう」と定義された。その目的は、「被験物質のヒトにおける薬物動態に関する情報を医薬品の臨床開発の初期段階に得ることである。具体的には、被験物質の吸収や血中動態、排泄特性、ヒトにおける代謝物プロファイル等を明らかにすること、分子イメージング技術を用いて被験物質の体内における局在に関する情報を得ること等である」とされた。MD試験を医薬品開発の初期に実施し、薬物動態学的に適正と思われるものを選択しておくことにより、その後の臨床開発において、体内動態が原因で開発が失敗する可能性を削減できるものと期待されている。この試験では許容される投与量が極めて低いことからAccelerator Mass Spectrometry（AMS: 加速器質量分析法）や高感度LC/MS/MS (Liquid Chromatograph/Mass Spectrometry/Mass Spectrometry)などの高感度分析法を使用する必要がある。また、Positron Emission Tomography (PET: 陽電子放射断層撮影法）等のイメージング技術と組み合わせることにより、ヒト体内分布を明らかにし、ヒト組織を用いた*in vitro*試験結果と組み合わせることにより、標的部位での薬力学的作用や意図せぬ部位での作用の推定

にも利用できる。

2）準薬効用量早期探索的臨床試験（早期探索的臨床試験Ⅱ型）：マイクロドーズ臨床試験の投与量よりは高いが、薬効や有害作用が現れることは期待されない用量（薬効用量のおよそ1/2を超えない量）を健常人あるいは軽症患者に単回投与することにより、薬物動態や薬効に関連する生物学的指標（バイオマーカー）を評価するための試験である（ここでいう「薬効用量」とは一般的な意味での薬効量であり、①の「薬効発現用量」とは異なる）。この試験では、通常の分析技術を用いても被験物質の体内動態の検討が可能であるとともに、薬効用量との乖離が少ないことから、臨床薬物動態の予測性が高い。更に、適切なバイオマーカーの変化を調べることにより、臨床での有効性予測が可能である。

3）薬効用量早期探索的臨床試験（早期探索的臨床試験Ⅲ型）：薬効は現れるが有害作用が現れることは期待されない用量を、健常人あるいは軽症患者に単回あるいは14日間を限度として反復投与し、薬物動態や薬物相互作用の検討、また、非臨床スクリーニング試験で推定された生物学的活性や薬効がヒトでも現れるか否かを確認することを目的とした試験である。

なお、現在進行中のICH-M3の議論ではこれらの試験を更に細分し、それらの実施に必要な非臨床試験の要件を検討中である（**表**）。

3―早期探索的臨床試験実施の意義

早期探索的臨床試験の意義は以下のように要約できる。なお、MD試験でも可能と思われるものには"(MD)"を付記した。

①早期に最適化合物を選択することによる、新薬開発の効率化とコスト削減(MD)

②薬効標的の妥当性の早期確認による合理的な医薬品開発の推進

③医薬品候補物質の価値を早期に見極めることにより、それ以後の資金調達が容易になる(MD)

④薬効発現における種差および個人差による問題への早期対応

⑤第1相以後における医薬品開発の成功確率の向上と、それにより志願者の協力に応えることができる(MD)

⑥動物使用数の削減による動物実験に関する3R原則への貢献(MD)

⑦患者への新薬供給の迅速化(MD)

⑧わが国における医薬品開発の効率化を図るとともに、国際的にfairな医薬品開発環境が整えられる。

また、上記以外にも、以下に示すような様々な目的への利用が期待される。

①ヒト薬物動態パラメータの複数化合物での比較(MD)

②ヒト特有な代謝物の早期発見とその意義の評価

表　ICH-M3合意文書案（Step 2）における早期探索的臨床試験の内容

臨床試験の型	内容	実施に必要な非臨床試験
マイクロドーズ臨床試験（アプローチ1）	総投与量を100μg以下とし、被験者1人あたり5回までで分割して投与するもの。	1）1つの動物種（通常、げっ歯類）における臨床投与経路を用いた拡張型単回投与毒性試験 2）薬理作用についての適切な評価
マイクロドーズ臨床試験（アプローチ2）	1回あたりの最高用量が100μgで投与回数が5回以下（被験者あたり総投与量500μg以下）の試験。	1）1つの動物種（通常、げっ歯類）における臨床投与経路を用いた7日間反復投与毒性試験 2）非標識化合物の遺伝毒性に関する構造活性相関評価 3）薬理作用についての適切な評価
準治療用量又は予定治療用量までの単回投与臨床試験（アプローチ3）	準治療用量（薬理作用発現用量）又は治療用量まで単回投与	1）げっ歯類及び非げっ歯類を用いた拡張型単回投与毒性試験 2）遺伝毒性試験（Ames試験） 3）薬理作用についての適切な評価及び安全性薬理試験のコアバッテリーが必要
反復投与臨床試験（アプローチ4）	最長14日間までの投与を、ヒトにおける薬物動態及び薬力学的特性を決定するために行うものであり、臨床における最大耐量を決定することを意図するものではない。	1）げっ歯類と非げっ歯類における標準的な2週間反復投与毒性試験。 2）用量設定は、最高用量にて予想される臨床AUCの倍数の暴露を基にする。 3）遺伝毒性試験（Ames試験及び染色体異常誘発能試験） 4）安全性薬理試験のコアバッテリーが必要
反復投与臨床試験（アプローチ5）	同上	1）げっ歯類を用いた標準的な2週間反復投与毒性試験（げっ歯類が適切な種であることの理由が必要）。 2）非げっ歯類（n=3）を用いた最短3日間で、少なくとも予定臨床試験期間の試験。 3）げっ歯類でのNOAELの暴露での確認試験。 4）遺伝毒性試験（Ames試験及び染色体異常誘発能試験） 5）安全性薬理試験のコアバッテリー

