laboratory. There was some variation of CV75 among laboratories. This might have been caused by differences of culture conditions between laboratories, because the same lots of serum and the same cell line were used at all laboratories. However, the CV75s at the individual laboratory were very close to the common CV75s used in the second trial. The coefficient of variation for each test chemical was between 0.07 and 0.4, and the range of CV value was good compared with that in another inter-laboratory study on cytotoxicity assay (Tani et al., 1999). The results of the third trial are summarized in Table 4. Among the five test chemicals, p-benzoquinone, glutaraldehyde and lactic acid were correctly evaluated at all laboratories. On the other hand, two laboratories missed the sensitizing potential of ethylene diamine or eugenol. These results are almost the same as those in the second trial.

Discussion

Several in vitro skin sensitization methods using cell lines have been reported in response to current trends in animal welfare and regulatory opinion (Casati et al., 2005), but final validation and regulatory acceptance have not yet been achieved. We have reported that h-CLAT using THP-1 cells was useful for predicting skin sensitization in vitro (Ashikaga et al., 2007; Sakaguchi et al., 2007). However, more data were needed, especially about the transferability of the protocol, and the inter-laboratory reproducibility of the test, in order to support formal validation activities (Hartung et al., 2004). Therefore, we organized a multi-laboratory study involving seven laboratories. As a first step, two well-known sensitizers (DNCB and Ni) and one non-sensitizer (SLS) were evaluated. The purpose of the first trial was to establish technology transfer of the h-CLAT protocol. Because all laboratories correctly evaluated the sensitization potentials of these three chemicals, transferability of the assay was judged to be basically good. However, some differences in dose-response relationship were observed. The reproducibility improved when re-evaluation was conducted with freshly cultured THP-1 cells. These results suggest that tight control of cell culture conditions is important, especially for good reproducibility of cell-based assay in which protein expression is used as an indicator.

Based on these results, we refined the standard operating procedure (SOP). We introduced the requirements that the viability of control cells should be more than 90 %, and that the viability in the positive control should be more than 60%. After the introduction of tighter control of cell culture conditions, the reproducibility of the dose-response relationship was improved. From the result of the first trial, we concluded that the h-CLAT protocol is easy to transfer, and to further confirm the reproducibility with various kinds of chemicals, we tested four sensitizers and one non-sensitizer in a second trial. In the total of 35 tests (seven laboratories, five chemicals), there were two false-negatives (ethylene diamine and eugenol). Therefore, inter-laboratory reproducibility of the assay was basically good. Ethylene diamine is known to be very reactive with organic compounds (Agius et al., 1991), and it evaporates at room temperature. Further, eugenol showed poor water solubility at the application doses, because oil drops were observed in the cell culture medium. It would be difficult for h-CLAT to evaluate the sensitization potential of such chemicals, so the false negative results may simply reflect the particular characteristics of these two chemicals. It will be necessary to clarify the extent of applicability of h-CLAT, particularly in relation to the physicochemical properties of target molecules. Some differences in CD86/CD54 expression pattern were also observed among laboratories. This confirms the importance of predicting sensitizing potential not just with one marker, but with two or more markers. Python et al. (2007) reported that a combination of at least two markers was needed to establish a reliable evaluation of dendritic cell activation potential. We also should mention problems of h-CLAT. Test chemicals are treated with THP-1 cells in cell culture medium. Therefore, if test chemical disperse non-equally in cell culture medium (e.g., sticky, water-proof particle, oil spill, etc.), h-CLAT may not evaluate these potential correctly. In addition, THP-1 is thought to almost not have metabolic enzymes such as P-450 (Prof. Yoshida, Showa Univ., personal communication). Therefore, h-CLAT might not be able to evaluate a potential of chemical that can be changed by metabolism. Study on the applicability domain of h-CLAT remains to be done.

Finally, chemicals tested in the second trial were re-evaluated with doses determined at each individual laboratory as a third trial, to see whether more appropriate application doses could be selected, depending on the precise test conditions. However, differences of the values of CV75 between laboratories were not large. Furthermore, the results (positive/negative judgment) were almost

the same as in the second trial with common application doses. In conclusion, further study is necessary, especially to clarify the limitations of the assay. Finally, all laboratories correctly judged the sensitization potential of six test chemicals among eight chemicals. These results suggest that the h-CLAT protocol is easy to transfer, and that inter-laboratory reproducibility is basically good. We consider that h-CLAT will be ready for formal pre-validation study after further minor improvements of the method.

