The 22nd Annual Meeting of Japanese Society for Alternatives to Animal Experiments

Date: November 13th (Friday) and 15th (Sunday), 2009
Venue: Icho kaikan, Osaka University (Suita Campus)
Conress President:

Tsutomu MiKi Kurosawa, DVM, M.Phil, Ph.D, DVCS, DJCLAM
The Institute of Experimental Animal Sciences
Osaka University Medical School

Symposium 1

International Symposium

Reception

Reception

Reception

General Session (Poster)

Symposium 2

Social Gatherin

Special International Session

General Meeting

General Meeting (Society Award Presentation)

Social Gatherin

Social Gatherin

Social Gatherin

Symposium 3

Symposium 4

Challenge Contest

Public Seminar

Special International Session (Nov 14, Sat.) 10:00－12:50

Contemporary Topics 1 (Nov 14, Sat.) 10:00－10:35

Chair person Atsushige Sato（Tokyo Medical & Dental Univ.）

CT－1 Introduction of KoCVAM for Alternative Test Methods in Korea

Soon Young Han[1,2]
[1]KoCVAM Director, [2]Toxicological Evaluation and Research Department, National Institute of Food and Drug Safety Evaluation,
Korea Food and Drug Administration

Special Lecture (Nov 14, Sat.) 10:30－11:30

Chair person Tsutomu Miki Kurosawa（Osaka Univ.）

SL Advancing Laboratory Animal Welfare and Public Health Science: The Role of Innovative Refinement, Reduction, and Replacement Strategies

William S. Stokes
National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park,
North Carolinal, USA

Contemporary Topics 2 (Nov 14, Sat.) 11:50－12:20

Chair person Makoto Hayashi
（Biosafety Research Center Food, Drugs and Pesticides)

CT－2 Revision of EU Council Directive 86/69/EEC on animal experimentation: an overview of its impact on animal welfare and science

Coenraad F.M.Hendriksen
Netherlands Vaccine Institute (NVI), Nilthoven,
The Netherlands & Netherlands Centre Alternatives to Animal Use (NCA),
Utrecht University, The Netherlands

Contemporary Topics 3 (Nov 14, Sat.) 12:20－12:50

Chair person Yuko Okamoto （KOSÉ Corp.）

CT－3 Three-Dimensional Reconstructed Human Tissue Models:Current Status of Regulatory Acceptance and Industrial Use

Roger Curren
Institution for In Vitro Sciences,Inc.

（Thank you very much for the contributors offer to organize this session）

Symposium 1 (Nov 13, Fri.) 9:30－11:00

Chair persons Noriho Tanaka（Hatano Research Institute, Food and Drug Safety Center）,
Hajime Kojima (National Institute of Health Sciences)

S1－1 Chemical management policy and development of short-term hazard assessment methods

Takuya Igarashi
New Energy & Industrial Technology Development Organization (NEDO)

S1－2 The Bhas 42 cell transformation assay: from basic researches to a guideline

Kiyoshi Sasaki[1], Dai Muramatsu[1], Shoko Arai[1], Nobuko Endou[1],
Sachiko Kuroda[1], Kumiko Hayashi[1], Ayako Sakai[1],
Shojiro Yamazaki[1], Yeon-mi Lim[1], Makoto Umeda[1],
Masanori Wada[2], Noriho Tanaka[1]
[1]Div. of Alternative Research, Food and Drug Safety Center, [2] ABLE

S1－3 Immunotoxicity: Development of a reporter gene assay system using human cell lines

Setsuya Aiba, Rumiko Saito, Yutaka Kimura, Ai Memezawa,
Ikuko Numata, Toshiya Takahashi
Department of Dermatology, Tohoku University Graduate School of Medicine

S1－4 Development of novel alternative tests for developmental toxicity:
1) Reporter gene assays using murine ES cells

Koichi Saito, Noriyuki Suzuki, Satoshi Ando Nobuyuki Horie
Environmental Health Science Laboratory, Sumitomo Chemical Co., Ltd.

S1－5 Improvement of the embryo culture method for developmental toxicity tests

Masaharu Akita[1], Noriko Ishizuka[2], Atsushi Yokoyama[3]
[1]Dept. of Nutrition and Dietetics, Kamakura Women's University,
[2]Dept. of Nutrition, Kiryu University,
[3]Life Science Laboratory of Kanagawa

Symposium 2 (Nov 14, Sat.) 14:00－17:45

Co-sponsoring symposium with the Japanese Tissue Culture Association
Chair persons Miho Kusuda Furue（National Institute of Biomedical Innovation ）, Isao Asaka（Kyoto Univ.）,
Yasuyuki Sakai（Tokyo Univ.）, Koichi Imai（Osaka Dental Univ.）

Opening （Chair persons）

S2－1 Standardization of human ES and iPS cells for drug discovery

Miho Kusuda Furue[1,2]
[1]JCRB Cell Bank, Laboratory of Cell Cultures,
Division of Bioresources, National Institute of Biomedical Innovation,
[2]Laboratory of Cell Processing, Institute for Frontier Medical Sciences, Kyoto University

S2－2 Establishment of Disease-specific iPS Cells and Challenges in The Standardization.

Isao Asaka
Center for iPS Cell Res. Appl., Kyoto Univ.

S2－3 Development of a novel drug toxicity testing system using human iPS cells

Hiroyuki Mizuguchi[1,2]
[1] Lab. of Gene Transfer and Regulation, National Institute of Biomedical Innovation, [2] Dept. of Biochemistry and Molecular Biology,
Graduate School of Pharmaceutical Sciences, Osaka Univ.

S2－4 Human ES cell-derived cellular models for drug discovery and development

Kazuhiro Aiba
Stem Cell and Drug Discovery Institute

S2－5 Application of ES cells to toxicology
- Current status and expected utilization: heart toxicity -

Tadahiro Shinozawa
Development Research Center, Pharmaceutical Research Division,
Takeda Pharmaceutical Company Limited

S2－6 A novel embryotoxic estimation method of drugs using ES cells differentiation system

Shinji Kusakawa, Akito Tanoue
Department of Pharmacology,
National Research Institute for Child Health and Development

S2－7 Regenerative tissues derived from stem cells and its application to pharmacological research

Yasuhiro Ogawa, Kazuhiko Oishi
Dept. of Pharmacology, Meiji Pharmaceutical Univ.

S2－8　Evaluating system using cells derived from ES/iPS cell
-Application of Cardiomyocite to safety evaluation of a medical device and quality evaluation of crude drug-

Yasuhiro Takagi, Sumiko Kawai, Kaori,Tsutomu Miki Kurosawa
The Inst. of Experimental Animal Sci., Osaka Univ. Med. Sch

S2－9　Alternatives to animal experimentation using ES/iPS cells
- Improvement of the EST -

Koichi Imai
Dept. of Biomaterials, Osaka Dental Univ.

Discussion

Symposium 3 (Nov 15, Sun.) 9:00－11:00

Chair persons　Yasuo Ohno (National Institute of Health Sciences),
Takashi Unno (Safety Evaluation Forum)

S3－1　Introduction

Takashi Unno
Safety Evaluation Forum

S3－2　Better safety evaluation using human and rat hepatocytes in spheroids as an *in vitro* model

Hisakazu Iwai
Safety Evaluation Forum Sanwa Kagaku Kenkyusho Co., Ltd.

S3－3　Use of *in silico* tool in the research and development of pharmaceuticals

Ichiro Naeshiro
Strategic Research Planning Dept.,
Pharmaceutical Research Division,
Takeda Pharmaceutical Company Limited

S3－4　Prediction of Embryotoxicity by Embryonic Stem Cell Test

Norihito Matsumoto
Investigative toxicology research group,
Discovery technology laboratory,
Ono Pharmaceutical Co., Ltd.

S3－5　Contribution of ICH on 3Rs in alternatives to animal experiments

Yasuo Ohno
National Institute of Health Sciences

Discussion

Symposium 4 (Nov 15, Sun.) 11:00－12:30

Chair persons　Tsutomu Miki Kurosawa（Osaka Univ.）,
Tadao Serikawa（Kyoto Univ.）

S4－1　Policy on animal experimentation of JALAS and alternatives to animal experiments

Tadao Serikawa
President of Japanese society for laboratory animal science
Inst. of Lab. Animals, Grad. Sch. of Med., Kyoto Univ.

S4－2　Contribution of experimental animal technologist to Refinement and Reduction

Yuji Sakamoto
Vice-president of The Japanese Association for Experimental Animal Technologists.
Senju Pharmaceutical Co., Ltd.

S4－3　Activities related to the 3Rs in the Japanese Society for Laboratory Animals and the Environment

Seigo Shumiya
President of Japanese Society for Laboratory Animals and the Environment

International symposium (Japan, China and Korea)

(Nov 13, Fri.) 15:00－18:00

Chair persons Isao Yoshimura (Science Univ. of Tokyo), Masaharu Akita (Kamakura Women's Univ.)

IS－1 Introduction of animal welfare education in colleges of veterinary medicine of Korea

Jae-Hak Park
Soeul National University

IS－2 The Current Status of 3Rs Research in China

Yue bingfei
National Institute for the Control of Pharmaceutical and Biological Products. Beijing. China

IS－3 Alternatives in China: Today and Tomorrow

Qiu Lu
Shanghai Entry-Exit Inspection and Quarantine Bureau of the P. R. China

IS－4 Development status of the laboratory animal industry in Beijing area

Lin Jianwei
Beijing Administrative Office of Laboratory Animal

IS－5 Bioartificial skin and cornea as screening models

Young Sook Son
Department of Genetic Engineering, College of Life Science
Kyung Hee University

Luncheon seminar

(Nov 15, Sun., 12:30－13:30, Offer of Natsume Seisakusho Co.,Ltd.)

Chair person Teppei Ogawa (Natsume Seisakusho Co.,Ltd.)

LA－1 Alternatives and Anesthesia

Tsutomu Miki Kurosawa
Laboratoy for Laboratory Animal Medicine, Osaka Univ. Med. Sch.

LA－2 The function of NARCOBIT - the inhalation anesthesia system for small laboratory animals.

Masaaki Inoue
S.K.I.Net,Inc.

Luncheon seminar: The box lunch ticket is issued for the first 150 persons at the reception from 8:50 Sunday.

The 1st International Research Promotion Session of Alternative Animal Experiments by Mandom Corp. (Nov 13, Fri.) 14:00－15:00

M-1 Jae-Hak Park
Dept. of Laboratory Animal Med., College of Veterinary Medicine, Seoul National Univ.

M-2 Kenji Sugibayashi
Josai Univ.

M-3 Jeong-Ik Lee
Tokai Univ.

M-4 Tsutomu Miki Kurosawa
Osaka Univ.

(Please see the separate volume)

Challenge contest

(Nov 15, Sun.) 14:00－15:00 Free

(Please see the separate volume)

Public Seminar

(Nov 15, Sun.) 15:00－17:00 Free

Chair person Tsutomu Miki Kurosawa（Osaka Univ.）

PS-1 Kiyonori Nishida
Osaka Aquarium KAIYUKAN

PS-2 Nobuhiko Hishi
Kobe Univ.

General Session (Oral Session 1, Nov 13, Fri. 13:00－14:00)

Chair persons Hidenobu Okumura (NOEVIA Co.,Ltd.), Takashi Ohmori (Kyoto Univ.)

O1－1 Availability of silkworm infection model for identification of environmental pathogens and virulence factors

Kimihito Usui[1], Shinya Miyazaki[1], Chikara Kaito[1], Kazuhisa Sekimizu[1, 2]
[1]Laboratory of Microbiology, Grad. Sch. Pharm. Sci. Univ. of Tokyo,
[2]Genome Pharmaceutical institute Co., Ltd.

O1－2 Investigation of the Test Method for Eye Irritation potential Using a Reconstructed Human Corneal Model

Masakazu Katoh, Fumiyasu Hamajima,
Takahiro Ogasawara, Kenichiro Hata
Japan Tissue Engineering Co., Ltd.

O1－3 Genotyping Using Amp-FTA Method With Buccal Swab sample

Satoshi Nakanishi, Takashi Kuramoto, Tadao Serikawa
Institute of Laboratory Animals, Graduate School of Medicine,
Kyoto University, Kyoto

O1－4 Embryonic stem cell-derived hepatic tissue micro-culture system

Sungho Ahn[1], Miho Tamai[1], Yu Toyoda[1, 2], Hisashi Okuyama[1],
Toshihiro Akaike[1], Takayuki Shindo[3], Yoichi Fujiyama[4],
Eiichi Ozeki[4], Yoh-ichi Tagawa[1, 5, 6]
[1]Tokyo Tech., Grad. School of Biosci. and Biotech., [2]JSPS Research Fellow,
[3]Shinshu Univ., Grad. School of Medicine, [4]SHIMADZU CORP.,
[5]Tokyo Tech., FCRC., [6]JST PREST

General Session (Oral Session 2, Nov 14, Sat. 9:00－10:00)

Chair persons Hiroshi Itagaki (Shiseido Co.,Ltd.), Takashi Sozu (Osaka Univ.)

O2－1 The characteristics of a Bhas 42 cell transformation assay and its predictability for the carcinogenicity of chemicals

Ayako Sakai, Kiyoshi Sasaki, Dai Muramatsu, Shoko Arai, Nobuko Endou,
Sachiko Kuroda, Kumiko Hayashi, Yeon-mi Lim,
Shojiro Yamazaki, Makoto Umeda, Noriho Tanaka
Laboratory of Cell Carcinogenesis,
Hatano Research Institute, Food and Drug Safety Center

O2－2 Investigation of a Minimally-Required Size for a Two-Dimensional Liver Micro-Tissue toward *In Vitro* Toxicity Tests

Kikuo Komori, Ippei Kameda, Tetsu Tatsuma, Yasuyuki Sakai
Institute of Industrial Science, University of Tokyo

O2－3 Inter-laboratory validation study of in vitro eye irritation test; Short time Exposure (STE) test

H. Sakaguchi[1], N. Ota[2], T.Omori[3], H. Kuwahara[4], T. Sozu[5], Y. Takagi[6],
Y. Takahashi[1], K. Tanigawa[7], M. Nakanishi[7], T. Nakamura[8], T. Morimoto[9],
S. Wakuri[10] Y. Okamoto[7], M. Sakaguchi[2], T. Hayashi[4], T. Hanji[6],
S. Watanabe[8] [1]Kao Corporation, [2]Pola Chemical Industries, INC.,
[3]Kyoto University, [4]Kanebo cosmetics INC., [5]Osaka University,
[6]Pias Corporation, [7]KOSÉ Corporation, [8]LION Corporation,
[9]Sumitomo Chemical Co., Ltd., [10] FDSC

O2－4 Classification for skin sensitization potency using human Cell Line ActivationTest (h- CLAT)

Yuko Nukada[1], Takao Ashikaga[2], Takayuki Abo[1], Sakiko Sono[2],
Hitoshi Sakaguchi[1], Hiroshi Itagaki[2], Naohiro Nishiyama[1]
[1]Kao Corporation, [2]Shiseido Co., Ltd.

General Session (Poster)
Poster session, 13, Fri. 11:00～13:00,
Posted period, 13, Fri. 9:00－14, Sat. 17:00

P－1 Cardiotoxicity evaluation of the anticancer drug using chick embryo

Hiroyuki Miyazaki, Toshimi Iizuka, Motohiro Okayasu, Toshio Kouzuki, Takeshi Homma, Takashi Ogura, Yohei Inada, Akane Kakuyama, Takenori Tamaki, Tomoko Miyazaki, Miyoshi Kido, Yuji Yoshiyama
Center for Clinical Pharmacy and Clinical Sciences, Kitasato University School of Pharmacy

P－2 Development of non-aqueous peptide binding assay for *in vitro* skin sensitization test

Teppei Nawa, Kazuhiro Hara, Masahito Usami, Masanao Niwa
Hoyu Co., Ltd

P－3 The agreement of assay results between a 6-well method and a 96-well method in the Bhas 42 cell transformation assay

Shoko Arai, Kiyoshi Sasaki, Ayako Sakai, Dai Muramatsu, Nobuko Endou, Shojiro Yamazaki, Yeon-mi Lim, Makoto Umeda, Noriho Tanaka
Division of Alternative Research, Food and Drug Safety Center

P－4 A Genotoxicity test system based on p53R2 gene expression in normal human skin cells.

Taisei Mizota, Katsutoshi Ohno, Toshihiro Yamada
The Food Safety Research Institute, Nissin Foods Holdings Co., Ltd.

P－5 Validation of an in vitro screening test for predicting the tumor promoting potential of chemicals based on gene expression

Hideki Maeshima, Katsutoshi Ohno, Toshihiro Yamada
The Food Safety Research Institute,

P－6 The value of hepatotoxicity assessment using primary cultured human hepatocytes

Yoshifumi Takenobu
Investigative toxicology research group, Discovery technology Laboratory, Ono pharmaceutical Co., Ltd.

P－7 Review of an alternative to animal testing for safety evaluation of Quasi-drug

Hajime Kojima[1], Masafumi IIjima[2], Kayoko Matsunaga[3], Hitoshi Sasa[4], Hiroshi Itagaki[4], Yuko Okamoto[5], Naohiro Nishiyama[6], Hiroshi Onodera[7], Iku Mita[7], Jun Washida[8], Koichi Masuyama[8], Mitsuteru Masuda[1], Yasuo Ohno[1]
[1]National Institute of Health Sciences, [2]Showa University, [3]Fujita Health University, [4]Shiseido Co. Ltd., [5]KOSE Corporation, [6]KAO Corporation, [7]Pharmaceuticals and Medical Devices Agency, [8]Ministry of Health, Labour and Welfare

P－8 Validation of LabCyte EPI-MODEL24, an *In Vitro* Assay for Detecting Skin Irritants

Hajime Kojima [1], Yoko Ando [2], Yoshihiro Yamaguchi [3], Tadashi Kosaka [4], Tamie Suzuki [5], Atsuko Yuasa [6], Yukihiko Watanabe [7], Shinsuke Shinoda [8], Kenji Idehara [9], Isao Yoshimura [10], Etsuyoshi Miyaoka [10], Kenya Ishiyama [10], Masakazu Kato [11], Takashi Omori [12] , NIHS[1], Aiken Co., Ltd.[2], KOBAYASHI Pharm. Co., Ltd.[3], Inst. of Environmental Toxicology[4], Fancl Res. Inst.[5], FUJIFILM Corp.[6], Maruishi Pharm. Co., Ltd.[7], Drug Safety Testing Center Co., Ltd.[8], Daicel Chemical Industries, Ltd.[9], Tokyo Univ. of Science[10], J-TEC[11], Kyoto Univ.[12]

P－9 An On-line Community of Stakeholders Interested in Non-animal Methods of Toxicity Testing

[1]AltTox.org

P－10 JaCVAM statement on new alternatives to animal testing

Hajime Kojima[1], Tohru Inoue[1], Mitsuteru Masuda[1], Masaharu Akita[2], Yasuo Ohno[1]
[1]JaCVAM Steering Committee, National Institute of Health Science (NIHS), [2]Kamakura Women's University

P－11 Investigation of sensitive cytotoxicity assay detecting drug-induced mitochondrial toxicity

Eriko Toudou
Investigative toxicology research group, Discovery technology laboratory, Ono Pharmaceutical Co., Ltd.

P－12 Bile Canalicular Formation and Hepatobiliary Transport are Enhanced in Hepatocyte Sandwich Culture on an Oxygen-permeable Polydimethylsiloxane Membrane.

Hitoshi Matsui[1, 3], Fanny Evenou[1], Masaru Sekijima[3], Teruo Fujii[2], Shoji Takeuchi[2], Yasuyuki Sakai[2]
[1]BEANS Laboratory, [2]Inst. of Ind. Sci., Univ. of Tokyo, [3]Mitsubishi Chemical Medience Co., Ltd.

P－13 Evaluation of three dimensional cultured skin models using membrane permeation test

Kenji Sugibayashi[1], Hiroaki Todo[1]
[1]Faculty of Pharmaceutical Sciences, Josai University

P－14 Development of a human corneal epithelium model utilizing a collagen vitrigel membrane and its application to eye irritation test

Kazunori Nishikawa[1, 2], Tomoko Yamamoto[1], Pi-Chao Wang[2], Toshiaki Takezawa[1][1]Transgenic Animal Research Center, National Institute of Agrobiological Sciences, [2]College of Agrobiological Resource Sciences, University of Tsukuba

P－15 Reproducibility of human 3-dimensional cultured epidermal model (LabCyte EPI-MODEL)

Fumiyasu Hamajima, Masakazu Katoh, Takahiro Ogasawara, Kenichiro Hata
Japan Tissue Engineering Co., Ltd.

P－16 The Skin Irritation Test using the Human Epidermal Model LabCyte EPI-MODEL24 : examination with 54 materials

Takahiro Ogasawara, Masakazu Katoh, Fumiyasu Hamajima, Ken-ichiro Hata
Japan Tissue Engineering Co., Ltd.

P－17 Statistical Issues in the Design and Analysis of Validation Studies

Takashi Sozu[1], Takashi Omori[2], Isao Yoshimura[3]
[1]Osaka University, [2]Kyoto University, [3]Tokyo University of Science

P－18 The effect of chemical compound on cultured rat embryos in S-9mix

Noriko Ishizuka[1], Masaharu Akita[2], Atushi Yokoyama[3]
[1]Kiryu University, [2]Kamakura Women's University, Japan, [3]Kanagawa Life Science Research Laboratory

P－19 Evaluation of eye irritation potential of 114 chemicals and orrespondence to GHS classification using in vitro Short Time Exposure STE) test.

Kazuhiko Hayashi[1], Yutaka Takahashi[1], Mirei Koike[1], Hitoshi Sakaguchi[1], Takumi Hayashi[2], Hirofumi Kuwahara[2], Naohiro Nishiyama[1]
[1]Kao Corporation, [2]Kanebo cosmetics INC.

P－20 Skin Sensitization Study by Quantitative Structure-Activity Relationships

Kazuhiro Sato[1], Tomohiro Umemura[1], Yukinori Kusaka[1], Kohtaro Yuta[2]
[1]Department of Environmental Health, School of Medicine, University of Fukui. [2]Fujitsu Limited (Present; National Institute for Environmental Studies)

P－21 Direct prediction of toxicity scores using the data obtained from an alternative test: A prediction model

Takashi Omori[1]
[1]Kyoto University School of Public Health

P−22 Whole embryo culture with minivaial on rat embryo of day 9.5.