について検討する。

③製造工程において、エンドトキシンその他の不純物が混入しないよう、必要な品質管理を行う。

④事前に同じ方法で製造した製品の無菌試験を実施し、同工程における品質に問題がないことを確認する。

⑤従来の工程に重要な製造工程の追加・変更を行う場合、当該製造工程により得られた被験物質による非臨床試験を再実施する。

3) 治験薬の交付手続き

治験薬の交付については、GCP省令においては、やむを得ない事由があるときを除き、「治験依頼者は治験薬について第三者を介在させることなく、直接実施医療機関に交付しなければならない」とされているが、MD試験においては、放射性標識体の合成等の被験物質の製造及び実施医療機関への交付について、外部事業者に行わせざるを得ない場合や被験物質の製造を実施医療機関において行わなければならない場合もある。そこで、「当該治験依頼者の責任のもとで、治験薬の品質確保、運搬及び受領を確実に行うことを前提に、外部事業者又は実施医療機関等の第三者に被験物質の交付を委託することが可能」とされた。

4) インフォームドコンセントについて

MD試験は医薬品のスクリーニングに使用されることから、インフォームドコンセントを受ける際に、通常の臨床試験とは異なる点、特に以下の点についてわかりやすい言葉で説明する必要がある。

①試験の目的

②事前に得られている動物実験等の非臨床試験データは第Ⅰ相試験の場合に比べ限定的であること。

③放射性標識体を投与する場合、放射性物質による内部被爆があること。

④MD試験の実施により生じた健康被害については補償されること及び具体的な補償方法。

6 ― MD試験ガイダンスの問題点

1) MD用量と臨床用量との間の線形性

MD試験での用量と臨床用量との間には100倍以上の用量差があることから、その間の関係が線形であるか否かについての疑問がある。しかし、日本薬物動態学会(2006)は「薬物濃度が代謝酵素、トランスポーターなどへのKm値に比べて十分に低いところでは、線形性が保たれる」ことは当然であり、それを否定する根拠はないとした。また、今日治療に用いられている医薬品の多くにおいて、臨床投与量では、溶解度が原因である場合を除けば薬物動態が原因で非線形性を生じる例は少ないとされている（杉山と加藤2007)。即ち、吸収段階でのトランスポーターが臨床レベルで飽和するなど特別な場合を除き、低用量レベルでは理論的に線形となると考えられる。実際、Lappinら（2006）による研究では5検体中3検体で線形性が見られた。しかし、このように実際の試験で証明した事例は少なく、今後、更に事例を積み重ねることが重要と思われる。

2) MD試験を行う体制

MD試験は開発早期における医薬品候補物質のスクリーニングであり、必ずしも最終的な承認申請につながることを意識した試験ではないことから、治験になじまないとの考えがあった。しかし、MD試験による志願者のリスクは極めて低いものではあっても、全くゼロとは言い切れない。治験とすることにより、公的な審査を経て、GCPの枠組みの中で行われることになり、被験者の安全や権利が保障されるとともに、思いがけない事故が起きた場合においても適切な対応が可能となることから、治験として実施すべきとされたものである。

なお、志願者の善意に応えるためには、医薬品開発のための臨床試験は事前に得られた情報に基づく科学的・倫理的考察の上で適切に行われるべきものでなくてはならず、その論理的経緯は治験届や承認審査の段階で明確に見える形で明らかにすべきものである。

3) 薬理活性発現用量の推定方法

MD試験では投与量は薬効発現量の1/100以下とされている。従って、MD試験実施のためには、科学的に妥当な薬効発現量推定方法が定まっていなければならない。ガイダンスには、①経験的な方法として、動物における薬効発現量をもとに体表面積換算することにより、ヒトでの薬効発現量を推定する方法、②薬物動態学的情報を用いる方法として、動物実験での薬理学的影響発現用量での最大血中濃度(Cmax)又は血中濃度時間曲線下面積(AUC)を基準に推定する方法、が示されている。

薬効発現量の予測は非臨床におけるモデル動物におけるPK/PD関係、ヒト組織やヒト酵素等の発現系を用いた*in vitro*試験等、ヒトへの外挿に適切と思われる評価系での結果を基に推定する必要がある。モデル動物においてAUCと薬効の間に良い相関関係があり、ヒトと動物の標的組織/臓器での*in vitro*の反応が同等であった場合、ヒトでも、同等のAUCの場合には同等の薬効が期待できることから、同等のAUCを示す投与量を薬効発現量とするのが適当であろう。また、濃度とレセプター占有率との関係や濃度－反応関係が明らかであり、これらの情報から有効血漿中濃度が明らかな場合は、この濃度にヒト推定分布容積を掛けた投与量が薬効発現量と推定できるであろう。

7 ―最後に

今回通知されたMD試験についてのガイドラインは現在の我が国内外の状況や経験に基づいて検討されたものである。ICHでは「医薬品の臨床試験のための非臨床安全性試験の実施時期についてのガイドライン」の見直しが行われており、2008年6月にステップ2案の合意がな