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Corresponding author:
Takao Ashikaga
Shiseido Co., Ltd., Quality Assurance Center,
2-12-1, Fukuura, Kanazawa-ku, Yokohama,
236-8643, Japan
Tel: +81-45-788-7308
Fax: + +81-45-788-7295
E-mail: takao.ashikaga@to.shiseido.co.jp

ORIGINAL ARTICLE

Validation of human skin models for skin corrosivity tests in Japan

Hajime Kojima[1,6], Tomoko Ando[2], Katsuhiro Inagaki[3], Mahito Ohhira[4], Tadashi Kosaka[5], Yosuke Nakamura[7], Hisashi Torishima[8], Noriyuki Morikawa[9], Jun Kanno[2], Mami Kuboki[4], Michiru Genno[8], Masaru Nokata[3], Takanori Harada[5], Takashi Morimoto[7], Isao Yoshimura[10] and Yasuo Ohno[11]

[1]Japanese Center for the Validation of Alternative Methods (JaCVAM), National Institute of Health Sciences (NIHS), [2]Div. Cellular and Molecular Toxicol. NIHS, [3]Res. & Develop., Div., Product Safety & Pharmaceutical Research Unit, Nihon Nohyaku Co., Ltd., [4]Toxicol. Res. Dep.,ODAWARA Res. Center, Nippon Soda Co.,Ltd., [5]Toxicol. Div. The Inst. Environ. Toxicol., [6]Res.Lab., Nippon Menard Cosmetic Co., Ltd., [7]Environ. Health Sci. Lab., Sumitomo Chemical Co., Ltd., [8]Bio-Medical Dep., Kurabo Industries Ltd., [9]Div. R&D, Gunze Ltd., [10]Fac. Eng. Tokyo Univ.Science, [11]NIHS

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^{TM}), a 3-dimensional cultured epidermal model and Vitrolife-Skin^{TM}, 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

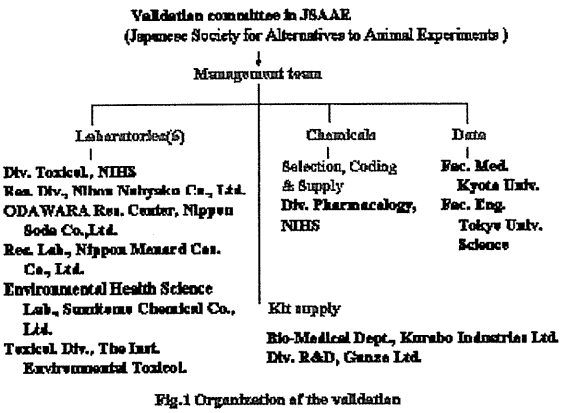
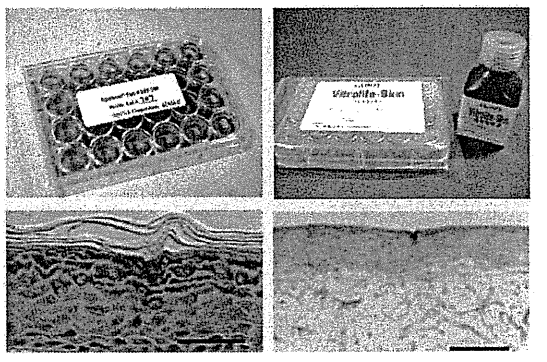


Fig.1 Organization of the validation



400μm 100μm

Fig.2 EpiDerm^{TM} **Fig.3** Vitrolife-Skin^{TM}

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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Corresponding author:
Hajime Kojima, Ph.D.
Japanese Center for the Validation of Alternative Methods (JaCVAM),
National Institute of Health Sciences (NIHS),
1-18-1, Kamiyoga, Setagaya-ku, Tokyo, 158-8501, Japan
Tel: +81-3-3700-9874
Fax: +81-3-3700-9874
E-mail: h-kojima@nihs.go.jp

医薬品安全性研究の動向
～マイクロドーズ試験を含めて～

漆谷　徹郎*

ポストゲノム時代，新薬開発は期待に反して加速されていない。ゲノム情報を活用しきれていない段階として安全性研究があるが，この分野もようやく新規技術開発の恩恵を受けるようになってきた。遺伝子型，遺伝子発現，タンパク質，代謝物を網羅的に解析するオミクステクノロジーの進歩は，「何が起こるかわからない毒性」を予測するための手がかりを与えてくれる。安全性研究者は，これらを臨床開発にまで利用できるバイオマーカーへと発展させる義務を負うが，それには多くの困難を克服する必要がある。マイクロドーズ試験は臨床治験を加速する手段として期待されているが，少なくともわが国の現状では安全性研究にインパクトを与えるには至っていない。

■キーワード：オミクステクノロジー，トキシコゲノミクス，バイオマーカー，マイクロドージング，早期探索的臨床試験

1 はじめに

ポストゲノム時代に入り，医薬品開発は新たな局面を迎えている。ヒトゲノムプロジェクトの進行中，ゲノム配列は宝の山であるという過大な期待が寄せられていたが，現在その熱は冷めつつある――というより，現実的な扱いになってきた。主要な医薬品としてGPCR（Gタンパク質共役型受容体）をターゲットとするものが多いことから，GPCR様配列がゲノム上に多数見出されたとき，新規医薬品開発を夢みた研究者は多かったが，そのほとんどは嗅覚受容体であることが判明し，期待はずれに終わった。しかしそうはいっても，遺伝性疾患の原因遺伝子に基づく薬物のターゲット策定や作用メカニズム解析において，ゲノム情報の寄与は大きい。毎週のように疾患関連遺伝子を同定したとの報道がなされ，それらの記事は「今回の発見は治療薬の開発につながる」と結ばれるのが常である。これが事実なら多くの難治性疾患があっという間に克服されるはずであるが，画期的な新薬が登場する数は年々減少の一途にある。