Yokoyama Atsushi[1,3], G.B.Vertrich[3], Hiroshi Yokoyama[3], Masaharu Akita[2]
[1]Kanagawa Life-Sciense Research, [2]Kamakura woman's college,
[3]Baltimore Life-sciense research

P−23 Evaluation of heterotypic cellular interactions using detachable substrates under controlled flow conditions

T. Kawashima[1], T. Yokoi[1], H. Kaji[1,2], T. Abe[1,2], M. Nishizawa[1,2]
[1]Tohoku University, [2]JST-CREST

P−24 The international validation study for the ER alpha STTA Antagonist Assay using HeLa9930

Atsushi Ono[1], Masahiro Takeyoshi[2], Susanne Bremer[3], Miriam Jacobs[4], Susan C. Laws[5], Takashi Sozu[6], Hajime Kojima[1]
[1]JaCVAM, NIHS, [2]CERI, [3]ECVAM, [4]EFSA,
[5]US-EPA, [6]MEI center, Osaka Univ.

P−25 Construction of a three-dimensional heterogeneous micro-tissue toward the valuation of a minimum required size

H. Suzuki, H. Kimura, K. Komori, T. Fujii, Y. Sakai
Institute of Industrial Science, University of Tokyo

P−26 A combined test skin irritation evaluation: Monolayer cell, human skin model tests and human patch test

Maki Nakamura[1], Yamaguchi Yoshihiro[1], Li Xiaolin[2], Li Jian[2], Xiong Wei[2], Qiu Lu[2]
[1]Kobayashi Pharmaceutical Central R&D Laboratory,
[2]Shanghai Entry-Exit Inspection and Quarantine Bureau of the P. R. China

P−27 Evaluating system using cells derived from ES/iPS cell
- Movie image analysis of beating cardiomyocyte-

Yasuhiro Takagi[1], Sumiko Kawai[1], Kaori Yabuuchi[1], Karin Shimada[1], Ayaka Iwao[1], Yuko Kotani[1],Ryoji Hashiba[1],Kyoko Shioya[2],Masaru Tajima[1] and Tsutomu Miki Kurosawa[1]
[1]The Inst. of Experimental Animal Sci., Osaka Univ. Med. Sch.,
[2] National Cardiovascular Center, Research Inst. Laboratory Animal Unit

P−28 Evaluating system using cells derived from ES/iPS cell - Microscopic observations for the differentiated multilayered cardiomyocyte -

Kaori Yabuuchi [1], Sumiko Kawai [1], Yasuhiro Takagi [1], Yuko Kotani [1], Ryoji Hashiba [1], Kyoko Shioya[2], Masaru Tajima [1], Tsutomu Miki Kurosawa [1]
[1] The Inst. of Experimental Animal Sci., Osaka Univ. Med. Sch.,
[2] National Cardiovascular Center, Research Inst. Laboratory Animal Unit

P−29 Evaluating system using cells derived from ES/iPS cell - Inspection of cardiomyocyte toxicity by index chemicals -

Sumiko Kawai[1], Yasuhiro Takagi[1], Kaori Yabuuchi[1], Yuko Kotani[1], Ryoji Hasiba[1], Kyoko Shioya[2], Masaru Tajima[1], Tsutomu Miki Kurosawa[1]
[1]The Inst. of Experimental Animal Sci., Osaka Univ. Med. Sch.,
[2] National Cardiovascular Center, Laboratory Animal Unit.

P−30 Evaluating system using cells derived from ES/iPS cell
-Application to a screening method of crude drug constituting a Chinese medical prescription-

Yasuhiro Takagi[1], Sumiko Kawai[1], Kaori Yabuuchi[1], Yuko Kotani[1],Ryoji Hashiba[1], Kyoko Shioya[2],Masaru Tajima[1] and Tsutomu Miki Kurosawa[1]
[1]The Inst. of Experimental Animal Sci., Osaka Univ. Med. Sch.,
[2] National Cardiovascular Center, Research Inst. Laboratory Animal Unit

P−31 Analysis of marker genes for prediction of embryotoxicity in neural differentiation using mouse embryonic stem cells

Noriyuki Suzuki, Satoshi Ando, Nobuyuki Horie, Koichi Saito
Environmental Health Science Laboratory, Sumitomo Chemical Co., Ltd.

P－32　Development of in vitro alternative method for developmental toxicity using mouse embryonic stem cells

Noriyuki Suzuki, Nobuyuki Horie, Satoshi Ando, Koichi Saito
Environmental Health Science Laboratory, Sumitomo Chemical Co., Ltd.

P－33　Investigation of an Eye Irritation Test Using a Human 3D Corneal Model

Satoshi Nakahara
Central Research Laboratories, Mandom Corporation

P－34　Training in anatomy using Japanese flying squid for college students majoring in nutrition and food –To increase the learning effects of clinical training in rat anatomy –

Michiko Sakamoto, Yukiko Nomura
Faculty of Food and Nutrition, Kyushu Nutrition Welfare University

P－35　Expansion of Short Time Exposure (STE) Test :Solvent Study

Mayumi Sakaguchi, Tomoko Kasahara, Shigemi Kinoshita,
Akio Shibamoto, Keiji Nishizumi, Naoko Ota

P－36　The influence of culture days of the reconstructed epidermis model (EPISKIN™) to the result of the skin irritation test with EPISKIN™

Koji Kurihara[1], Shoichi Yahagi[2], Yuri Okano[2], Hitoshi Masaki[2]
[1]Nikoderm Research Inc., [2]Cosmos Technical Center Co., Ltd.

P－37　A Modified Short-Time Exposure (mSTE) Test for Cosmetics: an Alternative to the Draize Eye Irritation Test

Yoshihiro Yamaguchi[1], Lu Qiu[2], Xiaolin Li[2], Junping Liu[2]
[1]Kobayashi Pharmaceutical Co., LTD. Central R&D Lab.,
[2] Shanghai Entry-Exit Inspection and Quarantine Bureau of the P.R.China

P－38　International validation study of the in vitro alkaline comet assay

M. Honma[1], K. Yamakage[2], B. Burlinson[3], P. Escobar[4], K. Pant[5],
A. Kraynak[6], M. Hayashi[7], M. Nakajima[7], M. Suzuki[7],
R. Corvi[8], Y. Uno[9], L. Schechtman[10], R. Tice[11], H. Kojima[1]
[1]National Institute of Health Sciences, Japan; [2]Food and Drug Safety Center,
[3]Huntingdon Life Science, UK, [4]Boehringer-Ingelheim, USA,
[5]Bio-Reliance, USA, [6]Merck, USA, [7]Biosafety Research Center,
[8]ECVAM, Italy, [9]Mitsubishi Tanabe Pharm,
[10]Innovative Toxicology Consulting, USA,
[11]NIEHS/ICCVAM/NICEATM, USA

P－39　Whole embryo culture with minivial on rat embryo of day 9.5

Yokoyama Atsushi[1, 3,] G.B.Vertrich[3], Hiroshi Yokoyama[3], Masaharu Akita[2]
[1]Kanagawa Life-Sciense Research, [2]Kamakura woman's college,
[3]Baltimore Life-sciense research

P－40　*In vitro* phototoxicity assessment of tattoo pigments using 3T3 fibroblast and reconstructed human skin model

Young Na Yum, Chae-Hyung Lim, Yong Kyoung Lee, Mi Jang,
Eun Jeong Kim, Soojung Son, Soon Young Han
Toxicological Evaluation and Research Department,
National Institute of Food and Drug Safety Evaluation,
Korea Food and Drug Administration

P－41　Integration of Micronucleus Assay into General Toxicity Testing for 3Rs

Young Na Yum, Hee Yun Kim, Joo Hwan Kim, Soojung Sohn,
Sue Nie Park, Seung Hee Kim, Soon Young Han
Toxicological Evaluation and Research Department,
National Institute of Food and Drug Safety Evaluation,
Korea Food and Drug Administration

P－42　Reduced numbers of fish used in acute toxicity testing

Marysia Tobor-Kaplon, D.F. de Roode, Ir. L.M. Bouwman
Institution NOTOX B.V.

P－43　Real-time evaluation of hemocompatible materials by substandard human blood for transplantation by blood bank

Satoshi Seki[2], Katsuko Furukawa[1],
Hanako Miki[1], Kazuyuki Mizuhara[2],Takashi Ushida[1]
[1] Department of Bioengineering, Department of Mechanical Engineering,
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CT-1
Introduction of KoCVAM for Alternative Test Methods in Korea

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Toxicological Test Methods are used to assess the safety or potential adverse effects of chemical substances. In recent years, there has been growing interest in the development of new or improved test methods, considering not only the concept of 3Rs but also new toxicity endpoints for the regulatory purpose.

In October, 2009, KoCVAM(Korean Center for Validation of Alternative Methods) was established at NiFDS(National Institute of Food and Drug Safety Evaluation), within Korea FDA, to promote the 3Rs in regulatory science for the safety assessment and to evaluate alternative test methods including validation, peer review and regulatory acceptance. In addition, KoCVAM will expand international activities with JaCVAM, ICCVAM/NICEATM, ECVAM and OECD. KoCVAM has formed the steering committee, the scientific advisory board, and ad hoc working groups responsible for the validation and peer review of each testing method. The Center continues to communicate with industry, academy, and public by holding symposiums and workshops and operating the KoCVAM web site. KoCVAM will make scientific contributions to the world-wide expansion of alternative test methods.

SL

Advancing Laboratory Animal Welfare and Public Health Science: The Role of Innovative Refinement, Reduction, and Replacement Strategies

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Objectives

Laboratory animals and *in vitro* methods are used to assess the safety, efficacy, and potential adverse health effects of new chemicals and products such as vaccines, medicines, food additives, pesticides, and industrial chemicals. Testing results are used for risk assessment decisions intended to safeguard the health of people, animals, and the environment. However, chemical toxicity and vaccine testing using animals can result in injury, disease, and mortality involving significant pain and distress, and can involve the use of large numbers of animals. Animal welfare concerns have led to national laws and policies in the United States and other countries to ensure the humane care and use of laboratory animals and to require the consideration of alternatives before animals can be used for testing or research.

Materials and Methods

Alternatives must be considered that can: reduce the number of animals required, refine animal use to lessen or avoid pain and distress, and replace the use of animals. The 3Rs concept of animal reduction, refinement, and replacement was first described by Russell and Burch in 1959. Since that time, numerous alternative safety testing methods and strategies have been developed and adopted that have significantly reduced and refined the use of laboratory animals. In some testing situations, safety and hazard decisions can now be made without the use of animals. Notable progress has been achieved, especially in the areas of pyrogenicity testing and ocular, dermal, allergic contact dermatitis, and acute systemic toxicity. Tiered testing strategies have been adopted that allow for hazard classification decisions using scientifically valid and accepted in vitro methods. The use of analgesics, anesthetics, and predictive earlier more humane endpoints can also be used to avoid or lessen pain and distress. Advances in science and new technologies are being used to identify potential predictive biomarkers for incorporation into *in vitro* test methods and strategies. Progress in the 3Rs has been aided by the development of national validation centers such as the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods and its Interagency Coordinating Committee on the Validation of Alternative Methods in the United States, the European Center for the Validation of Alternative Methods in Europe, and the Japanese Center for the Validation of Alternative Methods in Japan.

Results and Discussion

Enhanced international cooperation among these and other national centers will speed the international validation and acceptance of proposed new alternative methods. The continued development, validation, and adoption of innovative scientifically sound alternative methods and strategies is expected to advance the 3Rs and animal welfare while ensuring the protection of people, animals, and the environment.
Japan.

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CT-2

Revision of EU Council Directive 86/69/EEC on animal experimentation: an overview of its impact on animal welfare and science

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Animal experimentation in the Member States (MS) of the European Union (EU) has to be in line with the provisions specified in Council Directive 86/609/EEC. The main objective of the Directive, which came into force in 1986, was to guarantee an open market between the EU-MS. Already in the 90-s of the previous century discussions took place to revise the Directive. Ultimately, the EU Commission started the formal procedure in 2003. Revision was considered necessary to recognise the significant changes that had taken place since 1986: technical and scientific progress (e.g. availability of Tg animals), increasing societal concern about laboratory animal use, and, with the extension of the EU to 25 MS the need for a level playing field and a good functioning of the internal market. Revision was also seen as an opportunity to take into account the EU policy to consider animal welfare as an important ethical standard.

As a start of the revision process, four Technical Expert Working Groups were initiated to advise the Commission on aspects such as severity banding and the ethical review process. In addition, the Animal Welfare Expert group of the European Food Safety Agency (EFSA) was asked to provide an expert opinion on the inclusion of classes of non-vertebrate animals and embryonic and foetal forms in the Directive and on euthanasia.

The revision also included an impact assessment with a public and expert consultation. A total of 42,655 and 283 responses were received from the public and expert consultation, respectively. This ultimately resulted in a draft text that was send to the European Parliament in 2008.

There are several aspects in the revised document that will have an impact on research: basic research will be covered as well as the use of animals for tissue donation, 3Rs are given a prominent role, a ban is suggested on the use of great apes and on wild caught and F1 (animals born from wild caught) NHPs, the inclusion of certain classes of non-vertebrate species and foetal and larval forms, the ethical review process and the retrospective project assessment.

Following the publication of the draft, amendments have been suggested by the scientific community, animal welfare organisations and industrial platforms.

The revision is now in its final stage. After first reading, discussions have taken place between the European Parliament and the EU Commission. However, agreement is not expected before 2011.

This presentation will provide an overview of the revision process and will discuss the eventual consequences of the revised Directive on animal welfare and animal experimentation. It will be concluded that, although the revised Directive will clearly bring the 3Rs and animal welfare to the forefront, significant barriers to using laboratory animals in research and testing are not to be expected.

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CT-3

Three-Dimensional Reconstructed Human Tissue Models: Current Status of Regulatory Acceptance and Industrial Use.

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Toxicologists and product development researchers now have access to a great number of reconstructed human tissue models of both the eye (generally cornea) and skin. These models, produced in a number of countries around the world, have certain basic similarities, but also differ in a number of ways which means that the protocol for a specific endpoint often varies among the different commercial models. There are also differences in protocol design depending on whether the tissues are used for a regulatory application or a research application. In the former case generally only a single application of an undiluted material is required to determine a hazard category as defined by national or international standards. Research use, however, generally requires more detailed studies using multiple concentrations of material and/or various exposure times. These more detailed protocols allow differentiation between very mild products and can be used as a screening program for the "mildest" product. It is important for the success of any of these applications that the manufacturers' utilize strict quality control procedures to assure lot-to-lot consistency, and that they share this information with users.

Regulatory acceptance of these models has been progressing rapidly, especially for reconstructed skin. Four different commercial skin models can now be used under OECD Test Guidelines (TG) for identifying corrosive materials, and three models have been approved by ECVAM for skin irritation. An OECD skin irritation TG using skin models is moving forward rapidly and under the best circumstances might be adopted in 2010. Other regulatory applications now under development are the use of reconstructed skin for phototoxicity assessment and for genotoxicity determinations. Genotoxicity is important because the 7th Amendment to the Cosmetics Directive (EU) prohibits the use of animals for cosmetics evaluation, and up to now animal studies have often been used to clarify the meaning of a positive *in vitro* test. It is hoped that human skin models can provide an "in vivo-like" situation so that micronucleus and COMET endpoints can be used to verify or refute positive results in the traditional genetic toxicology battery of tests.

S1-1

Chemical management policy and development of short-term hazard assessment methods

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Objective(s)

Recent global chemical management policy shows four major changes: 1) life cycle-wide risk-based management is replacing hazard-based management; 2) industries are replacing governments as the major players in collecting hazard data; 3) the three Rs principle is carefully observed in animal testing for collecting hazard data; 4) risk analysis is deemed to be the foundation for prosperous nanotechnology industry development. Regarding hazard data collection, since data is required for tens of thousands of chemical substances, development of low-cost short-term hazard assessment methods is a matter of urgency. Furthermore, as an extension of the three Rs principle, transition from conventional toxicology is now anticipated, which has relied much on animal testing despite the issue of species difference [1]. The Japanese Government and industry should develop systems and methods for chemical risk analysis responding to such international movements.

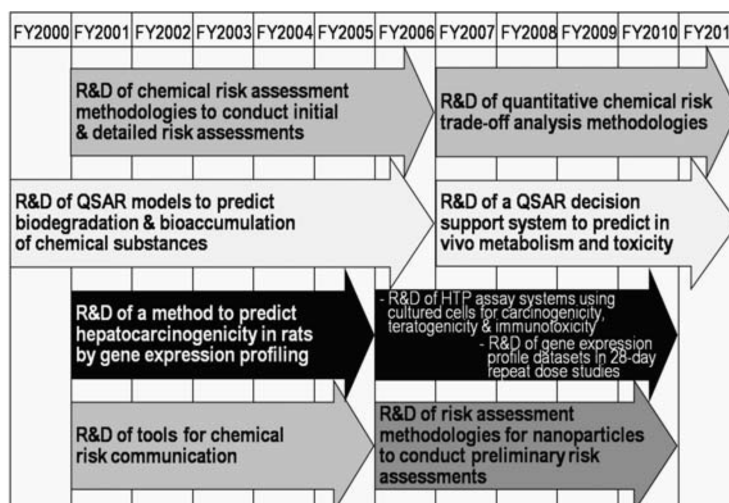

Fig. Strategic R&D of chemical risk analysis technologies

Materials and Methods

In conjunction with the above-mentioned movements, NEDO has been promoting R&D of chemical risk analysis technologies as shown in the figure [2].

As for short-term in vitro hazard assessment methods, focusing on carcinogenicity, immunotoxicity and reproductive toxicity, a cell transformation assay system and multicolor luciferase reporter gene assay systems, which are of high-sensitivity, have been developed, and a whole embryonic culture method has been improved to complement reproductive toxicity assessment in terms of metabolic activation.

Results and Discussion

Short-term hazard assessment methods being developed through NEDO projects are fruits of the Japanese forte, high-technology improvement and composition, and have advantages comparing to methods developed in Europe or America. It is a crucial task for project participants to finalize test protocols and complete international validation studies so as to offer the methods as international standards such as OECD Test Guidelines to the world's people involved in chemical management.

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S1-2

The Bhas 42 cell transformation assay: from basic researches to a guideline

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Objective

Bhas 42 cells were established from BALB/c 3T3 cells transfected v-Ha-ras gene by the selection of a clone which showed the contact inhibition of post-confluence but was transformable by 12-O-tetradecanoylphorbol-13-acetate. The two-stage transformation assay using Bhas 42 cells can detect both tumor initiating and promoting activities. The assay is superior in cost- and labor-performance, compared to the methods using parental BALB/c 3T3 cells. These characteristics suggest that the Bhas 42 cell transformation assay can be useful alternative method of carcinogenicity test. We present the data from the studies which we have carried out to propose the assay for a test guideline and to develop a high throughput transformation assay.

Materials and Methods

[Bhas 42 cell transformation assay]　The assay consists of an initiation assay and a promotion assay. The cells were treated with chemicals from day 1 to 4 in the initiation assay and from day 4 to 14 in the promotion assay after plating. On day 21, the cells were fixed and stained with Giemsa's solution and transformed foci were judged.
[Screening]　About 100 chemicals were evaluated using the 6-well method and the data were compared with those of in vivo carcinogenic activity (human or animals).
[96-well method]　The 96-well method (the same protocol of the 6-well method except using 96-well plates and different numbers of the cells plated) was developed for a high-throughput Bhas 42 cell transformation assay and the assay results were compared with those by 6-well method.
[Inter-laboratory collaborative study]　To confirm transfer and stability of technology, the 6-well method was performed using nine coded chemicals in six laboratories.
[Automatic system]　A robotic system, provides a series of the operation of transformation assay (plating cells, treating cells with chemicals, changing medium, fixing and staining cells), was developed.

Results and Discussion

[Screening]　The concordance with in vivo data showed more than 70%. Furthermore, the specificity and positive predictivity were high and exceeded 80%.
[96-well method]　The 96-well method yielded similar results as the 6-well method.
[Inter-laboratory collaborative study]　We confirmed reproducibility of the 6-well method. To evaluate by a third party, a validation is on going with the 96-well method using seven chemicals at four laboratories.
[Automatic system]　Transformation frequencies by this system were similar to those by the manual method.
A large amount of data concerning Bhas 42 cell transformation assay have been accumulated for proposing a guideline. And the 96-well method can be applied a high-throughput screening using the automatic system and/or a microplate reader.
This study was supported by the New Energy and Industrial Technology Development Organization (NEDO).

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S1-3

Immunotoxicity: Development of a reporter gene assay system using human cell lines

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Objective(s)

We are always exposed to enormous numbers of chemicals, which potentially impact our immune system. In such cases, dendritic cells (DC) sense these chemicals and trigger or modulate immune response. Epithelial cells in the route of entrance of chemicals affect DC and T cell function. Finally, T cell response themselves may be modulated by chemicals. The purpose of this study is to discover the common response patterns in DC, epithelial cells, or T cells, which are stimulated with different chemicals, by DNA microarray and to develop cell-based screening methods utilizing the obtained information.

Materials and Methods

We stimulated human monocyte-derived DC (MoDC), normal human epidermal keratinocytes (NHEK), and T cells with 5 different chemicals that affect our immune system, 2,4-dinitrochlorobenzene (DNCB), $NiCl_2$, formalin, diesel exhaust particles (DEP), $HgCl_2$, and analyzed their effects on mRNA expression by these three cells using DNA microarray and real-time PCR. We selected immune-related genes by analyzing the data from three independent experiments based on the bioinformatic data. Next, we established several reporter cells for evaluating the activity of chemicals to stimulate the promoter activity of the selected immune-related genes.