図1はしばしば引用される開発中止を余儀なくされた薬物の中止理由の変遷である。対外発表の場合必ずしも実情を反映していない場合があるにしても，特に減少が目立つのが薬物動態関連である。これは，その少なくとも一部にヒト型の薬物代謝酵素やトランスポーターの利用による予測性向上，すなわちヒトゲノム情報が寄与しているに違いない。一方，相対的に毒性関連の理由が増えており，さらに「有効性」を「安全性を考えるとそれ以上増量できないために目的の有効性が達成で

*同志社女子大学薬学部 医療薬学科病態生理学研究室　教授（うるしだに・てつろう）
（独）医薬基盤研究所 トキシコゲノミクス・インフォマティクス プロジェクトリーダー

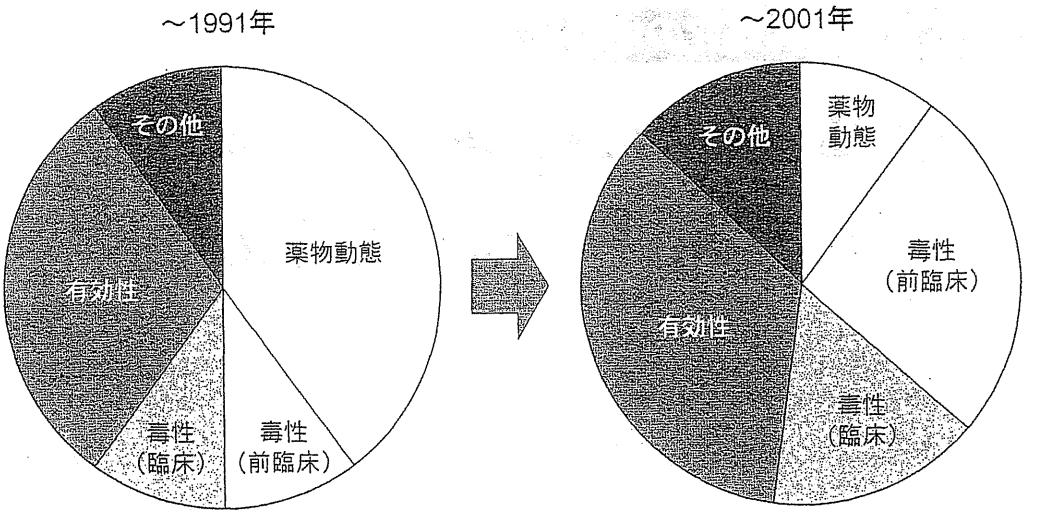

図1 米国における医薬品の開発中止理由の変遷
1991年の統計と，2001年の統計の比較で，この10年間に薬物動態を理由とするものが大幅に減った。
(Nature Rev. Drug Discov. 3：711-715 (2004) に記載のデータに基づき，筆者が改変)

きない」と考えると，ほとんどの理由が安全性関連と考えても差し支えないであろう。すなわち，安全性研究はまだゲノム情報解明の恩恵が薄いため，創薬プロセスから取り残された領域といえる。しかしながら，この分野にも着実な進歩の波が押し寄せている。

2 オミクステクノロジー

薬物開発の各段階，有効性の検討はターゲットが絞られている分，最新技術の応用がしやすい。一方，薬理作用の延長上にない範疇の毒性に関しては予めその対象を絞ることができない。サリドマイドの例を出すまでもなく，臨床で問題になった後にその有害作用を動物実験で再現することすら，かなりの努力を要するが，それを全く白紙の状態から予測しなければならない。薬物が生体に与える影響をすべて観測する方法があって初めて出発点に立てるのであるが，そんなことは不可能であると前世紀までの毒性学者は考えていたであろう。しかし，それに近いことを実現する技術，オミクステクノロジー (omics technology) が開発されてきたのである。

～ome とは「全体」を表す語尾，～ics とは「学問」を表す語尾であり，～omics とは「全体を扱う学問」ということになる。すなわち遺伝子全体(或いは発現遺伝子全体)，タンパク質全体，代謝物全体を網羅的に解析するものを，それぞれゲノミクス(genomics)，プロテオミクス(proteomics)，メタボロミクス (metabolomics) と呼び，これら技術を毒性学に適用した場合を，それぞれにトキシコ (toxico) という接頭語をつけて呼ぶ。

DNA マイクロアレイ技術の開発によって可能となったトキシコゲノミクス (toxicogenomics) には二つの方向性がある。ひとつは，遺伝子型と疾患，薬物代謝，薬物感受性などとの関連を見出すことによって，個別医療の道を開くことである。これは当然臨床の安全性確保にもつながることが期待される。個人の遺伝子型を決定するにはまだコストがかかるが，将来は，個々の患者が CYP の SNPs などの情報が入った IC チップを所持する時代が来るのであろうか。現在の段階では，個人差の大きい薬物代謝酵素には影響されないタイプの化合物を選択する，という戦略が主である。もう一つは，薬物投与後に生じる毒性学的変化を遺伝子発現の変化として捉えるものである。(mRNA の発現量の網羅的解析＝トランスクリプトミクス〔transcriptomics〕と呼ぶべきであるが，この語はあまり一般的でない)。非臨床安全

性研究において最も有効な使い道は，多種の既存薬物を投与した動物の各臓器における網羅的遺伝子発現変動をデータベースとして蓄えておき，開発候補品のそれと比較することにより毒性予測をするというものである。古典的毒性学的パラメーターの評価に比べて感度が高く，また毒性メカニズムに関しても同時に情報が得られることから，有用な方法である。ただし，予測性を高めるためには多量かつ均一なデータを蓄積する必要があり，これには膨大なコストがかかるため，欧米の巨大製薬企業でないと自前のデータベースをもつことはなかなか困難である。そこでわが国では，筆者も関係している，官民共同の「トキシコゲノミクスプロジェクト」が2002(平成14)年度から5年計画で行われ[1]，現在は第2期目「トキシコゲノミクス・インフォマティクスプロジェクト」に入っている。

トランスクリプトミクスの問題点として，

① 重要な遺伝子群が抽出されても，臨床に応用する場合バイオプシーが必要となり，現実的でない。

② たとえ毒性学的メカニズムが共通でも，個々の遺伝子発現でみると種差が大きい場合がある。

③ 翻訳後修飾が毒性学的に重要である場合が多く，mRNA量が直接機能と相関しない場合が多い。

などが挙げられる。種差を克服する手段としては，直接ヒトサンプルを対象にするのが理想であるが，バイオプシーが非現実的な選択である場合，遺伝子発現解析が可能な臨床サンプルとしては末梢血がほぼ唯一のものである。まだ成功例は少ないが，予測可能であるとの報告があり[2]，上記プロジェクトにおいてもデータ収集を開始している。

毒性学的機能に直結した因子を観測するには，タンパク質や代謝物の網羅的解析が必要となる。検出時に増幅が可能な遺伝子と異なり，特にタンパク質の検出感度は低い。現在，2D-DIGE(対照群と処置群を別々の蛍光色素でラベルしたものを混合し，2次元電気泳動で分離して，色調の変化で量の変動を定量する)で検出したタンパク質を質量分析で同定する方法が，最も効率がよいが，スループット性は低い。さらに，標的臓器において重要なマーカータンパク質が同定されても，これが臨床的に利用可能な侵襲度の低いサンプルに反映される確率は低いという，mRNA量と同様の問題がある。もちろん，他の方法で推定されたメカニズムの検証には，大いに利用価値があろう。

一方，低分子代謝物を検出するメタボロミクスは，高速液体クロマトグラフィーで分離後質量分析にかける方法と，核磁気共鳴スペクトルのピーク変化をデータベース化する方法に大別される。両方法とも，血清や尿という臨床に直結するサンプルに適応可能であるという大きな利点をもっている。その反面，変化する対象の同定が困難であり，必然的に毒性メカニズムに結び付け難いという問題が生じる。

結局，網羅的解析omicsといっても，複雑な生理反応に伴う変化のある一部を切り取ったものに過ぎず，それだけですべてを記述することには無理がある。そこで各網羅的解析結果を統合して解析する立場，トキシコパノミクス(toxicopanomics)の必要性が唱えられている。これは，安全性研究に限ったことではなく，すべての生命科学分野に当てはまることである。今世紀，重点的に発展させていく必要のある領域として，システムズバイオロジー(systems biology)が注目されているゆえんである。

3 バイオマーカーとレギュラトリーサイエンス

バイオロジストたるもの，客観的な指標なしに研究はできないのであり，バイオマーカーの概念は以前から存在していた。NIH(米国国立衛生研究所)の定義によれば，「客観的に測定され，評価される特性値であり，正常な生物学的プロセス，病理学的プロセス，または治療処置に対する薬理学的反応の指標として用いられるもの」であり，薬物開発に欠かせないことはいうまでもない。しかし創薬研究においてバイオマーカーという単語が注目されたのは，米国FDA(食品医薬品局)が明確な定義を発表してからである。「測定できる特性値であり，ヒトまたは動物における生理学的プロセス，薬理学的プロセス，または疾病プロセス