Results and Discussion

We found that DC augmented IL1A, IL1B, and IL8 by 4 chemicals, while NHEK augmented HMOX1, IL8, and sialophorin by 4 chemicals. These genes were not augmented by the stimulation with ATP, which is well known as a danger signal. T cells down-regulated IL5 and IL10 by 3 chemicals and IFNG and IL4 by 4 chemicals. By real-time PCR, we confirmed the response of IL1A, IL1B, IL4, IL6, IL8, TNF, and HMOX1 mRNA. These data suggest that several chemicals share some common mechanisms to affect our immune system irrespective of their chemical structure. In addition, these genes can be candidates as a biomarker to predict the immunomodulatory activity of chemicals. Based on these studies, we established the following reporter cell lines.

1) HaCaT-derived cell line, HR38H6, and U937-derived cell line, UR2H411, in which the expression of Luc2 and hRluc are regulated by HMOX1 and thymidine kinase promoters, respectively.

2) Jurkat-derived cell lines, 4A4, 2B12, and 10C6. 4A4 is a cell line transfected with two plasmids, one containing SLG the expression of which is regulated by IL4 promoter (IL4-SLG) and one containing SLR the expression of which is regulated by G3PDH (G3PDH-SLR). 2B12 is a cell line transfected with two plasmids, one containing SLO the expression of which is regulated by IFNG promoter (IFNG-SLO) and G3PDH-SLR. On the other hand, 10C6 are cell lines transfected with three plasmids, IL4-SLG, IFNG-SLO, and G3PDH-SLR.

3) U937-derived cell line, 6C12, in which the expressions of SLG and SLR are regulated by IL1B and G3PDH promoters, respectively.

These cell lines respond well to relevant stimuli. The response of these cell lines to chemicals is currently under investigation.

S1-4

Development of novel alternative tests for developmental toxicity: 1) Reporter gene assays using murine ES cells

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Objective(s)

A number of in vitro systems have been proposed as tests for developmental toxicity, however, it could be argued that these tests are unlikely to gain widespread acceptance and use. In the field of this toxicity one of the most promising in vitro models are based on embryonic stem (ES) cells. EST (Embryonic Stem cell Test） has received attention in recent years as an alternative test using murine ES cells. EST has already been validated in Europe, however, discussed the use, limitations and further needs for the tests to be fully suitable for regulatory acceptance.

To establish convenient and accurate in vitro short-term tests for developmental toxicity, here we report development of novel in vitro tests using mouse ES cells.

Materials and Methods

Our research plan is as follows:

1) Determination of suitable differentiation methods for mouse ES cells into cardiomyocytes and neurons
2) Analysis of gene expression of ES cells during differentiation into cardiomyocytes and neurons under the differentiation methods
3) Selection of candidates for marker gene by bioinformatics analyses
4) Identification of marker genes in ES cells by treatment with embryotoxic and non-embryotoxic compounds
5) Prioritization of the marker genes by gene function and expression profiles
6) Construction of luciferase reporter vectors containing the promoter site of each marker gene upstream of the firefly luciferase gene
7) Establishment of stable transgenic ES cells for detection of the marker gene expression
8) Development of basic protocols for reporter gene assays using the transgenic ES cells and investigation of efficacy using some standard compounds

Results and Discussion

In accordance with the research plan, we identified 13 and 22 marker genes during cardiomyocyte and neuron differentiation, respectively, by comparison of gene expression between embryotoxic and non-embryotoxic compound groups. We developed some stable transgenic ES cells to detect the chemical dependent changes in the marker genes easily and conveniently and have developed basic protocols for reporter gene assays. According to the basic protocol, preliminary studies are conducted using some standard compounds in order to clarify efficacy of our proposed tests.

This study was supported by a research grant from the New Energy and Industrial Technology Development Organization (NEDO) in Japan.

S1-5

Improvement of the embryo culture method for developmental toxicity tests

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Currently, ECVAM (European Centre for the Validation of Alternative Methods) in the EU appears to be at the forefront of the development of alternative methods for reproductive and developmental toxicity tests. Why is it difficult to develop alternative methods for developmental toxicity tests in comparison with other toxicity tests? In developmental toxicity tests, chemical substances first enter the blood stream and then reach the placenta via metabolism in the liver, and other organs. After further metabolism in the placenta, chemical substances finally reach to the fetuses where they affect fetal development. The difference in the *in vivo* route of chemical substances is an important reason for the difficulty in establishment of new methods for developmental toxicological tests in comparison with general toxicity tests.

According to the EU, the use of "*in silico*" techniques for developmental toxicity tests may be difficult, and I agree with this. The *in silico* technique is basically a method for prediction of toxicological effects from existing data, and can not predict new effects, because data obtained by developmental toxicological tests are too complex. Three techniques are now being examined to overcome with the difficulty in changing the method of developmental toxicological tests. They are the technique utilizing ES cells (EST), the micromass culture technique (MM) and the whole embryonic culture technique (WEC). It seems that EU is furthering development of EST and WEC in this time.

We are improving the WEC method for the purpose of using the WEC as an alternative method for developmental toxicity tests. Especially, we have tried to develop a novel WEC method with metabolic activation of chemicals using rat S-9mix. We also have developed new equipments to reduce serum volume in the WEC method.

In this symposium, we report progress of this research.

This work was supported by the grant P06040 from the New Energy and Industrial Technology Development Organization (NEDO).

S2-1

Standardization of human ES and iPS cells for drug discovery

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Human embryonic stem (ES) and induced pluripotent stem (iPS) cells are expected to be useful tool for the studies of drug efficacy or toxicity evaluation of new drugs. However, major limitations in the use of these cells are the current requirement for the use of feeder cells and our relatively poor understanding of their responses to growth factors or extracellular matrix to regulate undifferentiated and differentiated state. Furthermore, characteristics of hES and iPS cells are different among cell lines or clones. Generally, human ES and iPS cells are maintained on mouse embryonic fibroblast (MEF) cells with a medium supplemented with knock-out serum-replacement or alternatively on matrigel with an MEF-conditioned medium. These culture conditions hamper the cell biological analysis of human ES and iPS cells due to the presence of various unknown components. Although we are beginning to understand the environmental cues and the signaling pathways in the cells, our current knowledge is still fragmentary. There is a need to develop effective evaluation method and perform characterization of the cells prior to applying in the drug screening. With this as a basis, we previously developed a simple, defined, serum-free culture medium, hESF9 for culturing hES cells without feeders. We are making some progress towards developing elucidation method of the characteristics of hES and iPS cell using the conventional culture medium and also hESF9 medium.

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S2-2
Establishment of Disease-specific iPS Cells and Challenges in The Standardization

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iPS (induced pluripotent stem) cells are novel pluripotent cells which were established from differentiated somatic cells by a combination of Oct3/4, Sox2, Klf4, c-Myc genes (and/or Oct3/4, Sox2, Klf4 genes). Establishment of murine iPS cells was reported in 2006[1)], and human iPS cells were generated successfully in 2007[2)]. Since human iPS cells express some ES cell marker genes similar to human ES cells, and keep both pluripotency and self-renewal potency for a long period, iPS cells are thought very similar to ES cells.
Because even the differentiated somatic cells can be reprogrammed, iPS cell technology is expected to be applied for cell transplantation therapy, pathogenesis study and drug discovery. iPS cells have been also established from patient tissue, and the research is being advanced rapidly which aims at pathogenesis analysis for congenital disease and drug discovery or safety test by remodeling disease.
In Kyoto University, the establishment program of disease-specific iPS cells was started from June, 2008 under the approval of IRB, and many disease-specific iPS cells have been established until now.
However, to induce iPS cells we have to implement a lot of processes not only the preparation of somatic cells as the original raw materials, but also the processes such as the transformation of Oct3/4, Sox2, Klf4, c-Myc genes and the cloning of generated iPS cell colonies. And the total culture period takes 2 or 3 months. To steadily establish quality iPS cells from patient tissue for medical applications or drug discovery, monitoring the status of the cells which changes in every step and appropriately controlling the conditions is important. In addition, some special material and careful handling are also necessary for maintenance of established iPS cells. Their pluripotency would be weak or lost with wrong researcher treatment. Thus, the standardized maintenance system which evaluates the pluripotency or other characteristics of iPS cells to keep the total potential is also necessary to establish useful disease-specific iPS cells for drug discovery research.
In this presentation, I would like to explain the cell operation techniques that are necessary to establish and maintain iPS cells. Also, I would like to review the current subjects about the points of cell handling techniques in the standardization of iPS cell technology and the establishment of appropriate evaluation methods for iPS cells.

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S2-3

Development of a novel drug toxicity testing system using human iPS cells

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iPS cells, which have the capacity to turn into any cell type in the body and few ethical problems, may be an ideal source of cells for developing methods to evaluate drug toxicity. NIBIO (National Institute of Biomedical Innovation), in cooperation with various other national institutions, established the cross-institutional project 'Development of a novel drug toxicity testing system using human iPS cells'. This research consortium is centered at NIBIO, with principle investigator Hiroyuki Mizuguchi, in cooperation with the National Institute of Health Sciences, National Center for Child Health and Development, National Cancer Center, National Hospital Organization Osaka National Hospital and Kumamoto University. Different project work is assigned to these different institutions based on their relative research advantages.

In order to advance new pharmacology studies using iPS cells, in July 29, 2008, NIBIO created the 'iPS/stem cell basic drug project'. The project work is divided across members of laboratories in different fields, including the laboratory of gene transfer and regulation, the laboratory of cell culture, and the laboratory of genetic resources. Researchers are currently being appointed to this project. The research includes 1) the production of iPS cell lines from different gender, age, disease states and other variations, 2) the development of the method of quality control and quality evaluation for reproducible, stable culture of human iPS cell lines, 3) development of techniques capable of inducing highly efficient differentiation into the desired cell type, and a library of differentiated cells, and 4) development of new *in vitro* drug toxicity evaluation systems using toxicogenomics data base in NIBIO.

The development of this new *in vitro* toxicity evaluation system is expected to overcome the issues of cross-species differences encountered by other evaluation systems, predict and identify any drug side-effects unique to humans, improve the safety of therapeutic drugs and their development success rate, and contribute to shortening the development time of new drugs. In this symposium, I will introduce and summarize the current state of this special research project.

S2-4
Human ES cell-derived cellular models for drug discovery and development

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Reduction of drug attrition rates is desired in the drug discovery and development process. Major reasons of drug attrition during clinical trials are lack of sufficient efficacy and safety to human. Drug efficacy and toxicity are different between animals and human because of species difference. Therefore, human cell-based assay systems, which can mimic *in vivo* reaction in human, are demanded for efficient screening of drug efficacy and toxicity in pre-clinical stages.

Human ESCs are promising materials for construction of such *in vitro* systems. Genetic manipulation of hESCs and production of disease-model cells are useful strategy in drug discovery. Moreover, hESC-derived cardiomyocytes provide valuable screening tools for drug safety. Using hESC-derived differentiated cells with unlimited supply, potential drug side effects can be revealed before drugs enter clinical trials. Additionally, these *in vitro* models might reduce the amount of sacrificed animals.

In this presentation, I will discuss differentiation of hESCs into functional neurons, neurodegenerative disease models derived from hESCs after genetic modification and *in vitro* cardiotoxicity assay using hESC-derived cardiomyocytes.

S2-5

Application of ES cells to toxicology

- Current status and expected utilization: heart toxicity -

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Various *in vitro* tests employing human primary cells have been utilized in order to evaluate the possibility of adverse events in humans caused by candidate drug compounds. However, functional variation between each lot of primary cells and the unavailability of cells from some organs limit the development of toxicity tests. In addition, assays using recombinant cells carrying transfected human genes, such as the hERG assay, have been performed; however, it is considered that more functional cells which possess plural molecules related to the target function would be required for better assessment. Embryonic stem (ES) cells are expected to be a favorable source for the production of many kinds of cells *in vitro* and each of the differentiated cells should have the same genetic background and physiological functions in comparison with recombinant cells. Therefore, it is expected that ES cell-derived cells have potential to predict side effects in humans more effectively and exactly.

Recently, we analyzed the comprehensive gene expressions in cardiac marker positive cells derived from mouse ES cells to investigate their possible use for toxicity tests. In pathway analysis using Ingenuity Pathways Analysis™, we found that the differentiated cells had networks related to the heart function. In addition, gene expressions of various calcium and potassium channels in the differentiated cells were similar to those in the adult ventricle. We found that differentiated cells had the function of self-beating and the contractile function was affected by ion channel blockers. Moreover, many gene expressions of the structural components related to the contractile function such as troponin or actin in differentiated cells were also similar to those in the adult ventricle and transmission electron microscopy showed that myofibrils gradually matured along with the length of culture period. In this symposium, I would like to show the above results and discuss the expected utilization of ES cells and induced pluripotent stem (iPS) cells for the safety assessment of candidate drug compounds.

S2-6

A novel embryotoxic estimation method of drugs using ES cells differentiation system

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Recently, stem cells have become important a new tool for development of *in vitro* model systems to test drugs and chemicals and have been shown to be useful to predict or estimate embryotoxicity of teratogens. Among various stem cells, embryonic stem (ES) cells are some of the most valuable cells to develop *in vitro* model systems, because they are capable of self-renewing and differentiating into every cell types of the mammalian organism. The embryonic stem cell test (EST) is an *in vitro* toxicity assay that assesses the ability of drugs and chemical compounds to inhibit the differentiation of ES cells into cardiomyocytes, and has been validated as an *in vitro* developmental toxicity test [1]. The sensitivity and selectivity of this assay, however, must be characterized with a much larger number of toxins and non-toxic substances before one can safely assume that it is, indeed, a valid, accurate predictor of *in vivo* embryotoxic effects. Although EST is a simple and accurate test for toxicity, it is not sufficient for evaluating all chemicals because only two parameters of cytotoxicity and morphological change are assessed in the reported EST. We have developed an assay system based on the conventional EST, and tested this *in vitro* system by evaluating the embryotoxicity of antiepileptic drugs (valproic acid and carbamazepine) and antidepressant drugs (SSRIs) which have been reported as *in vivo* teratogens. In our studies, we added a molecular endpoint of differentiation to the conventional EST and attempted to characterize the tissue-specific embryotoxicity of these drugs by analyzing the gene expression of the tissue-specific markers as well as by conducting a histological and immunocytochemical study in the mouse ES cell differentiation system [2, 3, 4]. These analyses revealed that valproic acid, carbamazepine, and SSRIs have potent tissue-specific embryotoxicity. Since these corresponds with the known *in vivo* teratology of these drugs, we concluded that our assay system is useful for predicting the degree of embryotoxicity of teratogens, and that it can be used to estimate the *in vivo* embryotoxic effects of various medicines quickly and accurately. However, further improvements, such as addition of new endpoints and expansion of test system are necessary for optimized embryotoxicity test method.

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S2-7

Regenerative tissues derived from stem cells and its application to pharmacological research

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Induced pluripotent stem (iPS) cells are prepared by transducing several genes into somatic cells. Their features are similar to those of embryonic stem (ES) cells, and it is possible to differentiate iPS cells into various organ/tissue cells. These cells can be prepared using cells collected from patients, and they are able to proliferate without restriction while maintaining their pluripotent differentiation capacity. Therefore, they may replace ES cells as a cell resource that can be applied for regenerative therapy.

On the other hand, iPS cells may be useful for clarifying the pathogeneses of disorders and conducting pharmacological studies for the development of pharmaceutical preparations in addition to their clinical application including cell transplantation. For cell-based pharmaceutical screening, human primary cultured cells and cell lines have been employed. However, the use of somatic cells differentiated from human iPS cells may facilitate qualitatively stable cell-based assays, reflecting human tissues more accurately. If disease-specific iPS cells prepared using cells from patients are compared with normal iPS cells, the etiologies of refractory diseases may be clarified, contributing to the detection of new pharmaceutical targets. In addition, the utilization of somatic stem/precursor cells induced from iPS cells may facilitate the development of regenerative agents targeting patients' somatic stem cells. With the establishment of iPS cells, molecular mechanisms involved in cellular initialization and differentiation control have been clarified, facilitating control with low-molecular-weight compounds. Regenerative therapy with low-molecular-weight compounds without cell transplantation may be available in the future.

Using somatic stem/precursor/iPS cells, we have established regenerative tissues that reflect their essential functions, and can be applied for functional assessment. In this symposium, we will introduce our basic study on this function rearrangement system, and review its future application in pharmacological studies and issues.

S2-8
Evaluating system using cells derived from ES/iPS cell
-Application of Cardiomyocite to safety evaluation of a medical device and quality evaluation of crude drug-

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Objectives

The report of mouse iPS cell and human iPS cell development by Yamanaka offers a large possibility to widen variety fields such as pharmaceutical products, cosmetics as well as a medical field.
It is not an exception about application to safety evaluation of medical device and quality evaluation of Chinese crude drug.
In generally, the methods of evaluation gradually changed from the method utilizing animals to the method with culture cells. However, these methods utilized animals directly or cells and organs obtained from animals. These points suggest that the methods have been used are not good because these are derivated from the animal welfare.
In this regard, the iPS method developed by Yamanaka can let somatic cells differentiate to various types of cells / organs without the usage of animals, and so, these methods are quite unique epoch-making methods different from previous methods.
If these methods are used, it is thought that the new evaluation system which is suitable for various kinds of subjects can be established. The expectation for these methods is therefore extremely high.
From these viewpoint, we examined the movie image analysis of beating cardiomyocytes derived from ES cells to establish a new evaluation system.
This time, we would like to establish a new safety evaluation system for medical devices and quality evaluation of Chinese crude drugs using movie image analytical method developed. Obtained results were weighed against previous report results, and it will be clarified a novel evaluation system using the cells derived from ES/iPS cell. The method developed will be applied with a high expectation to various fields.
In addition, the main tools used for movie analysis were a stereoscopic microscope, a home video camera and software NIH-Image J for image analyses (free software), and all of them are easily purchased in the market.
Therefore it is thought that our novel method allows scientists to readily use as an alternative to animal experiment.

S2-9

Alternatives to animal experimentation using ES/iPS cells - Improvement of the EST -

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Spielmann *et al*. developed the embryonic stem cell test (EST), which is an *in vitro* embryotoxicity test method that can be used to estimate the risk of embryotoxicity of chemical substances relatively quickly compared to conventional methods that involve animal experiments. In a recent validation study in Europe, the EST was found to be reproducible, demonstrating an overall accuracy of 80% and 100% correct prediction of strong embryotoxicity for chemicals studied under blind conditions in four different laboratories.

However, in EST, there are some points involving the chemical characteristics of the sample that could be improved. That is, an EST protocol is not applied when a chemical substance is not dissolved in the culture medium. Furthermore, a mixture of two or more chemical substances is impossible. As one technique to resolve these issues, the three-dimension cultivating method was devised.

It is ethically difficult use a human embryonic stem cell for embryotoxicity screening. Moreover, the influence of human metabolic factors cannot be examined. Each chemical was cultured with TEST LIVER™ (TOYOBO), which can maintain human CYP3A4 and ammonia metabolizing ability for a prolonged period in vitro. As the culture medium for embryonic stem cells, fresh culture medium was further mixed with serum. By further decreasing the differentiation rate using thalidomide, findings closer to these of clinical data was obtained.

In addition, the cell recovery test was also developed similarly, allowing the examination of not only recovery of cell proliferation but also recovery from chemical disruption of cell differentiation. Stronger embryotoxicity is a concern when the recovery culture shows that the toxicity level of a chemical remains constant; therefore, it is necessary to consider the utility of examining recovery from embryotoxicity.

S3-1
Introduction

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It is said that the average life-span in Japan over the past 100 years has doubled, from about 40 to 80 years. The development of pharmaceuticals, including antibiotics has contributed to the increasing life span.
However, many patients still suffer from unmet medical needs. Therefore, companies aspire to develop novel medical treatment, including innovative medicine. Fifteen to twenty years and 15 to 30 billion yen for R&D are now required to launch one new pharmaceutical product.
Moreover, the probability it will be approved is considered to be 0.005%. It is therefore crucially important to determine the candidate with a high probability of success from the early stage of development.
This symposia will assert that the pursuit of the 3Rs (Replacement, Reduction, Refinement) principle, an ethical rule of animal experiments, is a key factor for the development of efficient novel medicine.

S3-2

Better safety evaluation using human and rat hepatocytes in spheroids as an *in vitro* model

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We conduct various toxicity studies and pharmacological evaluations using many kinds of animals for medical product development. A great number of animals are used in these studies. From the viewpoint of animal welfare, we have the ideal of the 3Rs, which stands for replacement, reduction and refinement. Development with alternatives to animal testing has been actively promoted, and the alternative method has especially been used as a screening system for evaluation of compounds at an early stage of drug discovery.

For *in vitro* studies, we have mainly used the monolayer culture method, but this method is reportedly not suitable for long-term evaluation because cell function is quickly lost. On the other hand, spheroid culture can supposedly maintain cell function for a comparatively long period due to its 3-dimensional structure. As a result, an *in vitro* examination by spheroid culture used for cells came to be utilized as an alternative method.

Our group members, who belong to a Safety Evaluation Forum, have used human and rat hepatocytes in spheroids as an *in vitro* model since last year, and have worked on the possibility of improvement in the quality of toxicity evaluation for compounds in the initial stage of development of medical supplies. We think we can evaluate toxicity with a chronic dosage of a compound by exposing a little compound every day.

Although joint experiments by our forum members have just begun, I introduce here our forthcoming forum approach in the days to come.

S3-3

Use of *in silico* tool in the research and development of pharmaceuticals

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In the pharmaceuticals research and development, the selection or optimization of the new candidates is one of the most important processes in the early stage of the discovery research. Currently, a lot of compounds have been synthesized and screened in a short period by introduction of new technologies of combinatorial chemistry and high throughput screening. It is necessary that many compounds have been evaluated toxicologically using a small amount of the test compound as the approach of the drug development changes. In the traditional synthesis and optimization of the lead compounds, the quantitative structure activity relationships method based on the efficacy or pharmacological effects is utilized. The concept becomes to be applied to toxicology and ADME. Computer systems for toxicity prediction using (quantitative) structure toxicity relationships have been developed in toxicology.
Toxicity prediction systems *in silico* are distinguished two categories by approach method: one is based on statistical or recognition method and the other is based on knowledge-base method. The former includes Toxicity Prediction by Multiple Computer Automated Structure Evaluation (MCASE), and the latter includes Deductive Estimation of Risk from Existing Knowledge (DEREK); they are used widely especially in United States and Europe. Use of the systems and device to improve predictive accuracy should be considered after the feature of the systems is understood fully.
In order to use the toxicity prediction systems effectively, it is necessary to construct the database of the chemical structural information and good quality data in the toxicity studies and to be provided with the systems for data search and data mining. In addition, it is important that the external database is merged into the internal database and the customized expert systems linked with the toxicity prediction tool are made up.
This symposium was organized to understand the present condition of the quantitative structure activity relationships and toxicity prediction and to consider the role of the toxicity prediction on the toxicological evaluation in the pharmaceuticals research and development.