を反映しているもの。治療に伴うバイオマーカーの変化は当該製品に対する臨床的反応を反映する」という定義は，内容的には大差ないと感じられるが，薬物開発におけるインパクトは大きかった。その立場は2004年3月にFDAが発表したレポート「クリティカルパス・イニシアチブ(CPI: Critical Path Initiative)」(真に有効で安全な新規医薬品をいち早く患者に提供するために，産・官・学が協力して従来の開発プロセスを見直そうという構想)で明らかとなる[3]。CPIについては他稿に譲るが，「医薬品の開発が遅れているのは先端技術の取り入れが甘い段階があるためである。最新技術を取り入れ，薬剤開発に当たっては基礎研究から市販後に至るまで利用可能なバイオマーカーを確立する努力をせよ」という，規制側から突きつけられた要求と解釈できよう。もちろん，基礎研究用のローカルなバイオマーカーはそれなりに利用価値がある。しかし，次のような例を考えてみよう。ある生活習慣病の治療薬を開発している。長期連用したラットの肝臓で，ある遺伝子Xの発現変化が起き，無視できない有害作用につながる可能性が示唆されているが，ヒトでこれが起こるかどうかは不明である。連続的にバイオプシーをして有害作用発現前に遺伝子変化を捉えることができれば治験で評価できるが，生活習慣病のために何度も肝臓のバイオプシーを施行するのは現実的でない。ここでの遺伝子Xは立派なバイオマーカーであるが，臨床に適応できず，臨床開発において無力である。ここで安全性研究者に求められているのは，「ラットの肝臓における遺伝子X」を知ってしまった以上，この薬物を臨床開発するための安全性バイオマーカーを開発する義務を負う，ということである。

この狭義でのバイオマーカーの開発は，がん治療の領域，特に分子標的薬の分野での進歩が著しい。バイオマーカーは，診断マーカーと患者選別マーカーに分けられる。診断マーカーは（抗体のように病因が消失したのちも存続するものもあるが）多くの場合，薬効を判定するマーカーともなりうる。患者選別マーカーは，特定の医薬品が奏効する患者群を選別する目的で用いられ，例えば特定の遺伝子変異を標的とする抗がん剤の場合などに威力を発揮する。がんの場合，適正な治療方針を決定するのが急務であること，無効な抗がん剤の使用は有害作用が大きいこと，患部のバイオプシーを行う科学的・倫理的理由がみたされることなど，ゲノム情報を活用した生物学的裏付けのある臨床利用可能なバイオマーカー開発に適した環境にある。現在安全性研究者に求められているのは，非臨床で得られる科学的知識を，臨床で評価可能な具体的な指標として提示することなのである。

4 マイクロドージング

臨床開発の各段階における臨床試験デザインとその目的については，これまでの紆余曲折を経ながら「臨床試験の一般指針」に示されている。しかし最近，欧米の規制当局は第Ⅰ相試験よりも前の前臨床開発の過程で，ヒトを対象とした探索的な臨床試験(早期探索的臨床試験)の導入を推進している[4]。

早期探索的臨床試験は，投与用量をヒトに有害作用が現れないと想定される用量以下，投与期間を短期に限定して行うものである。医薬品開発の初期段階で医薬品候補物質の絞り込みや開発可否の見極めが目的であり，診断や治療を目的とするものではない。通常の第Ⅰ相試験で行われる最大耐量を求めることも意図していないことから，これを実施するために必要な非臨床安全性試験は，通常よりも規模や用量，方法を縮小・簡略化できる。従って，目的は限定されるが，従来よりも早期に臨床試験を実施できるという利点があるとされる。

早期探索的臨床試験で得ることが期待されている情報としては，

① ヒトでの薬物動態情報
② ヒト体内分布に関する情報
③ *in vivo* あるいは *in vitro* の薬効スクリーニング系から期待される薬効がヒトでも得られるかに関する情報
④ 薬効に関連するバイオマーカーに関する情報

などがある。早期探索的臨床試験はその臨床投与量に基づき，(1)極めて低い用量を用いて薬物動態を検討する「マイクロドーズ臨床試験」，(2)