S3-4
Prediction of Embryotoxicity by Embryonic Stem Cell Test

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Objective

It is preferable for the drug discovery and development process to generate compounds that do not induce embryotoxicity including teratogenicity if the compounds are intended for use in women of childbearing potential. It is important to characterize and minimize the risk of compounds for unintentional exposure of the embryo or fetus when women of childbearing potential are included in clinical trials. The application of a predictive screen for embryotoxicity in the early drug development of lead optimization will open a gate for the generation of low-risk drug candidates for the embryo or fetus. For this purpose, we tried to examine the value of the embryonic stem cell test (EST)[1)] , which uses mouse ES cells but not the entire animal, for the screening test of embryotoxicity in the early drug development.

Materials and Methods

The EST was performed according to the test protocol[2)] published by the European Centre of the Validation of Alternative Methods (ECVAM), and the embryotoxic potential of test compounds were classified into three classes according to their in vivo embryotoxicity (not embryotoxic, weak and strong embryotoxic). The value of the EST on the drug development process was evaluated from results of the EST performed with drugs and drug candidates with known embryo-fetal toxicity in animals.

Results and Discussion

The EST results of six out of seven in-house compounds (86%) were consistent with the teratogenic potential in animal experiments. Subsequently, we applied the EST to various compounds in the early stage of drug development. The majority of the compounds tested were predicted to be weak embryotoxic, suggesting the need for further classification of the weak embryotoxic compounds to utilize the EST during the early stage of drug development. When the EST results of 32 compounds including commercial drugs and ECVAM's validation compounds were evaluated with the score that was calculated by subtracting linear discriminant function I from II, the compounds showing higher score had the trend of exhibiting teratogenic properties.

Applying this score, in addition to three classifications by ECVAM, to the prediction of embryotoxic potential of compounds, the EST as a predictive embryotoxicity screen would contribute to the generation of drug candidates that exhibit no embryotoxicity including teratogenicity. In the future, if we can establish the test system using human iPS cells, the detection of the human-specific teratogens whose effects are not detected in animal experiments is anticipated. The progress of animal testing alternatives for embryotoxicity will make it possible to reduce embryo-fetal toxicity studies using animals.

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S3-5

Contribution of ICH on 3Rs in alternatives to animal experiments

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For the purpose of international harmonization of regulatory requirements for the approval of pharmaceuticals for human use, both regulatory and industrial parties in Europe, Japan and US organized ICH (International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use). First ICH meeting was held in 1990 in Brussels. After that many meetings was held in general assembly and in separate meetings. Last June, ICH meeting was held in Yokohama. Topics for discussion were divided into 4 groups, Quality (Step 4&5/topics: 28/34), Safety (13/16), Efficacy (18/21), and Multidisciplinary (6/7). Until this 18 June, 2009, 78 topics and issues were discussed and final agreements (Step 5) were accomplished on 55 issues and Step 4 agreements on 10 issues. Toxicity testing including animal toxicity studies was discussed in "Safety" group. From my point of view harmonization in ICH contributed much to the promotion of Three Rs. Harmonization of guidelines on toxicity studies itself exclude the conduct of meaningless repetition of similar toxicity studies. In addition, decrease in requirement of animal toxicity studies was also achieved in ICH. For examples, it became possible to replace single dose toxicity studies in non-rodents with the other relevant data (ex. preliminary dose setting studies for repeated dose toxicity studies) (1993). Twelve months repeated dose toxicity studies in rodents were replaced with 6 months studies (1993) and number of animal species required for carcinogenicity evaluation was decreased from two to one (1999). Guideline on "Non-clinical safety studies for the conduct of human clinical trials for pharmaceuticals" indicates the harmonized timing of each safety studies (1997). Period of repeated dose toxicity studies before first clinical trial in Japan and for the evaluation of effects on male and female reproductive organs was decreased from 4 to 2 weeks (2000 and 2009, respectively). This harmonization helped to conduct clinical trials earlier than that before. They promoted rational and rapid development of new drugs and decreased total number of animal use.

S4-1
Policy on animal experimentation of JALAS and alternatives to animal experiments

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The Japanese Association for Laboratory Animal Science (JALAS) was established for the purpose of advancing laboratory animal science and related areas through the promotion of basic and applied research and the dissemination of relevant information concerning laboratory animals, thereby contributing to the development of science in Japan. Animal experimentation is an essential part of research, education and testing in such fields as medicine and the life sciences. JALAS members should comply with the Law for the Humane Treatment and Management of Animals as well as the Standards Relating to the Care and Management of Laboratory Animals and Relief of Pain. Furthermore, they should conform to the internationally accepted "3R" (Replacement, Reduction, and Refinement) principle in accordance with the Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities compiled by the related ministries as well as the Guidelines for Proper Conduct of Animal Experiments stipulated by the Science Council of Japan. JALAS began raising the awareness of its members as to the importance of laboratory animal welfare by promulgating the Guidelines for Animal Experimentation in 1987 and publishing a handbook for the Guidelines. JALAS will remain committed to the promotion of the proper conduct of animal experimentation under voluntary management in Japan. (Policy on animal experimentation of JALAS, November 1st, 2007) Thus, JALAS promotes the development of alternative methods and research on animal welfare.

S4-2

Contribution of experimental animal technologist to Refinement and Reduction

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The Japanese Association for Experimental Animal Technologists (JAEAT, about 1,300 members) are composed of people engaged in breeding of experimental animals at universities, research institutions, pharmaceutical companies, contract study institutions, providers of the animals, and companies occupied in temporary personnel services. The association was inaugurated in 1966 and the purpose of this establishment is for experimental animal technologists to gain greater understanding, to train techniques for experimental animals, to develop abilities of experimental animal technologists, and to contribute to progress of life sciences. Experimental animal technologists are becoming leaders in science of experimental animals, which is synthetic science of medicine, pharmacology, agriculture, veterinary medicine, biology, and they are indispensable for biomedical researchers to support them.

In recent years, the grasp of enrichment on laboratory animal welfare and ethical rationalization of animal experiment has accelerated. The former subject is related in “animal breeding”, which involved in “Enrichment” and “Well being” of laboratory animals and this subject is directly connected with experimental animal technologists. While, the later subject is related with “experiments using the animals”, this involved in the so-called 3 Rs (Reduction, Refinement, and Replacement of the animals), and this subject is connected with moral of researchers who use the animals. Among them, because “Replacement” of the animal use, seems a subject that the researchers should consider, it is difficult for experimental animal technologists to contribute the improvement of this “R”. On the other hand, they may contribute to improve the subjects, “Refinement” and “Reduction” of the animals, because experimental animal technologists, who stand at the intermediate position between the experimental animals and the researchers, may contribute suitable supports for improvement of them to the researchers, taking initiatives in development of novel techniques. Thus, when JAEAT can contribute to those “Refinement” and “Reduction” of the animals, the contributions will lead to improvement of animal welfare and obtainments of the experimental results with their qualities. Thus, it is required for JAEAT to train the experimental animal technologists who can provide polished techniques and not only breeding of the animals but also the experimental techniques.

The efforts of experimental animal technologists are indispensable for the enrichment of laboratory animal welfare. Experimental animal technologists should be people who promote the research using experimental animals with humanity and promote this enrichment as leaders. In future, JAEAT will continue to combat those subjects, as well as considering what the association can improve on the subject “Replacement” of the animal use.

S4-3

Activities related to the 3Rs in the Japanese Society for Laboratory Animals and the Environment

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1. The current state of the Japanese Society for Laboratory Animals and the Environment

The Japanese Society for Laboratory Animals and the Environment (JSLAE) aims to promote the study of laboratory animal facilities and the environment, and to conduct the exchange of information according to the rules of the society. Since July of 1992 when it was established, the society has continued activity for the promotion of environmental effects, setting up a proper environment, improvement of the environment, and standardization of the environment for laboratory animals under an artificial environment. In these situations, for the JSLAE, the care of laboratory animals is its main concern, and we have dealt with many subjects surrounding laboratory animals. As its aim is in the opposite direction for replacement of laboratory animals, the purpose of bioscience is in the same direction from different situations. It is hoped that the present symposium will be a good chance for understanding each other, so I intend to introduce the activities of JSLAE.

2. The variety of activities in JSLAE

For a long time, the environmental control of laboratory animals was intended to domesticate animals for artificial resources. This point of view has been firmly accepted at the present time. JSLAE intends to deal with laboratory animals as "living creature" for the advancement of animal welfare in Europe and America. Movements that are against animal experimentation are thought to arise from criticism of dealing with materials of laboratory animals. The relation to the 3Rs (replacement, reduction and refinement) has been specifically connected to refinement since establishment of JSLAE. This refinement has resulted in the connection of the reduction of animal use. Most past symposiums held by JSLAE included a theme related to refinement.

3. Complex systems of living body and replacement of alternative methods

When conducting animal experiments for the first time, it is necessary to consider alternative methods that do not require the use of animals within limits that allow scientific objectives to be achieved, and the significance of the research and the reasons why animal experiments are required must be explained. However, in the present state, animal experiments takes precedence, according to information of alternative methods which is insufficient for replacement. Adequate investigation and development are required, and limitation of replacement in relation to expressing the complex system of a living body to the simple system is also necessary.

4. Future direction of the 3Rs

The 3Rs were examined in the "Law for Humane Treatment and Management of Animals" and "Standards Related to the Care and Management of Laboratory Animals and Relief of Pain," both of which were enacted on June 1, 2006. These laws were considered to be implemented at international levels. However, the reality involves emphasize of only the necessity of animal experiments under insufficient information about replacement among the research workers of animal experiments. This is the major reason why complex systems do not change to simple systems. The approach to the 3Rs in Japan is composed of different scientific members. For the promotion of good bioscience, cross-understanding and cooperation are required for the groups of "Laboratory Animal Science" and "The Japanese Society for Alternatives to Animal Experiments."

IS-1
Introduction of animal welfare education in colleges of veterinary medicine of Korea

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Recently, animal protection law of Korea has been amended. In addition, new law about laboratory animals was made and will be acted from next year. These laws need the understanding of animal welfare from the companion animal owners, animal researchers and farm animal owners. Veterinarians may be in the middle of a net of duties: to their patients, client, other vets, society at large and themselves. These duties can conflict with one another sometimes. Veterinarians are often faced with ethically problems in their jobs. Ethics may be the tools to make these decisions good. If veterinarians are uneducated about ethics, they may decide routine affair by inadequate ethical principles without ever asking questions about them. A good and continuous ethical education will give also immediate practical help in appropriately resolving situations in which interests conflict between clients, general citizen and animals. Many veterinary colleges started to give lectures about animal welfare and veterinary ethics. Seoul national university serves animal welfare and veterinary ethics to 3rd grade student of veterinary college for 15 hours a year. Also laboratory animal welfare has been opened to graduated students 45 hours a year. Beside universities, department of agriculture of Korea and KFDA served animal welfare lectures and appropriate laboratory animal management knowledge. I will introduce the animal welfare lectures opened in the college of veterinary medicine in Korea.

IS-2
The Current Status of 3Rs Research in China

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Summary

In recent years, as awareness for animal welfare, China has made some progress in replacement of animals research. So, to sum up, it includes:

1. Regulations construction: leveraging the momentum of *Guideline on Humane Treatment to Laboratory Ani*06, *mals* issued in 20 universities and research institutions generally carried out ethics review for laboratory animal. In respect of laboratory animal circumstance, more consideration has been made for the need of animal welfare in *National Standard of Laboratory Animals 2009.*
2. Standard formulation: framing an array of standards for conducting cosmetics toxicology examination by the experiments in vitro.
3. Studies on Three Rs: Some new skills, such as Metabonomics, genomics, iPS and so on, have been applied in toxicology experimental study.

In a word, regulation system of animal welfare has been constantly improved in China, and Three Rs studies become boosting.

IS-3

Alternatives in China: Today and Tomorrow

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The ideas of alternatives started to be recognized in China in 1990s'. "Regulation for Administration of Laboratory Animals（1988）" is the first regulation for laboratory animals. It has been revised, which supplemented one chapter of laboratory animal's welfare. The Ministry of Science and Technology of the P.R.C issued "Guideline on Humane Treatment to Laboratory Animals" in October of 2006. Several local and national relative regulations and rules were released after 2000, including 3R concept. Some national and industrial technical standards for animal alternative test, e.g. Chemical - in vitro 3T3 NRU Phototoxicity test method, have been established, though, these standards almost depended on OECD Guideline completely. In China, the system of safety assessment is comparative complicated. More that twenty GLP laboratories developed medicine toxicity tests, CDC laboratories opened toxicity test items for functional foods and cosmetics for domestic market, some other agencies which got qualification from control ministries can be qualified in safety tests for chemicals, pesticide, environmental sample. Almost all the laboratories still use traditional animal tests to assess toxicity. For Entry-Exit Inspection and Quarantine Bureau (CIQ), its position and responsibility are for inspection of export and import products. So, we are more sensitive to international regulations, e.g. EU Directive 76/768/EEC as we probably have to face to approve when we choose the method to do experiment for those of cosmetics exporting or importing to EU. We are doing our best to make technical preparation now. Several validated alternatives by ECVAM have been set up in our labs. Some new approaches were explored. Some are in progress. We also started a good way which collaborated with a Japanese company, would be strategy to raise recognition and capability each other. We look forward to having more chances to cooperate globally. In China, of course, the present status of alternatives is still in initial level, less paid attention and no relative organization has been built. But tomorrow will be optimism as 3R is global reach. The 3R step will never stop. China will be in the way, today and tomorrow.

IS-4

Development status of the laboratory animal industry in Beijing area

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Summary:
According to industry development requirements, combined with the management characteristics of laboratory animals in Beijing and industry conditions, the research of the Beijing area 90% of the units engaged in the laboratory animals, statistical analysis of the development of these units in the past three years, including the personnel situation in the laboratory animal agency, expenditures on the operating costs of the laboratory animal facilities, the condition of the commonly used laboratory animal species production, usage condition of the commonly used laboratory animal species, laboratory animal management condition in Beijing area, the training of the people engaged in laboratory animal and the welfare of the laboratory animal. The development situation of laboratory animal units has been completely controlled in the Beijing area .If problem is found, we will be put forward countermeasures and suggestions.

IS-5

Bioartificial skin and biocornea as screening model

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Objective(s)

In order to evaluate the feasibility of 3D-bioartificial skin and biocornea as alternative screening models, we first established QC standards for the production 3D-bioartificial skin and biocornea and then we compared their sensitivity and specificity with other products according to the guideline of EcVAM and multicenter validation was performed.

Materials and Methods

For skin irritation test, 3-D bioartificial skin was reconstructed by primary keratinocytes isolated from newborn foreskins and stored in their master cell bank. The keratinocytes were directly plated on 12 mm millicell, which was inserted into the 6 well plates, submerge-cultured for three days, and exposed to air-liquid interface for different time points for optimization. Histological feature and reconstruction of epidermis architecture of 3D-bioartificial skin was evaluated in fixed tissue and degrees of keratinocyte differentiation and proliferation were examined by E-cadherin, filaggrin, p63, and PCNA immunohistochemical staining to set up quality control standards. Then to test their feasibility as alternative skin screening model, twenty chemicals which have been categorized as irritants or non irritants by EcVAM guideline were applied and the scores were analyzed based on their sensitivity and specificity. For 3-D biocornea construct, corneal epithelial cells derived from the remnant of corneal transplants were used and similar method to 3-D skin construct was applied except providing higher hydration rate than bioskin. For the reproducibility and QC of 3-D biocornea, ED50 to 0.3 % TritonX100, transepithelial electrical resistance (TEER) measurement, and histological data were compared with other products. And then we developed and optimized the irritation test method with PEG400, Glycerol. SLS, Triton X100, Sodium hydroxide and intralaboratory and interlaboratory validation were performed.

Results and Discussion

In three center validation of the skin irritation test, 78% of sensitivity and 67% specificity were obtained. In eye irritation test, we established QC standards; TEER (Ohm.cm2) > 150, 8<ET50<15 min, Thickness >20 micrometer, OD >0.7. We established eye irritation test protocol, which is now then under the multicenter validation. The data will be provided as a guideline for the standard test method to FKDA.
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M-1
Comparative studies on the ocular irritation potential of various alkyl polyglucoside surfactants

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The use of surfactant-based products is unavoidable in our everyday life. Due to the amphipathic property, surfactants can easily disrupt the skin barrier causing irritation to the skin and the eyes. For this reason, the selection of less irritant, less toxic surfactants are of general interest to the cosmetic industry. In the present work, we assessed the relationship between alkyl chain length and ocular irritation potentials using HET-CAM and BCOP assays using five commercial alkyl polyglucoside surfactants with different composition of alkyl chain lengths. There was a positive correlation between the eye irritation potential and percentage of C10-alkyl polyglucoside in alkyl polyglucoside surfactants on both HET-CAM and BCOP assays.

Key words: Eye or ocular irritation potential, Alkyl polyglucoside, HET-CAM, BCOP, Alkyl chain length

M-2
Permeation of several compounds through different three-dimensional cultured human skin models

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In vitro permeation experiments using excised human or animal skin are widely applied to evaluate the skin permeation profiles of many substances. The 3Rs' issue to the animal experiments as well as ethical problem to human tests, however, limits these experiments. The alternative permeation experiments using three-dimensional cultured human skin models have been paid attention under this background. Then, six kinds of three-dimensional cultured human skin models were selected and evaluated for their morphological properties and permeation profiles of model compounds, and the obtained results were compared with those through human and hairless rat skin, to examine the utility of the three-dimensional cultured human skin model as an alternative skin. The present experimental results suggest that only LSE-high and EpiDerm can be used as alternative membranes for the skin permeation test of drugs and cosmetic ingredients.

M-4
Establishment of the alternative method of animal experiment, using green fluorescent ES and iPS cells

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Objective(s)
Many animals have been conventionally used for safety evaluation. Recently, a safety test method using cells was developed. In Europe, the Embryonic Stem Call Test (EST) method which was examined the embryonic stem cell toxicity with ES cell was developed in 1993. And today, it became one of the indexes of an animal experiment alternative method. This is the method to examine of embryotoxicity. However, it does not examine the effect on cardiomyocytes. We developed an appraisal method using various differentiated cells derived from ES/iPS cells.The method could be an alternative to animal experimentation. In this study, we have received the Mandam award and with the award the establishment of applicable methods to basic and applied science using myocardiocytes derived from mouse ES cells were intended. This method can be applied in iPS cells and if successful, the basement of evaluating system using human and mouse ES/iPS cells can be established.

Materials and Methods
Mouse ES cells (EB3-Caa-eGFP-IP) were cultured in cell culture dishes without feeder cells in GMEM supplemented with FBS, NEAA, Sodiumu pyruvate, 2-Mercaptoethanol and LIF. Cells were differentiated as embryoid bodies (EBs) by the hanging drop method. EBs were transferred at day 2 from the hanging drop into a petri dishes. On day 5, one EB per well was plated into 24-well culture plates. Differentiation into cardiac cells was investigated by using a microscope. Various testing chemicals were diluted in medium They were applied on the palmic cardiomyocyte for a short period. Each palmic cardiomyocyte was recorded using a video camera Their picture images were analyzed by NIH Image J in terms of various pulsatile functions. The pathological analysis of these multilayered cell masses using a conventional histological method. Immunohitochemical analysis for various signals and gene expression with RT-PCR were conducted and the results were compared with HE stained cells..

Results and Discussion
EBs showed 100% beating rate in day 10, and continued beating. This result suggests that the continuous observation of can be applied at any time for various chemicals and medical devices. The beating was not influenced by non-cardiotoxic chemicals.. On the other, the beating was clearly inhibited by highly concentrated applications cardiotoxic chemicals. These results did not contradict to a report by a method with different exposure time. In histopathological examination, specimens were to be easily and properly examined as they have been examined using laboratory animals. The expressed signals in immunohistochemical analysis and genes expressed in RT-PCR were accord with the previous reports. The introduction of image analysis in this method allowed us to evaluate testing materials in a scoring system.
Although Tthe evaluation method developed in this study was focused on a pulsatile function of the palmic cardiomyocyte. we think that the method developed can be applied with any differenciated cells derived from ES/iPS cells in pharmacometrics of a variety of chemicals.
The results of this study indicate that the basement of evaluation system using human ES/iPS could be established.

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LA-1
Alternatives and Anesthesia

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The alternative research is internationally thought to be a research and practical activities for 3Rs. However, in Japan, the wording of “Daitaihou “may be superficially translated to be Replacement or Alternatives and then they are not interested in Refinement. There are many Japanese scientists in Replacement and Reduction in JSAAE but there are only few scientists who are involved in Refinement research. On the other hand, the research in Replacement and Reduction is at the its start line to solve the problems. There are so many hazards for human and animal health all over the world such as emerging infectious diseases, malignant cancer diseases, neurological disorders, cardiovascular diseases, liver failure and kidney disease. Further more, the health threat for elderly people and animals such as metabolic syndrome has not been delineated well yet. Others include endocrine disrupters of environmental hormones, environmental pollution, hazards of newly developed chemicals and also newly developed drugs and medical devices, food safety that may cause human and animal health disorders. The demand to confirm their biological safety is increasing and the immediate answers are demanded by the public. Therefore , we scientists should find practical ways to solve these problems and also we should think of the alleviation of the pain and distress of laboratory animals. Consequently, we are going to comply with Refinement. Although there are many aspects of refinement, generally speaking, refinement means to reduce pain and distress which may be felt by laboratory animals. It is concluded that we should seek the way to alleviate pain and distress in our research activities.

The best and only way to perform this task is anesthesia. Anesthesia in laboratory animals may be extended to anesthesia itself and analgesia and euthanasia. Unfortunately there are very few scientists carrying out their research in laboratory animal anesthesia , analgesia nor euthanasia in Japan due to the paucity of research funds for this area. Consequently the most of scientists in Japan are using anesthetic methods with old and disused anesthetics in clinical settings fro their animal experimentation though some of old anesthetics are proved not to have any analgesic effects.