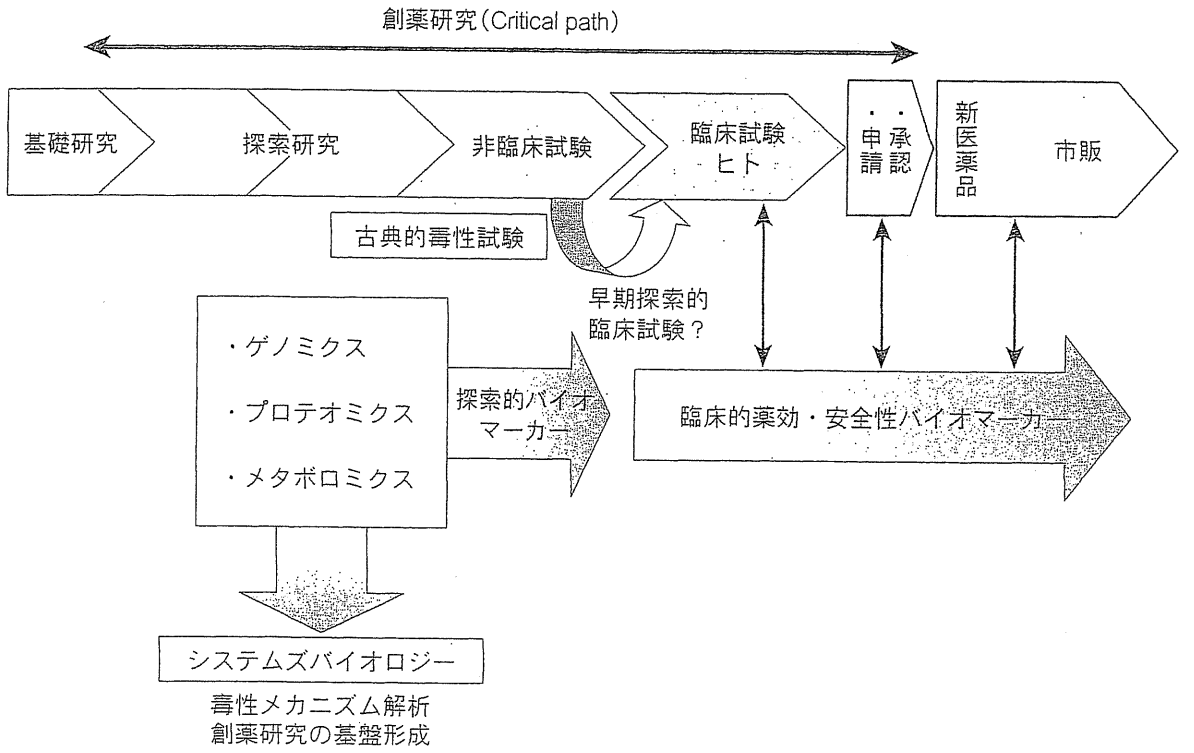

図2　創薬研究における安全性研究の関わり

古典的毒性学的研究が必要であることは変わりないが，ここにオミクステクノロジーを導入することによって毒性メカニズムの解析が進み，より成功確率の高い候補品が選択できる。望むらくはこの段階から，臨床の各段階での使用に耐えるバイオマーカーの創出につなげたい。動物実験と治験の間の大きなギャップを埋めるべく提案された早期探索的臨床試験は，現状の安全性研究に限っていえばまだ未知数である。

マイクロドーズ臨床試験よりは高いが，薬効用量以下の用量を用い，有害作用・薬効のいずれも現れないと想定される「準薬効用量早期探索的臨床試験」，及び（3）有害作用は現れないが，薬効は現れると想定される用量での「薬効用量早期探索的臨床試験」に大別される。現在わが国でガイダンス案が検討されているのは，このうち「マイクロドーズ臨床試験」であり，得られる情報もほぼ薬物動態に限られる。従って，安全性研究の立場からいえば，マイクロドーズ臨床試験で要求される安全性試験の妥当性，および将来，より高用量の試験に拡大されたときの安全性予測精度向上の可能性の2点が問題となり，現在はまだ臨床での安全性予測に直結するものとはいえない。

「マイクロドーズ臨床試験」とは，健常人に開発候補物質を「毒性試験におけるNOAEL[脚注]，及び薬効を発現するための予測投与量の1/100を超えない用量と，100μgのいずれか小さい方」を用いた極低用量（約2μg/kg）で単回投与する臨床試験である。本稿執筆の時点でまだガイドラインは確定していないが，欧米の例からみて，必要な前臨床試験としては拡大型単回投与毒性試験（一種の雌雄げっ歯類を用いた単回投与後2週間観察，各種毒性学的検査）を行って最小毒性量を求めればよく，かなり軽減されるようにみえる。問題は，臨床における薬効用量の推定と，放射性標識体による被験者内部被曝の安全性保証にあろう。特に後者は主目的が薬物動態であるため，極微量の試験薬を検出する必要から避けることが難しい。これはマイクロドーズ臨床試験を行わなければ生じない問題であり，放射性物質の規制が厳しく，一般人の心理的抵抗が高いわが国では，かなり高い

NOAEL：no observed adverse effect level；無毒性量

ハードルであると思われる。マイクロドージングを行えば通常の毒性試験や第Ⅰ相試験を省略できるのであればともかく，世界同時開発が通常である現在，最も規制のゆるい国で施行されることは確実で，わが国でわざわざマイクロドーズ臨床試験を行うメリットがあるとすればPET（ポジトロン断層撮影診断）用の診断薬ぐらいのものであろうという意見も聞かれる。