The seminar discussion will be included the meaning of laboratory animal anesthesia and practical methods of anesthesia.

O1-1

Availability of silkworm infection model for identification of environmental pathogens and virulence factors

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Objective

Many pathogenic bacteria are present in the environment, including unknown pathogens with the potential to cause infectious diseases. Efficient methods of detecting these environmental pathogens are therefore important. In general, the biologic aspects of pathogens are studied using *in vitro* methods, but to better understand the pathogenicity of bacteria in the living body, an animal infection model is needed. The use of mammals for infection experiments, however, is costly and raises ethical problems. Thus, the development of infection models using invertebrate animals is highly desirable. We previously reported that silkworms are sensitive to human pathogens such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* and resistant to nonpathogenic *Escherichia coli* and *Saccharomyces cerevisiae*. Here, we examined the utility of the silkworm infection model for detecting environmental pathogens and identifying virulence factors that are secreted from pathogens.

Materials and Methods

Bacteria were isolated from the soil and the content of fish intestines. Pathogenicity of these bacteria against silkworm was evaluated by injecting an overnight culture into the hemolymph. The bacterial species were determined by analyzing the 16S rRNA sequences. The pathogenicity of bacteria that killed silkworms was further evaluated by injection into the mouse peritoneal cavity. The exotoxin activity of a soil bacterium, *Bacillus sp.*, was purified with column chromatography by monitoring its toxicity in silkworms.

Results and Discussion

We isolated pathogens against silkworm from the soil and the content of fish intestines. We found that potent pathogens against silkworms also killed mice. *Staphylococcus pasteuri* and *Staphylococcus simiae*, which have not been recognized as pathogens against mammals, killed both silkworms and mice. Exotoxin of *Bacillus sp.* was purified with column chromatography using DEAE-cellulose and Mono Q and identified as sphingomyelinase C. These findings suggest that the silkworm infection model is useful for detecting pathogens against mammals. Bacterial toxins can also be identified by monitoring their toxicity in silkworms.

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O1-2

Investigation of the Test Method for Eye Irritation potential Using a Reconstructed Human Corneal Model.

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Objective(s)

In vitro eye irritation testing alternative to animal testing such as rabbit Draize test is required from an animal welfare standpoint. In order to develop an alternative method in vitro to Draize test, we studied about the tissue culture method of three-dimensional (3D) corneal models with normal human corneal epithelial cells and then an alternative method to eye irritation test using this model.

Materials and Methods

Human nomal corneal epithelial cells were proliferated with the 3T3-J2 feeder cells. These cells were seeded in culture inserts and cultured for 13days. Similarly to human cornea epithelial tissue, the stratification of the cells and squamous epithelial layer at the surface part was observed during the 3D-culturing process of cornea epithelial cells.

Results and Discussion

Both Claudin-1, a component of tight junction recognized in the superficial layer, and E-cadherin, a component of desmosome recognized in the pterygoid layer, were strongly expressed in the stratified squamous epithelial layer. Additionally, it was confirmed that Mucin-1 was expressed in the stratified squamous epithelium layer. These results suggested that this model was correlated with the tissue structure of normal human corneal epithelium. Since this result was correlated with the degree of in vivo eye irritation or the Draize score, it suggested that the eye irritation test using this model could be useful for variety of chemicals with irritant potency as an alternative method to Draize test.

O1-3
Genotyping Using Amp-FTA Method With Buccal Swab sample

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Objective

A novel genotyping method, the Amp-FTA method, has been developed by combining the advantages of the Ampdirect® Plus and FTA® technologies. This method allowed the direct PCR amplification of DNA from unpurified blood immobilized on the FTA® card. PCR templates were prepared only by punching out the discs from the FTA® card, which could lead to a foolproof way of genotyping. We tried sampling from the buccal swab which is a simple technique and minimizing distress to animals.

Materials and Methods

Buccal swabs were collected by scraping the inside of the cheek with tin cotton sticks with only 2 mm diameter buds and then smeared onto the FTA® card. Blood of the identical mouse used as a control sample. After drying, the discs were punched out and used directly as PCR templates. Un-treated sample discs were placed directly in a 15 µL PCR mixture containing 1×Ampdirect® Plus, 0.2µM of each primer, and 0.4 units of Nova™ Taq Hot start DNA polymerase. PCR was carried out in a thermal cycler using the following program: 30 seconds of 94 degree, 30 seconds of 60 degree, 60 seconds of 72 degree, 35 cycles. After PCR, 10µL of the reaction mixture was applied to a 4% agarose gel in 0.5×TBE buffer and electrophoresed. The bands were visualized with ethidium bromide staining.

Results and Discussion

PCR products from the buccal swab show the same pattern as those from blood. PCR bands from buccal swabs were weaker than those from blood with 35 PCR cycles, so an additional 5 cycles (total 40 cycles) is recommended to obtain clear bands from the buccal swabs. For animal welfare and because of technical advantages, the buccal swab may replace blood collected from clipped tail. These findings indicated that the Amp-FTA method is simple, safe for laboratory workers, eco-friendly, good for long-term storage, and non-invasive. The Amp-FTA method is ideal for the genotyping and genetic monitoring of laboratory animals.

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O1-4

Embryonic stem cell-derived hepatic tissue micro-culture system

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Objective(s)

The liver is an organ which plays a major role in drug metabolism and has many functions including glycogen storage, detoxification, and so on. The Liver is essential and necessary for living. A major cell population of the liver is hepatocytes which have the multiple liver functions as described above. But, single culture of hepatocytes is difficult to keep multiple liver functions for a long period. We consider hepatic reconstruction including parenchymal cells, as hepatocytes, and non-parenchymal cells, as endothelial cells, should be necessary for maintaining multiple liver functions *in vitro*. So, we are trying to develop a novel microfluidic chip system for culturing reconstructed hepatic tissue and evaluating multiple liver functions.

Materials and Methods

Human hepatocellular carcinoma cell line, HepG2, and embryonic stem (ES) cell-derived hepatocytes, human umbilical vein endothelial cells (HUVEC), GFP-positive vascular endothelial cell line, GH7 are used for hepatic tissue reconstruction. Cells were seeded on extracellular matrices, such as collagen, laminin and so on. To induce hepatocytes from murine ES cells, hanging drop method was used for embryoid body formation. Embryoid bodies were harvested and cultured in differentiation medium.

Liver specific gene expression, such as Hepatocyte Growth Factor, alpha-fetoprotein, albumin, was detected by reverse transcription-polymerase chain reaction (RT-PCR) analysis.

To analyze multiple liver functions, reconstructed hepatic tissue was cultured in medium contains ammonium chloride and testosterone. The medium was aspirated and used for urea synthesis assay. Also, CYP isozyme reaction was terminated by using high-performance liquid chromatography(HPLC).

The reconstructed hepatic tissues were cultured in PDMS-based microfluid chips. The medium was injected into the chip using a syringe pump located outside of the incubator at a controlled rate of 40 μL/hour. Using collected medium, multiple liver function was analyzed as mentioned above.

Results and Discussion

Each reconstructed hepatic tissues were cultured in PDMS-based microfluid chips. In the result, higher liver functions were detected than that of single culture of hepatocytes. It suggests that our culture system of reconstructed hepatic tissue could maintain hepatic functions *in vitro*.

In the next step, we want to succeed in human embryonic stem cell-derived hepatic tissue reconstruction as an application for toxicity evaluation.

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O2-1

The characteristics of a Bhas 42 cell transformation assay and its predictability for the carcinogenicity of chemicals

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[Objective]

The carcinogenicity of chemicals has been predicted with genotoxicity assays. A cell transformation assay (CTA) can also detect chemical carcinogens but is classified into a category different from a genotoxicity assay[1]. BALB/c 3T3 is one of cell lines which have been used for CTAs. The Bhas 42 cells were established from the BALB/c 3T3 cells through transfection of an oncogenic murine *ras* gene (v-Ha-*ras*) and regarded as initiated cells in the two-stage carcinogenesis theory[2]. Using the Bhas 42 cells, a short-term CTA was developed[3]. Bhas 42 CTA is superior to conventional CTAs in cost and labor performance. Bhas 42 CTA has previously been reported to be capable of detecting initiating and promoting activities of carcinogens and consists of an initiation assay and a promotion assay to detect initiating activity and promoting activity[4-6]. In the promotion assay, the initiating treatment with a known carcinogen is not required. We applied this short-term assay to 92 chemicals to characterize the assay and evaluate its performance for the prediction of chemical carcinogenicity.

[Materials and Methods]

In the initiation assay, the Bhas 42 cells were seeded at a density of 2 x 10^3 cells/mL onto 6-well microplates (Day 0), treated with test chemicals from Day 1, when the cells were sparse, to Day 4, and thereafter cultured in normal medium until Day 21. In the promotion assay, the cells were seeded at a density of 7 x 10^3 cells/mL, treated with chemicals from Day 4, when the cells were sub-confluent, to Day 14, and maintained in normal medium until Day 21. The cultures were fixed and stained with Giemsa's solution. The transformed foci were judged on the basis of morphological characteristics. A statistical analysis for the number of transformed foci per well was performed by the multiple comparison using the Dunnett method.

[Results and Discussion]

Bhas 42 CTA could detect Ames-negative and Ames-discordant chemicals, and the promotion assay detected most of them, confirming that the Bhas 42 cells act as initiated cells in the CTA. The concordance, sensitivity, specificity, positive predictivity, negative predictivity, false positive and false negative were calculated from the assay results for known carcinogens and non-carcinogens. Among these performance characteristics, the specificity and positive predictivity were high and exceeded 80%. For the overall evaluation, the other performance characteristics were equivalent to those of genotoxicity tests and conventional CTAs. From these results, we concluded that the accuracy of prediction for chemical carcinogenicity would be increased by including Bhas 42 CTA in the battery of in vitro assays.

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O2-2

Investigation of a Minimally-Required Size for a Two-Dimensional Liver Micro-Tissue toward *In Vitro* Toxicity Tests

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Objective(s): To miniaturize cell-based biochips and devices, micro-tissues, which exhibit physiologically-relevant responses identical to those of their corresponding original tissues/organs, should be embedded. However, the minimally-required size of such micro-tissues has not consistently been determined to the best of our knowledge. We therefore determined the minimally-required size for two-dimensional micro-tissues of human hepatocarcinoma cell line Hep G2, which preserves various hepatic functions as well as growth capacity, and evaluated their responses to several model chemicals. In addition, a rapid formation of cell spots was performed using an avidin-biotin binding system (ABBS).

Materials and Methods: To control size of the micro-tissues, we employed photocatalytic lithography, which is a novel and convenient patterning technique. After collagen was adsorbed to micro-patterned adhesive regions (63, 200, 630, and 2000 μm in diameters) treated with photocatalytic lithography, followed by adsorption of avidin, cells modified with biotin were seeded on the avidin/collagen-coated glass surface (1.0×10^5 cells cm^{-2}). The cell morphology was evaluated with microscope images. Cytotoxicity was evaluated by a cell viability assay after 48 h exposure to adriamycin (ADM), aflatoxicin B_1 (AFB_1), and TritonX-100 as typical examples of direct and indirect mutagens and surfactant, respectively. In addition, the observed cytotoxicity was compared with that obtained in the conventional 96-well plate-based assay (ca. 6 mm in diameter).

Results and Discussion: The biotin-modified cells were seeded on the avidin/collagen coated glass surface, resulting in immobilization of cells via ABBS within 10 min. The cells stably adhered and proliferated in the micro-patterned cell-adhesive regions for at least 6 days of cultivation. Thus, this technique is useful for the rapid formation of cell spots and/or array.

Using the 3-day cultured cell array, cytotoxicity tests were performed. ADM is known to intercalate into an intracellular double strand DNA and cause DNA-strand breakage, resulting in cell apoptosis. The cytotoxicity of ADM was independent of the size of micro-tissues examined and was equal to that observed in the plate-based assay. This is presumably due to the fact that the growth capacity per cell was the same for all sizes of micro-tissues. In contrast, AFB_1 is known to be metabolized by intracellular enzyme cytochrome P450 (CYP) 1A1/2, resulting in formation of an ultimate strong toxicant. It binds with guanine in DNA, followed by cell apoptosis. The cytotoxicity of AFB_1 significantly increased in the micro-tissue of 630 μm in diameter (ca. 1000 cells) or larger and reached the level observed in the plate-based assay. This agreed well with the enhancement of their CYP1A1/2 capacities per cell. TritonX-100 is known to cause cellular membrane disruption. Interestingly, the cytotoxicity of TritonX-100 gradually increased with increase in the size of the micro-tissues. Although the reason is unclear, the stability of the cellular membrane might increase due to the cell organization. In any case, the cytotoxicity of TritonX-100 depended on the size of micro-tissues.

In conclusion, the present evaluation is necessary for miniaturizing cell-based biochips and devices giving physiologically-relevant responses.

Acknowledgement: This work was supported in part by 2nd Mondom International Research Grants on Alternative to Animal Experiments.

O2-3

Inter-laboratory validation study of in vitro eye irritation test; Short time Exposure (STE) test.

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Objective(s)

Short Time Exposure (STE) test is an easy in vitro eye irritation test using cell viability as an end point, in SIRC (rabbit corneal cell line) cells following a one 5 minute dose treatment. The Japanese Society for Alternative to Animal Experiments organized an Executive Committee and conducted a validation study with 5 laboratories to assess transferability, inter-laboratory reproducibility, and predictive capacity of the STE test.

Materials and Methods

This study was conducted based on the test protocol of the STE test. At first, three standard chemicals (sodium lauryl sulfate (SLS), calcium thioglycolate (CT), and Tween 80 (TW80)) were evaluated to confirm transferability. Three experiments for each chemical were evaluated using 5%, 0.5%, and 0.05% test chemical in vehicle (saline). Both the mean cell viability and STE rank classification (1, 2 or 3) based on the STE prediction model using cell viability of 5% and 0.05% solution were compared with their background data from the lead laboratory of the STE test. Next, using 25 blinded chemicals, three experiments for each chemical were evaluated using 5% and 0.05% test chemical in saline or mineral oil as a vehicle. STE irritation category (NI or I) based on cell viability of 5% solution was compared with GHS category (NI or I: Cat. 2 and 1). Moreover, STE rank classification was compared with GHS rank (NI, Cat. 2 or Cat. 1).

Results and Discussion

For three standard chemicals, the mean cell viability of each chemical was similar to the lead laboratory's background data and the rank classifications for these chemicals were SLS: 3, CT: 2, TW80: 1, respectively, in all 5 laboratories and the lead laboratory. From these data, a good transferability of the STE test was obtained. STE irritation categories (NI or I) for 25 blinded chemicals showed good correlation with GHS category (NI or I) and nearly the same results were obtained by each laboratory. Moreover, STE rank classification (1, 2 or 3) also showed good correlation (above 80%) with GHS rank (NI, Cat. 2 or Cat. 1) in all laboratories. Based on these findings, the STE test was useful as an alternative eye irritation test.

O2-4

Classification for skin sensitization potency using human Cell Line Activation Test (h-CLAT)

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Objective(s)

We have been developing an in vitro skin sensitization test, the h-CLAT, using THP-1 cells (human monocytic leukemia cell line). This test is based on the augmentation of CD86 and CD54 expression in THP-1 cells following 24 hours exposure to skin sensitizers. We previously showed that the h-CLAT protocol is easy to transfer and that good inter-laboratory reproducibility was observed in an inter-laboratory study in Japanese, European, and US laboratories. In this study, we updated the h-CLAT database to 106 chemicals. Moreover, we evaluated the utility of the sensitization potential classification by various calculated values obtained in the h-CLAT.

Materials and Methods

THP-1 cells were exposed to each test chemical for 24 hours in 24 well flat-plates ($1x10^6$ cells/1 mL). After incubation, cells were collected and then stained by FITC-conjugated anti-CD86 and CD54 antibodies and propidium iodide. CD86 and CD54 expression and cell viability were measured by flow cytometry. Two of 3 independent assays at any dose should exceed the positive criteria (CD86 $\geq$ 150 or CD54 $\geq$ 200) in order to be considered a 'positive' outcome. 106 test chemicals, including 75 sensitizers and 31 non-sensitizers, were chosen from LLNA database. Estimated concentration required to induce the cell viability (CV75) and estimated concentration required to produce a RFI of 150 or 200 for CD86 or CD54 (EC150 or EC200), respectively, were used to evaluate sensitization potential classification of each chemical in the h-CLAT.

Results and Discussion

The accuracy of the h-CLAT vs. LLNA was 84%. Most chemicals were evaluated correctly, but there were 9 false negative and 8 false positive outcomes. Comparison to human data, the accuracy of the h-CLAT vs. human data was 80%. The h-CLAT especially showed good positive predictivity, which is the accuracy of h-CLAT detecting known positive allergens as "positive", for LLNA or human data (89% and 90%, respectively). We also evaluated the utility of h-CLAT to classify the skin sensitization potential by using various calculated values. From the data of 66 chemicals, which was both positive in h-CLAT and LLNA, several values were calculated as follow: 1) CV75, 2) maximum RFI of each chemical, 3) EC150, and 4) EC200. Correlational analyses between LLNA EC3 and the four values were performed. A statistically significant correlation was observed between CV75, EC150, and EC200 values with LLNA EC3. The EC150 value showed the better correlation compared to other values. From EC150 and EC200, Minimum Induction Threshold (MIT) was determined as a minimum value, smallest of either EC150 or EC200. MIT also show the good correlation with EC3. From these data, the h-CLAT values might be useful to predict the allergic potency of chemicals after improving the detailed conditions.

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P-1
Cardiotoxicity evaluation of the anticancer drug using chick embryo

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Objective(s): Alternative test, which comes from mind of animal welfare, is replacement of an animal test with one that uses non-animal systems for purpose of research, education, toxicity test or production (Replacement), and includes reduction of animal use (Reduction) and to lessen or eliminate pain or distress to animals (Refinement). Moreover, alternative test is also useful to the abolition of useless animal experiment, economic evaluation of a large number of new compounds, risk evasion with searching a toxic unknown compound in animal experiment, study of the mechanism of action for extrapolation to human. In recent years, from the viewpoints of animal welfare and promotion of efficiency of research and development, the reconsideration of animal experiment, reduction of the used animal number, and international development of the alternative experimental animal which can replace it from mammals, are studied positively. We reported that chick embryo was useful as an alternative of mammals. We were aimed at demonstrating investigative potency to relate to cardiac failure induction and aggravation by anticancer drug by the chick embryo electrocardiographic findings that were alternative experimental animal.

Materials and Methods: Fertile eggs of White Leghorns were purchased from Saitama Experimental Animal Supply. These were incubated under 37.6±0.2°C, 65.5RH% in an incubator with automatic turn device (Showa Furanki), and were used on day 16 of incubation. The tested drug was arsenic trioxide (ATO), which is anticancer drug for refractory leukemia concerned about as the adverse response that cardiotoxicity limited clinical use. The electrocardiograms (ECGs) were measured using three-needle electrode. Two electrodes were inserted into diagonal holes on the equator for using as a bipolar lead, and other 1 was inserted hole on south pole for using as a ground. This bipolar potential was led to a memory oscilloscope (VC-11, Nihon Kohden) via an input box and passed PowerLab, and was recorded as ECGs consecutively. ECGs were recorded for 60 minutes after drug administration, and the heart rates (HRs) were calculated from R-R intervals.

Results and Discussion: In low dosing of ATO, ECGs of chick embryo have no change; however, the HRs were decreased accompanied with arrhythmia by high dosing. There are many methods of alternative test that is using a culture cell in the *in vitro* test as an alternative experimental animal; however, it is impossible to form a tertiary structure of *in vivo*. Moreover, the accurate extrapolation of reaction to *in vivo* was difficult, because there is not interaction with other tissue/organ. Chick embryo is located in the middle position of in vitro and in vivo, and has the circulatory system, endocrine system, nervous system and interaction of tissue/organ which is impossible of reproduction by in vitro test, thus, elucidation of multifaceted pharmacological action is possible. Furthermore, chick embryo has advantage that management as experimental material is simple, and an experiment under a constant condition is practicable. The evaluation method of toxicological/ pharmacological action utilizing chick embryo is the pharmacological study technique all of above-mentioned 3Rs (Replacement, Reduction and Refinement) is possible. We emphasize that this method can become an alternative test method of very high utility is expected.

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P-2

Development of non-aqueous peptide binding assay for *in vitro* skin sensitization test

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Objective

Skin sensitization is initiated by the formation of hapten-protein complexes. Some methods for the evaluation of sensitizers based on measurements of protein reactivity to test articles have been proposed[1)]. These methods require use of a buffer for pH adjustment to obtain appropriate reactivity, limiting test materials to aqueous substances. We therefore developed an in vitro skin sensitization test applicable to non-aqueous substances.

Materials and Methods

Hexapeptides, APH(C) and APH(K), were used as reactive substrates[2)]. A total of 20 chemicals with octanol/water partition coefficients above 2.0 and for which sensitizing potential has been reported were used as test materials. A peptide and a test chemical were dissolved in DMSO to make the molar concentration of the test chemical 50 times that of the peptide. The mixture was placed in the dark at 40oC for 24hr. The reactivity of test materials was determined by depletion of peptides as measured by HPLC.

Results and Discussion

For 90% of the test substances, the results of this assay and LLNA or human patch tests agreed. This method is thus useful for predicting the sensitizing potential of compounds that are practically insoluble in water. However, prediction of sensitizing strength is difficult, and further improvement of this method is necessary.

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P-3

The agreement of assay results between a 6-well method and a 96-well method in the Bhas 42 cell transformation assay

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Objective(s)

The cell transformation assay using Bhas 42 cells (Bhas 42 CTA) was developed as an *in vitro* method for predicting the carcinogenicity of chemicals. The Bhas 42 cell line was established by introducing the v-Ha-*ras* oncogene into Balb/c 3T3 mouse fibroblasts. The Bhas 42 cells retain the contact inhibition in cell growth but lose the characteristic by carcinogens to form the transformed foci with aberrant morphology. Thus, in Bhas 42 CTA, the formation of transformed foci was defined as the endpoint. Previously we have performed an interlaboratory validation study[1)] or intralaboratory screening study by a method using 6-well plates (6-well method), and the high predictability of Bhas 42 CTA has been demonstrated. In the automation system we are currently developing, it is necessary to alter the test method to the one using 96-well culture plates (96-well method). This study was carried out to confirm the correspondence of test results between the 6-well method and the 96-well method.

Materials and Methods

The 96-well method was performed in manual operation for 33 chemicals (positive: 20, negative: 13) that had already been evaluated with the 6-well method. The test procedures of the 96-well method were done as follows. For detecting the tumor initiators (initiation assay), 200 cells were seeded in each well with 100 µL of culture media by using an 8-channel pipette. Similarly, 400 cells were seeded for detecting the tumor promoters (promotion assay). In the initiation assay, the cells were treated with a test chemical for 3 days from the next day of seeding (: cells were sparse). In the promotion assay, the cells were treated with a test chemical for 10 days from 4 days after seeding (: cells were sub-confluent to confluent). The post-cultivation was continued for 17 days in the initiation assay and for 7 days in the promotion assay. Thus, the total culture periods were 21 days in both assays. The transformation frequency was expressed as the number of wells having foci in a plate. These procedures were the same as those of the 6-well method except for the number of cells seeded, the volume of culture media and the expression of transformation frequency. The statistical analysis was performed by a chi-square test with Bonferroni's adjustment.

Results and Discussion

For all the chemicals, the assay results obtained from the 96-well method were similar to those from the 6-well method. In addition, the manner of medium exchange and the judgment of transformation have been found to be more convenient in the 96-well method than in the 6-well method. These results showed that the use of 96-well plate is rational in the automation system and manual operation. The chi-square test with Bonferroni's adjustment was appropriate for statistical analysis of the data from 96-well method.

This study was supported by the New Energy and Industrial Technology Development Organization (NEDO).

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P-4

A Genotoxicity test system based on p53R2 gene expression in normal human skin cells

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Objective(s)

In vitro genotoxicity assays play an important role in predicting potential carcinogenic activity of chemicals. Ames test is the most commonly used assay and some in vitro mammalian cell assays have been used. However, there are some problems of throughput, the frequency of false positive results, or species difference in these assays. For the purpose of constructing an easy-operating genotoxicity test system suitable for a primary screening of reagents for human genotoxicity, we developed a p53R2-dependent luciferase reporter gene assay using human cell lines (MCF-7) expressing wild-type p53 gene. DNA repair gene p53R2 plays a pivotal role in cell survival by repairing damaged DNA and is activated by DNA damage in a p53-dependent manner. In this study, we investigated its reactivity using normal human skin fibroblast (NB1RGB) for assessing the human skin carcinogens.

Materials and Methods

The p53BS-Luc reporter plasmid was constructed by inserting the sequence for the p53 binding site derived from human p53R2 gene. NB1RGB cells were plated in 96-well plate. The cells in each well were transiently co-transfected with p53BS-Luc plasmid and pRL-SV40 internal control plasmid in each assay. 6 h after transfection, test samples or solvent were added into triplicate wells. After 16-18 h incubation, the cells were washed and lysed. Luciferase activities are measured and normalized. Relative p53R2-dependent luciferase activity after treatment with chemicals was expressed as the % of the control cell activity. When the relative luciferase activity of cells treated with the test sample was over 150% of that treatment with vehicle only in a dose-dependent manner, the test sample was judged to be positive for genotoxicity.

Results and Discussion

The p53R2-dependent luciferase activities induced by typical genotoxins, food additives, and cosmetic ingredients were evaluated. The results of this reporter gene assay were in good agreement with the Ames test, and can detect some Ames-negative carcinogen such as Acrylamide, and $CdCl_2$. It was also showed that this assay could be applied to the evaluation of the UV-induced mutagenicity. It is suggested that this genotoxicity test system, based on p53R2 gene expression, is applicable to normal human skin cell that expressed wild-type p53, and can become a valuable tool for predicting the human skin cancer risk.

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P-5

Validation of an in vitro screening test for predicting the tumor promoting potential of chemicals based on gene expression.

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Objective(s)

Chemical carcinogenesis is a multifactorial process comprising two main stages; initiation and promotion. Tumor promoters cause the development of tumors in initiated cells and the majority of them are non-genotoxic carcinogens. The identification of tumor promoters is important for preventing cancer. We previously identified 22 specific gene markers using a global gene expression analysis of chemically induced tumor promotion and established an in vitro real-time PCR screening assay for the assessment of the tumor promoting potential of chemicals in BALB/c 3T3 cells. Our in vitro tumor promoter screening test, based on these marker genes, enables earlier assessment, and is easier to conduct than classical methods. The general applicability of these markers, however, was unknown. In this study, to evaluate the performance of a set of markers, we independently validated a separate sample set, which had various structures and properties.

Materials and Methods

To validate the reliability and robustness of this screening assay, the 22 gene markers were tested on the 63 independent test chemicals (test set). The in vitro screening test for predicting tumor promoting potential of chemicals based on gene expression was carried out as follows. The test chemical, as the promotion treatment, was added to the medium on day seven of a short-term two-stage cell transformation assay, using BALB/c 3T3 cells. Total RNA was extracted from cells, 48 h after test chemicals or solvent were added, and was reverse transcribed. By real-time quantitative RT-PCR analysis, mRNA expression of 22 genes was investigated. The number of marker genes that were up-regulated more than 1.5 fold by a chemical treatment was used as the gene expression score. We judged a chemical to be positive if more than two genes responded (gene expression score). The performance of this assay was measured by examining how well the classifier predicted the non-tumor promoter (cell transformation assay negative) or tumor promoter samples (cell transformation assay positive) in the test set.

Results and Discussion

Sixty-three chemicals were tested by QRT-PCR and a cell transformation assay. Thirty-three chemicals were positive in the cell transformation assay, and the others were negative. Our gene expression assay judged 32 to be positive among 33 chemicals that were positive in the cell transformation assay, and judged 29 to be negative among 30 that were negative in the cell transformation assay. For severe or moderate tumor promoters, our assay showed dose dependent increases in the gene expression scores. There was a correlation between the results of our assay and the cell transformation assay results, in BALB/c 3T3 cells. This assay consistently achieved high accuracy, sensitivity, and specificity (96.8%, 97.0% and 96.7%). These results indicate that the tumor promoting activity assay, based on the expression of 22 marker genes, will become a valuable tool for rapid screening of potential tumor promoters.

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P-6

The value of hepatotoxicity assessment using primary cultured human hepatocytes

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Objective(s)

The liver is an extremely powerful organ that elicits detoxification and removal of toxic chemicals, and is also frequently the target organ of toxins. The primary cultured hepatocytes are useful tools for investigating drug metabolism, induction of drug-metabolizing enzymes and cytotoxicity studies of chemicals. Studies have been performed in order to know whether the primary cultured hepatocytes are useful as compared with HepG2 cell line using the compounds that are known to cause hepatotoxicity by CYP-mediated metabolism.

Materials and Methods

HepG2 cell line and human hepatocytes (plateable, cryopreserved) were suspended in Hepatocyte Culture Medium and were seeded in collagen-coated 96-well plates. After overnight incubation, the culture medium was replaced with that containing various concentrations of test compounds. After 24 hours exposure, cellular ATP levels were measured using the Promega Celltiter-Glo luminescent assay kit to evaluate cytotoxic effect of test compounds.

Results and Discussion

The first example was perhexiline maleate whose toxicity is thought to be mediated by the cationic amphiphilic structure of the molecule, but not by CYP metabolism[1)]. Perhexiline maleate exhibited strong cytotoxicity in both HepG2 cells and human hepatocytes. Flutamide is metabolized by CYP1A and CYP3A to generate reactive metabolites[2)]. Ticlopidine is a selective mechanism-based inhibitor of human liver CYP2C9[3)]. Liver toxicity caused by aflatoxin B1 is mediated by toxic epoxide formation[4)]. These three compounds exhibited significantly higher cytotoxicity in human hepatocytes than in HepG2 cells. The cytotoxicity of Aflatoxin B1 in human hepatocytes was evaluated in the presence and absence of a nonspecific, irreversible P450 inhibitor, 1-aminobenzotriazole or a CYP3A4 inhibitor, ketoconazole. Both 1-aminobenzotriazole and ketoconazole reduced the cytotoxicity of Aflatoxin B1 in human hepatocytes. Ethosuximide, which is mainly metabolized by CYP3A4[5)] and has no reports on reactive metabolite formation and liver toxicity in humans, did not exhibit cytotoxicity in human hepatocytes. The cytotoxicity assay using metabolically-competent hepatocytes that express phase I and phase II drug-metabolizing enzymes is suitable to predict potential hepatotoxicity risk of compounds.

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P-7
Review of an alternative to animal testing for safety evaluation of Quasi-drug

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Since 2007, the research project H19-Drug-003 “Research on establishment of a system of safety evaluation using alternatives to animal testing and its international cooperation (Chief researcher: Hajime Kojima, NIHS (the National Institute of Health Sciences)” has been coordinated by the MHLW (the Ministry of Health, Labour and Welfare). One of the purpose of this project is to review of alternatives to animal testing for the safety evaluation of Quasi-drug. To accomplish this purpose, the chief researcher organized an *ad hoc* research committee which consisted of dermatologists, regulators, representatives of the JCIA (Japanese Cosmetic Industry Association), and researchers of the NIHS. Furthermore, six task forces were organized under the research committee to clarify merits and demerits of alternative methods used for safety evaluations of the Quasi-drug. These task forces separately reviewed alternative methods for skin irritation, skin sensitization, eye irritation, phototoxicity, genotoxicity, and skin penetration & absorption. This year, research group will make discussions to integrate the reports submitted from the task forces.

P-8
Validation of LabCyte EPI-MODEL24, an *In Vitro* Assay for Detecting Skin Irritants*

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The *in vitro* test system, employing reconstructed human epidermis model (RhE: LabCyte EPI-MODEL24), has progressed through protocol optimisation as in vitro skin irritation test. The multi-laboratory assessment of this system performed based on the a few ESAC performance standards [1-3)] and this assay performed as me-too assay.

The present objective was to conduct a validation study to assess the reliability (reproducibility within and between laboratories) and relevance (predictive capacity) of this test system with a challenging set of coded test chemicals for which high quality *in vivo* data were available. The validation study was undertaken in accordance with the principles and criteria documented in the OECD *Guidance Document No.34*[4)] .

Based on the GHS-EU classification, 12 irritants and 13 non-irritants in the ESAC Performance Standards were tested by the 7 labs using **LabCyte** EPI-MODEL24. The assay demonstrated high Reproducibility within and between laboratories, and acceptable reliability of the positive control (100%) and accuracy (78.0% overall accuracy, 83.3% overall sensitivity, 71.1% overall specificity) on the MTT assay for use as a stand-alone assay to distinguish between skin irritants and non-irritants. IL- 1 α did not significantly contribute to the performance of the assay.

*Supported by the Japanese Society for Alternative to Animal Experiments.

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P-9

An On-line Community of Stakeholders Interested in Non-animal Methods of Toxicity Testing

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Introduction

Toxicity testing has begun to undergo a paradigm shift away from *in vivo* methods and towards non-animal approaches. This shift is being driven by advances in science and technology and societal concern for animal welfare. A full-fledged transformation will take coordinated efforts by diverse stakeholders in government, industry, academia, and non-governmental organizations.

As this paradigm shift gains momentum, replacement alternatives are likely to play an increasingly large role in reducing animal numbers (and consequently animal suffering) in toxicity testing, with proportionately less of a role for reduction alternatives or refinement alternatives. Consequently, the Three Rs community is likely to increasingly invest its efforts into replacement strategies as the primary means of addressing animal use in toxicity testing.

For all these reasons, the time is ripe to strengthen the community of stakeholders interested in advancing replacement (non-animal) testing methods. Our response to this need was create an online platform—AltTox.org—for this community.

Unlike other alternatives websites, AltTox is devoted exclusively to progress in *in vitro* and *in silico* methods (i.e., the “R” of replacement) and to the field of toxicity testing, as well as being interactive, which enhances information exchange among stakeholders. In addition, AltTox covers science policy as well as technical aspects of advancing non-animal methods of testing.

AltTox’s aim is to stimulate progress internationally in the development, validation, acceptance, and implementation of non-animal testing methods.

Conclusion

AltTox.org users are encouraged to contribute to the site and interact with each other in several ways, including:

- Participating in the interactive forums
- Providing invited commentaries on The Way Forward
- Suggesting or submitting content, monthly features, data, and graphics
- Demonstrating the need for a new “Community of Practice” forum
- Providing website feedback to the AltTox Management Team
- Becoming a sponsor, either as an individual or a corporation

Members of the AltTox community can also sign up to receive the *AltTox Digest*, a monthly newsletter summarizing what’s new on the website. To sign up, visit http://www.alttox.org/e-newsletter/.

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P-10

JaCVAM statement on new alternatives to animal testing

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Introduction

The Japanese Center for the Validation of Alternative Methods (JaCVAM) has a framework for peer review and regulatory acceptance of alternative methods. After JaCVAM has received a request for peer review from a researcher or developer, the JaCVAM steering committee meets to deliberate on the proposal methods. Upon the receipt of permission for peer review, JaCVAM organizes the oversight committee in order to evaluate a new test method. Based on the background review documents and references prepared by the oversight committee, a ad hoc peer review panel evaluates a new or revised test method. The members of the oversight committee and the peer review panel assigned to evaluate a new test method are selected by the JaCVAM steering committee. JaCVAM and its steering committee have a regulatory acceptance board for new or revised methods. This board reviews new or revised test methods based on the reports of the peer review panel and prepares a report and statement on the test method for regulatory agencies.

Conclusion

At 2008 at the National Institute of Health Sciences (NIHS), Tokyo, Japan, the members of the JaCVAM Regulatory Acceptance Board unanimously endorsed the following statement:
1) Vitrolife-Skin™, a 3-dimensional cultured skin model can be used for distinguishing between corrosive and non-corrosive chemicals within the context of the OECD testing guidelines No. 431 on In Vitro Skin Corrosion: Human Skin Model Test.
2) LLNA (Local Lymph Node Assay) -DA can be used for distinguishing between sensitizer and non-sensitizer chemicals within the context of the OECD testing guideline No.429.

We are preparing other statements for this year. We will continue to publish these statements and work on the regulatory agencies. These statements can be seen on JaCVAM web site (http://jacvam.jp/).

P-11

Investigation of sensitive cytotoxicity assay detecting drug-induced mitochondrial toxicity

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Objective(s)

Drugs that exhibit no mitochondrial toxicity are desirable because mitochondria are subcellular organelles that generate almost all the energy in a living body. Drugs that exhibit mitochondrial liabilities can cause side-effects including death, and in some cases, are withdrawn from the market. Replacing glucose with galactose in assay media makes cells yield no net ATP production from glycolysis, which impels cells to yield ATP by mitochondria. Consequently, this culture system with galactose media is anticipated to be ultrasensitive to the cytotoxic effects of mitochondrial toxins[1)]. In the present study, we examined the values of this galactose culture system as cytotoxicity assay by comparing with standard glucose culture system using classic inhibitors of mitochondrial oxidative phosphorylation and drugs that cause liver toxicities via the mitochondrial effects.

Materials and Methods

HepG2 human hepatocarcinoma cells, which were maintained in the galactose media for greater than 10 passages, were suspended in the same media and seeded in collagen-coated 96-well plates. After overnight incubation in 37℃, 5% CO_2-95% , the culture medium were replaced with those containing various concentrations of test compounds. After 24 hours exposure, cellular ATP levels were measured using the system of enzyme activity of Luciferin-Luciferase. The cytotoxic effects of the test compounds were expressed by 50% **i**nhibitory **c**oncentration (IC50) of ATP levels. For the control experiment, cells maintained in the standard culture media containing glucose were used.

Results and Discussion

Galactose-grown cells were ultrasensitive to the cytotoxic effects of classic inhibitors of mitochondrial oxidative phosphorylation, rotenone (complex I inhibitor), antimycin (complex Ⅲinhibitor), oligomycin (ATP synthase inhibitor), and carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP, uncoupler). The cytotoxicity of troglitazone（complexⅢ,Ⅳ,Ⅴinhibitor, MPT inducer）was pronounced in galactose-grown cells as compared with glucose-grown cells. In contrast, the cytotoxic effect of benzbromarone (BSEP inhibitor, reactive metabolites formation) was parallel in glucose- and galactose-grown cells. Consequently, this test system is valuable for the detection of toxic effects of compounds that affect mitochondrial electron transport chain.

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P-12

Bile Canalicular Formation and Hepatobiliary Transport are Enhanced in Hepatocyte Sandwich Culture on an Oxygen-permeable Polydimethylsiloxane Membrane.

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Objective

Previously, Nishikawa and colleagues developed culture plates fabricated with polydimethylsiloxane (PDMS) membranes covalently bound with collagen[1)]. We therefore established a sandwich culture of rat primary hepatocytes on a PDMS membrane, and examined the effects of direct oxygen supply through a PDMS membrane on the formation of bile canaliculi and the measurement of uptake and efflux transport activity.

Materials and Methods

On day 0, hepatocytes were seeded on a PDMS cell culture plate fabricated as described previously[1)] or on collagen-coated plates. On next day, the sandwich configuration was established by overlaying collagen gel. 5-(and-6)-carboxy-2’,7’-dichloro-fluorescein diacetate (CDFDA; Invitrogen, Carlsbad, CA) was used for detecting the formation of bile canaliculi. 5-(and-6)-carboxy-2’,7’-dichloro-fluorescein (CDF) produced from CDFDA in hepatocytes and excreted into bile canaliculi were detected by confocal laser microscopy using a Zeiss LSM7 DUO microscope (Carl Zeiss Inc.,). Digital images were analyzed by ZEN software (Carl Zeiss Inc.,). Transporter function assays were performed as reported previously[2)].

Results and Discussion

The development and maintenance of bile canaliculi were analyzed by imaging the accumulation of CDF in polarized hepatocytes. The accumulation of CDF in the bile canalicular structures between hepatocytes on PDMS was initially detected on culture day 2, whereas almost all fluorescence of CDF remained inside hepatocytes on culture day 2 in the sandwich culture on polystyrene coated with collagen. The quantification of the CDF fluorescent area of the bile canaliculi after completion of bile canalicular formation at culture day 4 on both PDMS and polystyrene coated with collagen clearly showed that a more wide area of bile canaliculi were formed on PDMS than on polystyrene coated with collagen, and this increase in bile canaliculi was maintained until culture day 10 (Fig. 1).

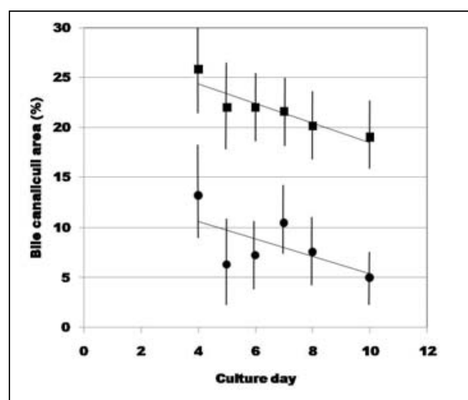

Fig. 1. Quantification of CDF-labeled bile canaliculi area of sandwich culture on PDMS (squares) and polystyrene (circles).

To determine the timing of appearance of the biliary excretion of CDF and biliary excretion activity in sandwich-cultured hepatocytes, time-dependent CDF accumulation incubated in standard or Ca^{2+}/Mg^{2+}-free buffer and BEI was determined on culture days 2 and 4.

The culture system described in this article is promising approach to easily and inexpensively facilitate the design and fabrication of liver tissue microdevices for testing phramcodynamics and drug hepatotoxity.

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P-13

Evaluation of three dimensional cultured skin models using membrane permeation test

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Objective(s) Skin permeation test is a useful tool to develop and evaluate topical drug formulations and cosmetics. Recently, three dimensional cultured skins as well as animal and human cadaver skins have been utilized as skin membranes. In the present experiment, six kinds of three-dimensional cultured human skin models (Vitrolife-skin and LSE-high are of full thickness model, and Episkin, EpiDerm, Neoderm-E and LabCyte EPI-MODEL are of epidemis model) were selected and morphological properties and permeation profiles of model compounds of and through the skin models were evaluated.

Materials and Methods Seven substances; antipyrine, isosorbide-5-mononitrate, caffeine, aminopyrine, isosorbide dinitrate, benzoic acid and flurbiprofen were used as penetrants. These have almost the same molecular weight (ca 300 Daltons) and different hydrophilicities. Six kinds of three dimensional cultured human skin models, having different characteristics, were used. Permeability coefficients (P), diffusion parameters (D/L^2) and partition parameters ($K \cdot L$) were calculated from the obtained permeation profile of 7 compounds through the cultured membranes, where D, K and L are diffusion coefficient, partition coefficient and barrier thickness, respectively. One-layered diffusion model was used for theoretical calculation. Furthermore, histological observation using H.E. stained tissues and transmission electron microscopy was done for each sample.

Results and Discussion Three-dimensional cultured human skin models showed different skin permeation profiles, so that we should have a great attention to select a three-dimensional cultured human skin model for the permeation test of drugs and cosmetic ingredients. MTT assay has been used as a skin irritation test, since linear relationships were obtained between MTT assay results and drug concentration in skin[1,2)]. Furthermore, substance metabolism in human and three dimensional cultured skin models were different[3)]. Thus, MTT assay using LSE-high and other cultured human skins might be sometimes false-negative in the skin irritation. Only a few studies were found for the skin permeation of drugs and cosmetics ingredients through cultured human skins except for LSE-high. Present results would have a great information of permeability through several cultured skin models.

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P-14

Development of a human corneal epithelium model utilizing a collagen vitrigel membrane and its application to eye irritation test

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Objective(s)

A collagen vitrigel membrane is composed of high density collagen fibrils equivalent to connective tissues in vivo and is easily handled with tweezers. Also, it possesses excellent transparency and permeability of protein with high molecular weight and consequently the various researches utilizing it as a cell culture substratum advances so well[1, 2). We have established a reconstruction method of rabbit corneal epithelium model by culturing normal rabbit corneal epithelial cells on the collagen vitrigel membrane substratum and inducing differentiation to form multilayers of the cells. However, to estimate eye irritation and permeability of chemicals towards human it is required to reconstruct a corneal model with barrier function utilizing human cells. In this study as a first step, we aimed for establishing a reconstruction method of human corneal epithelium model with barrier function utilizing both a human corneal epithelium-derived cell line (HCE-T) and the collagen vitrigel membrane substratum. Further, to confirm the utility of the human corneal epithelium model the changes of its barrier function induced by exposing eye-irritant chemicals were measured.