筆者の主観であるが，少なくともわが国の安全性研究においてマイクロドーズ臨床試験が大きなインパクトをもつことはまだないと思われる。将来的な問題は，用量を増加した早期探索的臨床試験が行われる場合の安全性研究である。2006年3月にロンドン近郊の病院において実施された完全ヒト化抗CD28モノクローナル抗体（TGN1412）の事件[5]はまだ記憶に新しい。第Ⅰ相試験において，投薬を受けた6名全員がICU（集中治療室）に搬送されるという，絶対に起きてはならない事態であり，「所詮動物実験による安全性予測精度が低いのならば，早期に人体実験に移行した方がよい」という一部の意見に，冷水を浴びせることになった。治験のプロトコールに見出された問題や，前臨床試験の充実でこれが予測できたか否かなど論点が多く，この場でこの問題を論じるのは避けるが，最大のポイントは，臨床における有害作用を時間的・用量的に予測可能なバイオマーカーが利用可能かどうかというところに帰着する。その意味から，これまで述べてきた種々の技術の応用がますます重要になってくることは明らかである。

以上，安全性医薬品開発における安全性研究の最近の動向を概観してみた。かつての創薬研究において安全性がネックになっていたことは否めないが，近年のオミクステクノロジーの進歩を取り入れることによってブレークスルーが可能になってきたとの感がある。現在の最大の課題は，これを臨床開発で活用できるバイオマーカーとして結実させることであるにちがいない。（図2）。

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GENE EXPRESSION PROFILING OF RAT LIVER TREATED WITH SERUM TRIGLYCERIDE-DECREASING COMPOUNDS

Ko OMURA[1], Naoki KIYOSAWA[1], Takeki UEHARA[1],
Mitsuhiro HIRODE[1], Toshinobu SHIMIZU[1], Toshikazu MIYAGISHIMA[1],
Atsushi ONO[1], Taku NAGAO[2] and Tetsuro URUSHIDANI[1,3]

[1]*Toxicogenomics Project, National Institute of Biomedical Innovation,*
7-6-8 Asagi, Ibaraki, Osaka 567-0085, Japan
[2]*National Institute of Health Sciences,*
1-18-1 Kamiyoga, Setagaya-Ku, Tokyo 158-8501, Japan
[3]*Department of Pathophysiology, Faculty of Pharmaceutical Sciences,*
Doshisha Women's College of Liberal Arts,
Kodo, Kyotanabe, Kyoto 610-0395, Japan

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ABSTRACT — We have constructed a large-scale transcriptome database of rat liver treated with various drugs. In an effort to identify a biomarker for interpretation of plasma triglyceride (TG) decrease, we extracted 218 probe sets of rat hepatic genes from data of 15 drugs that decreased the plasma TG level but differentially affected food consumption. Pathway and gene ontology analysis revealed that the genes belong to amino acid metabolism, lipid metabolism and xenobiotics metabolism. Principal component analysis (PCA) showed that 12 out of 15 compounds were separated in the direction of PC1, and these 12 were separated in the direction of PC2, according to their hepatic gene expression profiles. It was found that genes with either large or small eigenvector values in principal component PC 2 were those reported to be regulated by peroxisome proliferator-activated receptor (PPAR)α or constitutive androstane receptor (CAR), respectively. In fact, WY-14,643, clofibrate, gemfibrozil and benzbromarone, reported to be PPARα activators, distributed to the former, whereas propylthiouracil, omeprazole, phenobarbital, thioacetamide, methapyrilene, sulfasalazine and coumarin did to the latter. We conclude that these identified 218 probe sets could be a useful source of biomarkers for classification of plasma TG decrease, based on the mechanisms involving PPARα and CAR.

KEY WORDS: Triglyceride, Liver, CAR, PPAR, Toxicogenomics

INTRODUCTION

The toxicogenomics project was a 5-year collaborative project by the National Institute of Biomedical Innovation (NIBIO), the National Institute of Health Science (NIHS), and 15 pharmaceutical companies in Japan that started in 2002 (Urushidani and Nagao, 2005). Its aim was to construct a large-scale toxicology database of transcriptome for prediction of toxicity of new chemical entities in the early stage of drug development. About 150 chemicals, mainly medicinal compounds, were selected, and gene expression in liver (also kidney in some cases) was comprehensively analyzed by using Affymetrix GeneChip®. In 2007, the project was finished and the whole system, consisting of the database, the analyzing system and the prediction system, was completed and named as TG-GATEs (Genomics Assisted Toxicity Evaluation System developed by Toxicogenomics Project, Japan).