Materials and Methods

HCE-T cells purchased from RIKEN cell bank (Tsukuba, Japan) were seeded on the collagen vitrigel substratum and cultured for 2 days to the confluent stage. Subsequently, the reconstruction of a corneal epithelium model was examined by culturing it on air-liquid interface to induce the cell differentiation for multilayer formation. The corneal model in the reconstruction process was analyzed histologically by the light-microscopic observation of cross-section stained with hematoxylin and eosin. Also, the formation of its barrier function was analyzed by measuring transepithelial electrical resistance (TEER). Further, time-dependent changes of TEER after exposing chemicals (NaOH, EtOH, Tween 20, etc.) to the model were measured.

Results and Discussion

HCE-T cells proliferated well on the collagen vitrigel substratum and gradually differentiated into multilayers on air-liquid interface culture, resulting in the time-dependent increase of TEER. The corneal epithelium model possessing five cell layers was well reconstructed after one week-culture on the air-liquid interface. The exposure of chemicals to the model induced the time-dependent changes of TEER in response to the characteristic of each chemical. These results suggest that eye irritant chemicals could be estimated by the barrier function of reconstructed human corneal epithelium model as an indicator.

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P-15
Reproducibility of human 3-dimensional cultured epidermal model (LabCyte EPI-MODEL)

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Objective(s)
The validation study of *in vitro* skin irritation testing using a 3-dimensional reconstructed human skin model has been conducted by the European Centre for the Validation of Alternative Methods (ECVAM). As a result, three protocols were approved by ECVAM. In the Performance Standard[1)] (PS) announced by ECVAM along with the approval, the structural and performance criteria of the human cultured skin models for testing are defined.
We have performed some quality control testing, according to the method described in PS, of the new human epidermis reconstructed model, LabCyte EPI-MODEL24 (Japan Tissue Engineering Co., Ltd., JAPAN). In this study, the reproducibility between continuous batches is analyzed by using the quality control examination result of these LabCyte EPI-MODEL24.

Materials and Methods
The following quality control testings described in PS have been conducted using LabCyte EPI-MODEL24. The data acquired was analyzed.
Tissue viability: The tissue viablility of LabCyte EPI-MODEL24 was measured by the MTT method (n=3).
Barrier function: Various concentrations (0.1-0.4% (w/v)) of cytotoxic marker chemical sodium lauryl sulfate (SLS) were applied to the LabCyte EPI-MODEL24, and the tissues were incubated for 18 hours. The tissues were then washed with PBS and subjected to the MTT assay. The concentration at which SLS reduces the viability of the tissues by 50% (IC_{50}) was calculated.
Morphological examination: Tissue sections of LabCyte EPI-MODEL24 were prepared and stained with hematoxylin and eosin for light microscopic examination.

Results and Discussion
Tissue viability from the evaluation of continuous batches (179 batches) of LabCyte EPI-MODEL24, has been fabricated every week, were 1.46±0.26 (OD_{570nm}) and barrier function (IC_{50}) of that (51 batches) was 0.26 ± 0.03 %. Tissue viability and barrier function of LabCyte EPI-MODEL24 have remained constant and that indicate their reproducibility is high (low CV: 17.4% and 9.8%, respectively).
Additionally, IC_{50} of barrier function was maintained at an acceptable range (lower limit: 0.14 %, upper limit: 0.4%), established by prior examination, in all measured batches.
It was observed that all batches of LabCyte EPI-MODEL24 tested were composed major epidermal layer, including cornified layer, which resembled the normal human epidermis morphologically.
Reproducibility of tissue viability, barrier function, and morphology of LabCyte EPI-MODEL24 was high between continuous batches tested, indicates that LabCyte EPI-MODEL24 is applicable to the skin irritation testing protocol according to PS.

References
1)Performance Standards for In-Vitro Skin Irritation Test Methods based on Reconstructed Human Epidermis(RhE): ECVAM 2009-08-24.

P-16

The Skin Irritation Test using the Human Epidermal Model LabCyte EPI-MODEL24 : examination with 54 materials

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【Objective】

The European Centre for the Validation of Alternative Methods (ECVAM) validated some protocols of *in vitro* skin irritation testing using 3-dimensional human reconstructed skin model and approved three kinds of protocols. According to the Performance Standard[1)] (PS) by ECVAM, the human cultured epidermal model, LabCyte EPI-MODEL24 (Japan Tissue Engineering Co., Ltd., JAPAN) has been validated, and the results will be announced in this workshop. Here, we also report the evaluation of *in vivo* correlation in LabCyte EPI-MODEL by MTT test and the skin irritation test with 54 materials.

【Materials and Methods】

We first selected 54 materials (17 irritants and 37 non irritants) and performed some experiments (details in the poster) to assess the effect of these materials to MTT test. Then, the materials were applied on the surface of LabCyte EPI-MODEL24 for 15 minutes. Cells were washed to remove materials completely and cultured with medium for 42 hours. Cell viability was measured by MTT assay for the evaluation of skin irritation.

【Results and Discussion】

7 of 54 materials showed the influence on the MTT test. We also applied these 7 materials on the LabCyte EPI-MODEL24 whose cells were killed by freeze-thawing and performed MTT assay to see the reactivity. Interestingly, the materials reacted to MTT by 13-145% compared with the negative control. However, we decided the reactivity of these 7 materials to MTT had no influence on the assessment of skin irritation because all of 7 materials were determined as the skin irritants. *In vitro* skin irritation test with 54 materials showed a high correlation to *in vivo* stimulation test; 79.6% as for Accuracy, 88.2% as for Sensitivity, and 75.7% as for Specificity. These results indicate that our LabCyte EPI-MODEL24 would be useful to assess the skin irritation.

【References】

1) Performance Standards for In-Vitro Skin Irritation Test Methods based on Reconstructed Human Epidermis (RhE): ECVAM 2009-08-24.

P-17

Statistical Issues in the Design and Analysis of Validation Studies

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Objective

As statisticians, we have been involved in the planning, conducting, and evaluation of multi-laboratory validation studies (VS) for alternatives to animal experiments. For more than ten years now, we have faced many statistical issues in the design and analysis of VS on eye irritation, skin irritation, skin corrosion, skin sensitization, photo-toxicity, and so on. Here, we show examples of statistical methodologies and strategies to be used to deal with the issues encountered in the design and analysis of VS.

Materials and Methods

We consider six issues that we deem important: (1) randomized allocation of chemicals to multiple laboratories, (2) calculation of sensitivity and specificity, (3) data management, (4) evaluation of transferability, (5) evaluation of inter-laboratory reproducibility, and (6) use of confidence intervals.

Results and Discussion

For issue 1, we investigated the adequacy of the allocation design, using the D-optimality based on the fixed effects model and the inter-laboratory variability based on the mixed effect model. With regard to issue 2, we confirmed that the method used to calculate sensitivity or specificity should be discussed during the planning of VS, and described in the report on VS. Regarding issue 3, we developed a pre-formatted MS-Excel sheet for the inputting of data and information in order to correctly collect the values observed in the laboratories. For issue 4, we proposed the use of the intra-class correlation coefficient based on the random effect model as the measure of transferability, and the use of a flowchart to evaluate transferability. To deal with issue 5, we showed that the inter-laboratory reproducibility could be evaluated in consideration of the variation of the response, even when multiple experiments for a chemical are not conducted within the laboratory. As for issue 6, we showed that the use of confidence intervals along with scatter plots is useful to assess the toxicity of a chemical. These statistical works have contributed much to VS. However, further investigations are needed to generalize our solutions. In addition, some of our results need to be provided in a form that is easy to use for non-statisticians.

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P-18
The effect of chemical compound on cultured rat embryos in S-9mix

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Objective(s)
We are attempting an improvement to optimize the embryo culture method as an alternative developmental toxicity test. In the present study, we investigated the effect of a chemical compound on cultured embryos in S-9mix.

Materials and Methods
(1) Added to 4ml of culture medium (Rat Serum : RS) supplemented with rat S-9mix was 10 µg/ml(RS) of imipramine (IMP). Concentration of desipramine (DEP : a metabolite of IMP) in the culture medium was measured of time trend. (2) Rat embryos on day 11 (plug day = 0) of gestation were cultured for 48 hours. And 10 µg/ml (RS) of IMP was administered at 2 hours after culture for comparison in fetal abnormality between S-9mix group and no-S-9mix group. The rat S-9 concentration in the culture medium was 3% in all tests.

Results and Discussion
(1) As time passed, concentration of DEP increased. (2) For the effect of IMP on cultured embryos, deformity rates were 67% in the S-9mix group and 54% in the no-S-9mix group after 48 hours of culture. Types of deformity included cleft lip, short tail and abnormal maxillary formation in the no-S-9mix group, while deformity in the S-9mix group had many cases with fetal atrophy, hematoma and edema; each group had different types of abnormality. The difference in types of abnormality seemed to be due to a difference in types of abnormality induced in the cultured embryos between IMP and its metabolite DEP.
These findings suggested that embryo culture method combining metabolic system may be a test method more likely to be able to confirm abnormality due to a chemical, as well as its metabolites, than the existing embryo culture methods.
This work was supported by the grant P06040 from the New Energy and Industrial Technology Development Organization (NEDO).

P-19

Evaluation of eye irritation potential of 114 chemicals and correspondence to GHS classification using in vitro Short Time Exposure (STE) test.

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Objective(s)
Testing of any cosmetic in animal eye irritation tests has been banned in EU. Although some alternative methods of eye irritation using various cell lines are being developed around the world, none of them are yet accepted as an OECD test guideline. The Short Time Exposure (STE) test, an alternative eye irritation test, involves exposing SIRC (rabbit corneal cell line) cells for 5 min to test material. A good correlation has been confirmed between the eye irritation category by STE test and the eye irritation score in the Draize test. In the present study, we evaluated the eye irritation category of 114 chemicals using STE test. Then, we examined the association of STE irritation classification with the Globally Harmonized System (GHS).

Materials and Methods
The SIRC cells were exposed to 200 μL of 5% and 0.05% test chemical solutions for 5 minutes. After exposure, cell viability (%) was measured by the MTT assay. Category classification of eye irritation by STE test was determined based on the relative viability assessed for 5% test concentrations. Moreover, the STE rankings of 1, 2, and 3 classified by the prediction model (PM) based on the relative viability at two concentrations (5% and 0.05%). The GHS classifications of the chemicals were estimated as NI（not classified), category 2 (Cat. 2) or category 1 (Cat. 1) based on the Draize data listed in mainly the ECETOC Data Bank.

Results and Discussion
A good correlation was confirmed between the eye irritation category determined based on the relative viability assessed for 5% and GHS category of non-irritant or irritant (Cat. 2+Cat. 1); accuracy obtained was 87%. Moreover, the STE rankings of 1, 2 and 3 classified by the PM highly correlated with the GHS rankings of NI, Cat. 2, and Cat. 1 (accuracy was 71%). The STE ranks based on PM was allowed to discriminate between eye irritation rankings of the GHS for Cat. 2 and 1 accompanied by high prediction accuracy. Accordingly, it was demonstrated that STE test possessed a good predictive performance of eye irritation and it might be a promising alternative eye irritation test.

P-20

Skin Sensitization Study by Quantitative Structure-Activity Relationships

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Objective(s)

Skin sensitization (Positive / Negative) prediction models (discriminant function) had been generated and parameter analysis had been discussed based on QSAR technology.

Materials and Methods

Samples used in this research were obtained from DFG（Deutschen Forschungsgemeinschaft） for Positive data, and NITE's open database for negative data. Total 291 compounds (Posi;122, Nega;169) were used in this study.

Used parameters are generated from 2-D and 3-D structure of used compounds. All generated about 800 parameters are reduced to 47 parameter set and 32 parameter set. Various linear and non-linear discriminant analysis methods are applied by using those 2 parameter set. All data analysis was done on The ADMEWORKS/MODELBUILDER software.

Results and Discussion

Perfect classification ratios were achieved by Support vector machine and AdaBoost (32 parameters), except the linear discriminant analysis by least squares method. The highest prediction ratio (81.44%; Leave 10 out method) was achieved on NN method (47 parameters).

The LogP parameter has been an important parameter on QSAR approach. In this study, the LogP parameter is not so much important and some other parameters were important for Skin sensitization.

The "Secondary sp3 carbon count" and the "Environment molecular connectivity of substructure (-O-C)" parameters have large positive coefficient, and the "All-path calc for substructure (-C-)" and the "Count of substructure (DMPATH) (-ester-)" have large negative coefficient.

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P-21

Direct prediction of toxicity scores using the data obtained from an alternative test: A prediction model

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Objective(s)

Ohno (2004) proposed a method for classifying the toxicity of substances by using the cytotoxicity test as an alternative test for the Draize eye irritation test. In his proposal, 3 concurrent positive controls were required in the assessment of the cytotoxicity of unknown substances. We consider such an approach, which involves several reference substances for the assessment of an unknown substance. In this approach, if the score of the target test is directly predicted by the score of the alternative test, the procedure would yield considerable information about the substance rather than providing data on the category of toxicity. In this study, we aimed to develop a prediction model using the direct-prediction approach and discuss the features of this model.

Materials and Methods

To directly predict the scores of the target test method, we proposed the use of calibration. The calibration is typically performed in 2 stages: the calibration stage and prediction stage. In the calibration stage, we used a dataset of positive controls to regress the scores of the target test to the scores of the alternative test. In the prediction stage, the scores of the target test were estimated using the constructed regression line and the scores for unknown substances.
Since the number of substances used as positive controls is usually less, the regression line constructed in the calibration stage will be unstable. To solve this problem, it is possible that Bayesian regression is used in the calibration stage, thereby allowing the incorporation of data and prior knowledge. Further, the application of the Bayesian approach in the prediction stage will yield the predicted score with its distribution.

Results and Discussion

We have presented a numerical example in this study. We used the data obtained from a validation study of a cytotoxicity test performed using SIRC cells and crystal violet staining (Itagaki et al., 1995). In accordance with Ohno's proposal, we used Tween 20, SLS, and Triton X-100 as the positive controls and then fitted the linear regression line to the data. Next, we used this regression line to estimate the predictive values of the maximum average score (MAS) for other substances. The predicted MAS for the EC50 of 329.9 (polycthyleneglycol monolaurate) was 7.7, and the corresponding score for 454.1 (sodium N-lauroyl sarcosinate) was 3.1.

This approach is based on a linear relationship. Since complete linear relationship is usually not obtained, the approach will only yield roughly predicted values for toxicity. However, predicting these quantitative predictive values has several merits. First, the values are interpretable using the same scale used for the target test method. Second, this approach does not require any cut-off value, thereby bypassing the difficulties involved in the identification of valid cut-off values.

This study was supported by the Health and Labour Science Research Grant, Japan.

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P -22
Whole embryo culture with minivaial on rat embryo of day 9.5 .

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The conceptuses were transferred into 25ml-bottles or 15ml-bottles containing immediatery centrifuged and heat-inactivated male rat serum (5ml or 2ml). Then, the bottles were rotated at 20 rev. / min in an incubator at 38℃. At the end of the culture period, the conceptuses were transferred to Tyrode solution and examined under a stereoscopic microscope. The degree of differentiation and development of each conceptus was evaluated by a morphological scoring system. Rat embryos of day 9.5 of gestation were cultured for 48 hours. The embryo toxic effects were not observed on cultured rat embryos.

P-23

Evaluation of heterotypic cellular interactions using detachable substrates under controlled flow conditions

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Objective(s)

Model systems designed to accurately reflect tissue architectures and functions are urgently needed given the recent campaigns against animal experiments. We have reported a detachable substrates-based coculture system to investigate the interactions that occur between HeLa cells and human umbilical vein endothelial cells (HUVECs)—acting as a tumor/endothelium model—by monitoring their movements in the controlled system[1]. Our results suggest that soluble signaling-factors secreted from both cell types affect the migration of HUVECs in particular. Here, we attempt to integrate the coculture system with a microfluidic device in order to control the diffusing direction of the soluble factors.

Materials and Methods

The detachable substrate system consists of two parts—one of polystyrene (PS) and the other of PDMS—with complementary edges. These substrates were fabricated by replica molding. To initiate a coculture, complementary substrates on which either HeLa cells or HUVECs had been grown to confluency were assembled. Microfluidic device was composed of glass substrate and PDMS having microchannel structures. The device was gently put on the cell-cultured deatachable substrates and cramped. And medium flow was controlled using a syringe pump connected to the microchannel.

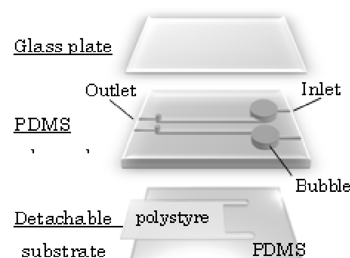

Fig.1 Schematics of the components of the detachable substrate with microfluidic device.

Results and Discussion

Microfluidic device could be integrated with the cell-cultured detachable substrates without peeling the cells from the substrates. Fig.2 shows diffusional simulation of soluble factor secreted from cells at various flow rates, indicating that the diffusion region would be controlled. We found that the shear stress ($<\tau = 0.05$) at these flow rate does not affect the migration behavior of HUVECs. We are now conducting the coculture under controlled flow conditions.

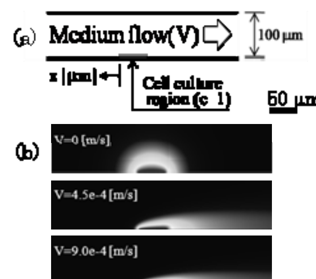

Fig.2 (a) A schematic of diffusion and laminar flow model using the simulation. (b) images of diffusion and laminar flow simulation at various flow rates.

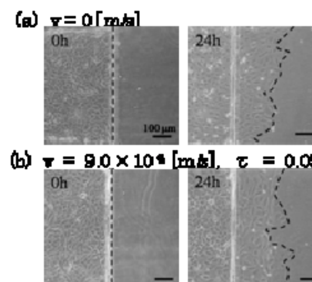

Fig.3 Micrographs of HUVECs cultured for twenty-hours on detachable substrates with microfluidic device at (a) 0 m s^{-1} flow rate, and at (b) $9.0x10^{-4}$ m s^{-1} flow rate

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P-24

The international validation study for the ER alpha STTA Antagonist Assay using HeLa9930

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Objective(s)

The new *in vitro* OECD test guideline for "the Stably Transfected Human Estrogen Receptor Transcriptional Activation Assay (STTA) for Detection of Estrogenic Agonist-Activity of Chemicals[1)]" using humanERalpha-HeLa-9903 (HeLa9903) Cell Line was approved at the 21th OECD Working Group of National Coordinators meeting. Comments received from the Peer Review Panel (PRP) [2)] included: 'The STTA assay can at this point only be used for estrogen agonist testing and further studies would be needed if also estrogen antagonists could be tested.' The STTA for detecting anti-estrogenic activities is a rapid *in vitro* screening method for identifying compounds which have the potential to inhibit the estrogenic response of the endogenous ligand, 17beta-Estradiol. Therefore, to develop a reproducible assay protocol suitable for international regulatory use, the STTA antagonist validation study was initiated and is coordinated by JaCVAM, with membership of the management including representation of ECVAM, EFSA and the US EPA.

Materials and Methods

The protocol of the antagonist assay was designed to ensure the reliability and sensitivity of the assay by including testing of "Control chemicals" at a defined concentration in each assay plate and testing "Reference chemicals" once each day the assay is run.

Five participating laboratories are following three tasks, [Task-1]: Set up of the test system including testing of edge effects on the 96-well plate and demonstration of the basic skills of the participating laboratory by testing the reference chemicals (17beta-Estradiol, 17alpha-Estradiol, Corticosterone) in the agonist "estrogenic" assay according to the draft test guideline; [Task-2]: Testing of un-coded reference chemicals was conducted based on the provisional performance standard for antagonist assay using the antagonist "anti-estrogenic" assay protocol; [Task-3]: Coded chemicals will be tested by the laboratories to demonstrate proficiency with the antagonist assay in Task-2, to evaluate the intra- and inter-laboratory reproducibility.

Results and Discussion

Results of Task1 of the all participant labs indicated that there is no edge effects and their proficiency for the agonist assay with the results which met the assay criteria with repeat testing of reference chemicals. For Task 2, three labs successfully conducted the antagonist assay and provided reproducible results. However, loss of responsiveness of the HeLa9903 cell was suggested from the results of two other labs. The performance standards for antagonist assay have been revised based on the Task-2 results of three labs. The updated performance criteria will be further evaluated in Task 3 which is currently on-going.

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P-25

Construction of a three-dimensional heterogeneous micro-tissue toward the evaluation of a minimum required size

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Objectives

Micro-tissues, which exhibit physiologically-relevant responses identical to those of their corresponding tissues/organs, should be minimized to miniaturize cell-based devices. Previously, we constructed different sizes of a two-dimensional monolayered micro-tissue of liver cells and examined its minimum required size. Liver specific functions per cell dramatically increased in the two-dimensional (3D) micro-tissues of 200-630 μm in diameter or larger. In contrast, it is known that three-dimensional organization of liver cells or and co-culture with fibroblasts further enhance the activity of liver cells due to homogenic or heterogenic cell-to-cell interactions. We therefore constructed different sizes of a three-dimensional heterogeneous micro-tissue using micro-grooves toward the evaluation of the minimum required size.

Materials and Methods

To control the size of the 3D heterogeneous micro-tissue, we fabricated a polydimethylsiloxane (PDMS) sheet with different sizes of micro-grooves (63, 200, 630, and 2000 μm in diameters and 20 μm in depth) using the photolithography techniques. The top surface of the sheet was coated with MPC polymer, which is a non-adhesive material, followed by local adsorption of collagen only to the micro-groove surface. Human hepatocarcinoma cell line, Hep G2 cells were seeded on the surface-treated PDMS sheet at a high density of 2.0×10^5 cells cm^{-2} and were cultured for 24 h, followed by seeding of mouse fibroblasts NIH-3T3 cells (2.0×10^5 cells cm^{-2}). The cell morphology was evaluated by optical microscopy.