In toxicity studies, plasma triglyceride (TG) decrease is often observed. Because plasma TG level can be influenced mostly by nutritional status, decrease in food consumption is one of the factors for its change. For activator of peroxisome proliferator-activated

Correspondence: Tetsuro URUSHIDANI (E-mail: turushid@dwc.doshisha.ac.jp)

receptor (PPAR)α, it is a hot field of drug development, and this drug facilitates the expression of genes related to fatty acid β–oxidation (Schoonjans *et al.*, 1996), subsequently lowering plasma TG level. Phenobarbital (PB), an antiepileptic barbiturate derivative, decreases plasma TG level but increases plasma total cholesterol without decrease in food consumption in rats (Kiyosawa *et al.*, 2004; Hall *et al.*, 1990). Kiyosawa *et al.*(2004) proposed a mechanism of serum cholesterol elevation via up-regulation of hepatic cholesterol synthesis. So far, PB is not reported to be a PPARα activator, and the mechanism of plasma TG decrease by PB is not well understood. In these cases, plasma TG decrease could be a target of the drug in one case, or a sign of toxicity in another case, each with different mechanisms. Thus, identification of the mechanisms behind plasma TG decrease during drug treatment would enable both seed discovery and interpretation of toxicity.

One of the main purposes of TG-GATEs is to identify biomarkers for toxicity evaluation. Although there have been various reports describing strategies to extract marker genes from the transcriptome data (Hibbs *et al.*, 2004; Mutlib *et al.*, 2006; Tan *et al.*, 2006), the best way has not been established. In the present study, we have started to identify candidates of potential biomarker genes for interpretation of the fundamental mechanism(s) of plasma TG decrease, since our database contains several drugs that cause plasma TG decrease.

MATERIALS AND METHODS

Animals and treatments

Male Crj:CD(SD)IGS rats were purchased from Charles River Japan Inc., (Kanagawa, Japan) at 5-weeks of age. After a 7-day quarantine and acclimatization period, the animals were divided into groups of 5 animals using a computerized stratified random grouping method based on the body weight for each age. The animals were individually housed in stainless-steel cages in a room that was lighted for 12 hr (7:00-19:00) daily, ventilated with an air-exchange rate of 15 times per hour, and maintained at 21-25°C with a relative humidity of 40-70%. Each animal was allowed free access to water and pellet food (CRF-1, sterilized by radiation, Oriental Yeast Co., Japan). Rats in each group were orally administered with various drugs suspended or dissolved either in 0.5% methylcellulose solution (MC) or corn oil according to their dispersibility. At the time when the present analysis was performed, 15 compounds in our database were found to decrease the plasma triglyceride level during repeated administration (Table 1). Of these, isoniazid (INAH, 50, 100, 200 mg/kg; MC), phenobarbital (PB, 10, 30, 100 mg/kg; MC), thioacetamide (TAA, 4.5, 15, 45 mg/kg; MC), benzbromarone (BBr, 20, 60, 200 mg/kg; MC), methapyrilene (MP, 10, 30, 100 mg/kg: MC), amiodarone hydrochloride (AM, 20, 60, 200 mg/kg; MC), gemfibrozil (GFZ, 30, 100, 300 mg/kg; corn oil) and sulfasalazine (SS, 100, 300, 1000 mg/kg; MC) were purchased from Sigma Aldrich (St. Louis, MO, USA). Alpha-naphthylisothiocyanate (ANIT, 1.5, 5, 15 mg/kg; corn oil) was purchased from Kanto Chemical (Tokyo, Japan). Coumarin (CMA, 15, 50, 150 mg/kg; corn oil), propylthiouracil (PTU, 10, 30, 100 mg/kg; MC) and WY-14,643 (WY, 10, 30, 100 mg/kg; corn oil) were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Carbon tetrachloride (CCL4, 30, 100, 300 mg/kg; corn oil), clofibrate (CFB, 30, 100, 300 mg/kg; corn oil) and omeprazole (OPZ, 100, 300, 1000 mg/kg; MC) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Body weights were recorded every day while food consumption was recorded every 4 days during repeated dosing and expressed as g/day. The animals were treated for 3, 7, 14 or 28 days, and they were sacrificed 24 hr after the last dosing. Blood samples were collected to heparinized tube under ether anesthesia from the abdominal aorta after which the animals were sacrificed.

Blood chemistry analysis

Heparinized blood samples were centrifuged at 1,600 × g for 20 min to obtain plasma and the concentration of TG was determined using an automated clinical analyzer (Japan Bioassay Research Center and Anpyo Center; HITACHI 7070, Hitachi Ltd., Food and Drug Safety Center: COBAS MIRA plus, Roche Diagnostics, Bozo Research Center: TBA-120FR, Toshiba Lab Medical, Tokyo, Japan).

Microarray analysis

After collecting the blood, the animals were euthanized by exsanguinations from abdominal aorta under ether anesthesia. An aliquot of the sample (about 30 mg) for RNA analysis was obtained from the left lateral lobe of the liver in each animal immediately after sacrifice, kept in RNAlater®(Ambion, Austin, TX, USA) overnight at 4°C, and frozen at –80°C until use. Liver samples were homogenized with the buffer RLT supplied in RNeasy Mini Kit (Qiagen, Valencia, CA, USA), and total RNA was isolated according to the