Results and Discussion

Hep G2 or NIH-3T3 cells were seeded on the surface-treated PDMS sheet having groove-like structures, resulting in adhesion of cells only within all sizes of micro-grooves and formation of two-dimensional homogeneous micro-tissues in them. Next, Hep G2 cells were seeded, followed by seeding of NIH-3T3 cells on the sheet. Both cells similarly adhered and proliferated in all sizes of the micro-grooves. However, in the case of such co-culture, the cells hemispherically formed 3D heterogeneous micro-tissues in all sizes of the micro-grooves. This result indicates that the interaction between Hep G2 and NIH-3T3 cells is higher than that between the cells and the sheet surface. As such, using such a micro-groove structure, 3D heterogeneous micro-tissues can successfully be formed and stably cultured even in the smallest groove whose diameter is 63 μm. Therefore, the proposed micro-groove-based patterned surfaces are sufficient to allow biochemical evaluation of the minimum required size.

Currently, we are examining the internal structure of all sizes of the 3D heterogeneous micro-tissues from fluorescence microscope images after staining of Hep G2 and NIH-3T3 with different fluorescence dyes, respectively. In addition, we also examine intracellular activities of cytochrome P450 1A1/2 (involved in hepatic detoxification) as an index for liver specific function of each 3D micro-tissue using the EROD assay.

Acknowledgment

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P-26

A combined test skin irritation evaluation: Monolayer cell, human skin model tests and human patch test

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Objective(s)

Many cosmetics, including foreign products, have to be tested before be allowed into Chinese market. Till now, we have been conducting rabbit tests to evaluate skin irritation for safety vigilance. However, we have recently evaluated cosmetics for skin irritation using a monolayer cell test, where upon detection of irritation, test-samples were subsequently subjected to the rabbit test and human patch test for reevaluation. Although the monolayer cell test method drastically reduces animal use (35th Japan Toxicological Sciences Annual Meeting), it is not a reliable alternative for dermal irritation test. Consequently, we examined a combined evaluation system comprising the monolayer cell test, human skin models test and the human patch test.

Materials and Methods

Mouse (BALB/3T3) fetal fibroblasts incubated in 96-well plates were exposed to test-samples diluted with PBS (-) for 5 min to derive the LC50. Test-samples with LC50 <10% were defined as suspicious irritants, which were then confirmed with human skin models. The pre-incubated human skin models were exposed to test-samples for 15 min, and cell viability (CV) was assayed at post-incubation 42 h; test-samples yielding CV ≥ 50% were defined as non-irritants, otherwise were irritants. As a final confirmation, the 24 h human patch was required.

Results and Discussion

Test-samples yielding LC50 ≥ 10% shouldn't induce irritation in the human patch test. Test-samples with LC50 <10% and those yielding CV ≥ 50% would indicate no irritation in the human patch test. This combined evaluation system manifested a concordance rate of 92% with the human patch test. In evaluating skin irritation of cosmetics without animal use, the present battery evaluation may serve a replacement assay for 3Rs.

P-27

Evaluating system using cells derived from ES/iPS cell - Movie image analysis of beating cardiomyocyte-

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Objective(s)

The report of mouse iPS cell and human iPS cell development by Yamanaka offers a large possibility to widen variety fields such as pharmaceutical products, cosmetics as well as a medical field. We investigated methods using the cardiomyocytes derived from ES/iPS cell for evaluating system.

In this report, particularly, a beating image analysis method was examined using the beating cardiomyocytes derived from ES cells as an evaluating system. Furthermore, if the cardiomyocyte have a long beating period, it can be used many time for evaluation repeatedly. Therefore, the time to differentiate to cardiomyocyes and beating periods were examined.

Materials and Methods

Mouse ES cells (EB3-Caa-eGFP-IP) were cultured in the absence of feeder cells. 750 cells in droplets of 15 ul were seeded into the lid of a culture dish and grown for 2days in a "hanging drop" culture to produce EBs. After that, the EBs were transferred to petri dishes. On day 5 EBs were seeded singly in one well of a 24-well culture plate. Differentiation to beating cardiomyocyte was observed by a stereoscopic microscopy.

Beating of cardiomyocytes in 24well plate werer recorded with a digital video camera.

A recorded images of beating cardioyomyocytes were analyzed with NIH image J.

Furthermore, the duration between the commencement of beating and the decease of beating were recorded in some cells. The results were compared with previous reports.

Results and Discussion

The recording of the beating cardiomyocyte in 24well plate with a video camera was always successful. However, the recording was often failed when the EBs were not centrally located in wells.

The EB cells which transfered into 6cm dish at hanging drop 2day showed 100% rate of beating, and the beating persisted for more than 20 day.

The image analysis of beating cardiomyocyte was always successful.

The main tools used in this report were a digital video camera, NIH image J (free software), and a stereoscopic microscope. These are easily purchased.

Therefore, the evaluation system developed by us can be used for various kinds of evaluation systems, and it is thought that the new evaluation system is very convenient.

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P-28

Evaluating system using cells derived from ES/iPS cell - Microscopic observations of the differentiated multilayered cardiomyocytes -

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Objective(s)

The basic and applied studies of stem cells are watched by scientists all over the world with intrests. They also apply the evaluating system using stem cells for the efficacy of drugs and the biological safety of medical devices. The traditional evaluating methods depend on the evaluation of cell reactions on the affected live tissues. The histological evaluation of cardiomyocytes derived from stem cells are not exceptions. We thought that the evaluating methods using stem cells could be alternative to the traditional methods.

This study was aimed to develop the evaluating system for the efficacy of drugs and the biological safety of cosmetics and medical devices. The objective of this study was to develop the histological evaluating methods with differentiated multilayered cardiomyocytes.

Materials and Methods

Mouse ES cells (EB3-Caa-eGFP-IP) were cultured in the absence of feeder cells. Seven hundred and fifty cells in droplets of 15 ul are seeded into the lid of a culture dish and grown for 3days in a “hanging drop” culture to be EBs. Then, the EBs were transferred to petri dishes for another 2days. On day 5, EBs were singly seeded into *UpCell* (*CellSeed*) dishes and one well of a 24-well tissue culture plate to allow the development of contracting myocard. The cell differentiation was assessed by microscopic observation of the EBs on day 15, and the contracting cells were recorded and analyzed. The expression of marker genes of ES cells and EBs were analyzed with RT-PCR.

On day 15, contracting EBs which were cultured on *UpCell* were detached with *CellShifter* according to the manufacturer’s instructions. They were directly fixed in 10% neutral buffered formalin and embedded in paraffin. The serial sections were examined with HE and fluorescent immunohistochemistry.

Results and Discussion

Day 10 EBs showed 100% contracting rates and they kept the contraction until day 15. We examined the expression of marker genes of ES cells and EBs with RT-PCR.

We confirmed the contraction of EBs on day 15, which were cultured on *UpCell,* with recorded movie analysis. We observed multilayered day 15 EBs in serial sections stained with HE. We detected troponin positive signals on EBs in fluorescent immunohistochemistry.

We could show that the multilayered contracting cardiomyocytes from mouse ES cells could be observed with detaching and directly fixing methods without the usage of living body. One of the general methods of the observation of the cell sheet was the transplantation to the live heart and the fixation transplanted cells with the heart. We could observe and analyse the relation between the layered cells with this new methods. We need to examine whether the traditional histological methods can be applied to the sections with this method in future.

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P-29

Evaluating system using cells derived from ES/iPS cell - Inspection of cardiomyocyte toxicity by chemicals -

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Objective(s)

Many animals have been conventionally used for safety evaluation. Recently, a safety test method using cells was developed. In Europe, the Embryonic Stem Call Test (EST) method which was examined the embryonic stem cell toxicity with ES cell was developed in 1993. And today, it became one of the indexes of an animal experiment alternative method. This is the method to examine of embryotoxicity. However, it does not examine the effect on cardiomyocytes. We developed an appraisal method for a pulsatile function of the palmic cardiomyocyte which differentiate from mice ES cell. In this paper, we examined the influence of chemicals with our evaluation system.

Materials and Methods

Mouse ES cells (EB3-Caa-eGFP-IP) were cultured in cell culture dishes without feeder cells in GMEM supplemented with FBS, NEAA, Sodiumu pyruvate, 2-Mercaptoethanol and LIF. Cells were differentiated as embryoid bodies (EBs) by the hanging drop method. EBs were transferred at day 2 from the hanging drop into a petri dishes. On day 5, one EB per well was plated into 24-well culture plates. Differentiation into cardiac cells was investigated to contain one or more beating areas by using a microscope. Caffeine, Dexamethasone, Digoxin, Diphenylhydantoin, 5-FU and Saccharin were diluted in medium with a final concentration of 1% DMSO. They were applied on the palmic cardiomyocyte for 10 minutes. Each palmic cardiomyocyte was recorded at before application, after application, 10 minutes later and after wash. Their picture images were analyzed by NIH Image J.

Results and Discussion

EBs showed 100% beating rate in day 10, and continued beating. In Saccharin, the beating was not influenced by the concentration tested. On the other, the beating was clearly inhibited by highly concentrated applications of Dexamethasone, Digoxin, Diphenylhydantoin and 5-FU. These results did not contradict to a report by a method with different exposure time.

The evaluation method developed in this study was focused on a pulsatile function of the palmic cardiomyocyte. We think that the method developed is capable in pharmacometrics of a variety of chemicals such as the cardiotonic drug. Currently, we are improving the method to be simpler and easier and trying the development of evaluation system by the human iPS cell. We would like to advocate that this evaluation method would be a new test method that evaluates medical supplies and chemicals by pulsatile function of cardiomyocytes.

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P-30

Evaluating system using cells derived from ES/iPS cell -Application to a screening method of crude drug constituting a Chinese medical prescription-

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Objective(s)

Many Chinese medical prescriptions consist of plural crude drugs. Each constitutional crude drugs has a subtle difference each other for efficacy due to various kinds of factors such as locality. Therefore efficacy stabilization of constitutional crude drug is necessary for efficacy stabilization of a Chinese medical prescription. An animal experiment and a culture cell have been used for pharmacometrics of Chinese crude drugs. Our quality screening method of crude drug has changed from methods using an animal (mouse) to methods using cell culture such as spleen cells. The development of iPS cells by Yamanaka offers a large possibility in various kinds of fields. One of the expected field is application to a screening method of Chinese crude drug. We investigated the methods using movie analysis of beating cells derived from ES/iPS cell for quality screening of Chinese crude drug. The obtained results were weighed against results of quality evaluation by the culture cell system which was already reported. And a possibility to use an ES/iPS cell for quality screening of Chinese crude drug was examined.

Materials and Methods

Mouse ES cells (EB3-Caa-eGFP-IP) were cultured in the absence of feeder cells. Seven hundred and fifty cells in droplets of 15 ul were seeded into the lid of a culture dish and grown for 2days in a “hanging drop” culture to produce EBs. After that, the EBs were transferred to petri dishes. On day 5, EBs were seeded singly one well of a 24-well culture plate. Differentiation to beating cardioyomyocytes was observed by a stereoscopic microscopy. Several kinds of Aconiti Tuber which varied in locality were evaluated. Each Aconiti Tuber was diluted to five phases by 10 times and added to the wells in which beating cardiomyocytes were observed for 10 minutes. The beating of cardioyomyocytes were recorded with a video camera, just before application of testing drugs, just after application, 10 minutes later and after washout 10 minutes later. The recorded movie images of beating cardioyomyocytes were analyzed with NIH image J.

Results and Discussion

Many of Aconiti Tuber promoted the beat of cardioyomyocytes, but there were Aconiti Tubers which did not have any influence on the beat. These results compared to the previous results obtained using cell cultures. The results of these two methods were almost identical. Therefore, it was thought that the movie image analysis method developed here was thought to be useful for screening of Chinese crud drugs. In addition, the main tools used for this analysis were a stereoscopic microscope, a video camera and NIH-Image J (free software). Because these tools are purchased very easily, thus these method can be applied as an evaluation system of chemical compounds in various fields, and it is thought that this method is extremely useful.

P-31

Analysis of marker genes for prediction of embryotoxicity in neural differentiation using mouse embryonic stem cells

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Objective(s)

Embryonic stem cell test (EST) is one of the most useful tests for developmental toxicity using mouse embryonic stem cells. However, it is said that the EST has to be improved because other major target tissues such as nervous system and skeletal tissues have to be included in order to get precious information about the embryotoxic potential of chemicals. Here we report investigation of novel gene makers for evaluation of embryotoxicity in neural differentiation using mouse ES cells.

Materials and Methods

Analysis of gene expression of ES cells during differentiation into neural cells

Differentiation of mouse ES cells into neuron was carried out under serum-free condition. The differentiated cells were collected periodically for 10 days and total RNA was isolated from the cells. To isolate the candidate marker genes involved in differentiation into neural cells, global changes in gene expression were measured by microarray analysis using the isolated RNA.

Treatment with test compounds

The cytotoxic effects of twelve test compounds on ES cells were determined by measuring the inhibition of undifferentiated ES cell proliferation. The mouse ES cells were differentiated into neural cells in media containing the appropriate concentration of six embryonic compounds, six non-compounds or vehicle, and the differentiated cells were collected periodically.

Gene expression analysis of the candidate genes

Total RNA was isolated from the cells treated with the test compounds and cDNA was synthesized. Gene expression of the candidate marker genes was analyzed by quantitative real time PCR.

Results and Discussion

In the DNA microarray analysis of ES cells during differentiation into neural cells, genes which were substantially up-regulated during the differentiation process were isolated as candidate marker genes. Approximately 83 genes were determined as the genes for neural cells differentiation in this study. Gene expression profile of the genes was confirmed by quantitative real time PCR analysis. Expression of 22 genes was noted remarkably by comparison of gene expression between embryotoxicants and non-embryotoxicants-treated groups suggesting that these genes were considered to be useful markers for predicting embryotoxicity. This study was supported by a research grant from the New Energy and Industrial Technology Development Organization (NEDO) in Japan.

P-32

Development of in vitro alternative method for developmental toxicity using mouse embryonic stem cells

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Objective(s)

Almost all developmental toxicity tests are currently conducted as in vivo animal tests in accordance with some test guidelines. To reduce the in vivo tests, several in vitro tests have recently been developed and validated. Embryonic stem cell test (EST) is one of the most useful validated in vitro tests and employs mouse embryonic stem cells. The endpoint of EST is ability of ES cell differentiation into cardiomyocytes by measuring alteration in beating of the differentiated cells following drug exposure. However, it is said that the EST has to be improved because the measurement was difficult to standardize and could only be carried out by experienced personnel.

To establish convenient and accurate in vitro short-term assays, we reported the identification of putative marker gene related to embryotoxic potential, using mouse embryonic stem cells in last annual meeting. Here, we report the establishment of stable transgenic ES cells to detect the chemical dependent changes in the 3 candidate genes using the reporter-gene easily and conveniently.

Materials and Methods

The reporter plasmids were generated by insertion of the promoter region of Hand1, Smyd1 and Cmya1 gene up-stream of the firefly luciferase reporter-gene, and the plasmids were transfected into ES cells to develop stable transformant cells. After these ES cells were differentiated into cardiomyocyte, the expressions of marker gene were analyzed by real-time PCR method, and luciferase activities were measured periodically in differentiated cells.

To establish appropriate basic operating protocol for in vitro short-term assay for prediction of embryotoxicity, we optimized the assay method about various conditions. Then, we evaluated several embryotoxic and non-embryotoxic chemicals using our transgenic ES cells following the basic protocol.

Results and Discussion

We established three stable transgenic ES cells with the luciferase reporter gene to detect gene expression of the Hand1, Smyd1 and Cmya1 genes easily and conveniently. These cells were named as Hand1-ES, Smyd1-ES and Cmya1-ES, respectively. During differentiation into cardiomyocyte of the transgenic ES cells, changes in marker gene expression were coincident with those in luciferase activities, and the luciferase activities in the Hand1-ES, Smyd1-ES and Cmya1-ES elevated from day 6, 8 and 6, respectively. We developed basic protocols for reporter gene assays using these transgenic ES cells, and preliminary studies are conducted using some standard compounds in order to clarify efficacy of our proposed tests.

This study was supported by a research grant from the New Energy and Industrial Technology Development Organization (NEDO) in Japan.

P-33

Investigation of an Eye Irritation Test Using a Human 3D Corneal Model

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Objective

In general, cosmetic products are generally not used directly in the eyes, some are used around the eyes or enter the eyes by improper use. It is therefore necessary to assess irritation when such cosmetic products or cosmetic integrants enter the eyes.

Until now, rabbit Draize tests have been conducted generally; however, *in vitro* eye irritation assessment methods using cultured cells are recently being discussed as an alternative, from the perspective of animal protection. Such assessment methods have the problem that direct exposure to samples is not possible. In this study, we examined an eye irritation test using a human 3D corneal model to address this problem.

Method

We tested the viability of the model for 34 samples of chemical substances and cosmetic integrants with known eye irritation scores by the rabbit Draize test, using a 3D corneal model (Japan Tissue Engineering Co., Ltd.) prepared from human corneal epithelial cells, according to the method of Arai et al.[1)] and Ogasawara et al.[2,3)] (sample exposure time, 1 min; post-incubate time, 24 h; irritation, cell viability rate of 70% or less).

Furthermore, the inflammatory cytokine IL-1α in the culture was analyzed by ELISA and examined as part of the *in vitro* eye irritation assessment method together with the viability of the model described above.

Results and discussion

The results indicated a high correlation of 86.2% to the irritation classification based on the result of the rabbit Draize test. In particular, the alcohol that had difficulty with evaluation, it was enabled in this evaluation method. Moreover, this method is considered to be more useful than traditional alternatives, because it can apply to it in test substance itself directly.

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Human corneal tissues used in this study were provided by the NPO HAB Research Organization, after approval of the ethics committee.

P-34

Training in anatomy using Japanese flying squid for college students majoring in nutrition and food –To increase the learning effects of clinical training in rat anatomy-

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Objective(s)

In colleges specialized in food and nutrition, designed to train dietitians, "training/experiments in anatomical physiology" courses provide clinical training in rat anatomy to help students improve their knowledge of the structure and function of organisms. In recent years, however, decreasing numbers of elementary, junior high, and high schools provide training in anatomy, and, as a result, most students enter college without any experience of performing a dissection. In this context, the Faculty of Food and Nutrition, Kyushu Nutrition Welfare University, has introduced a new approach to its training in anatomical physiology to increase the learning effects of clinical training in rat anatomy. As it is important for students to become familiar with the anatomical structure of organisms, food ingredients, such as chicken wings and eggs as well as squid, are used in training sessions. We then provide education on animal welfare and other ethical issues prior to clinical training in rat anatomy. This paper reports training in squid anatomy provided on this course, and the results of questionnaire survey showing students' responses to the training session.

Materials and Methods

Two lessons (90 minutes x 2) were used for the training session. Japanese flying squid (one of every three persons) and checklists including the items for observation were distributed to students. They: 1) observed the outer surface, 2) cut and opened the mantle to examine the internal organs, and 3) removed the organs from the mantle to examine the digestive system. Kyushu Nutrition Welfare University provides "Training in Anatomical Physiology" in the second term of the first year, and "Experiments in Anatomical Physiology" in the first term of the second year. We conducted a questionnaire survey involving about 100 students at the end of training in squid anatomy in the second term of the first year, and on completion of the one-year training/experiments course.

Results and Discussion

Fourteen types of clinical training and experiment were conducted over one year of the Training/Experiments in Anatomical Physiology course. A large number of students became "very interested" or "interested" in "training in squid anatomy". Squid had the following merits as a training material: 1) the students did not have an aversion to touch them because they were a common food ingredient; 2) they showed many structural similarities to humans, including muscular, respiratory, cardiovascular, urinary, digestive, nervous, sensory, and genital systems; 3) their physical structure and mechanisms were complex, which stimulated students' interest and increased their motivation for learning. The students were impressed with and became interested in the anatomical structure of the squid which had been a very familiar creature but not a subject of interest for them. We believe that Japanese flying squid are an appropriate training material to help students majoring in food and nutrition achieve a better understanding of the structure and function of organisms, and increase their interest in clinical training in rat anatomy scheduled in a higher academic year as well as their motivation for learning.

P-35
Expansion of Short Time Exposure (STE) Test
:Solvent Study

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Objective(s)
Short Time Exposure (STE) Test is an in vitro eye irritation test that assesses cytotoxicity in SIRC (rabbit corneal cell line) cells following a single 5-min treatment. Recent validation study supported by Japanese Society for Alternative to Animal Experiments showed good result. Three solvents can be used in this study. For better assessment of insoluble substances, applying other solvents should be effective. Thus, we selected several solvents including cosmetic bases and investigated the application of this test method.

Materials and Methods
*Materials
Validation study chemicals (25 chemicals), GHS Category 2 substances (19 chemicals)
*Methods
We selected the solvent based on cell viability, solubility of substances (products and chemicals), and operativity. We diluted test materials to 5% and 0.05% in the new solvent and treated SIRC cells for 5 min. Then cell viability (%) was calculated, and test materials were classified as irritants or non-irritants depending on cell viability. Test substances were classified as mentioned below;
MAS ≥ 15 on Draize test or cell viability ≤ 70% by the STE method : irritants
MAS < 15 on Draize test or cell viability > 70% by the STE method : non-irritants
Furthermore, we compared STE rank classification using the STE prediction model for GHS eye irritation categories.

Results and Discussion
We selected 'SALINE including 30% DMSO' as the test solvent (selected from PEG-400, BUTYLENE GLYCOL, CETHYL ETHYLHEXANONATE and SALINE including 10-40% DMSO). 30% DMSO is suitable for STE test because of the low cytotoxicity in short contact time.
Eye irritation STE class showed good correspondence with eye irritation class based on Draize test data. Sensitivity, specificity and accuracy also showed good correspondence between STE rank classification and Draize eye rank classification. Thus, 30% DMSO is useful as a solvent in STE test.

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P-36

The influence of culture days of the reconstructed epidermis model (EPISKIN™) to the result of the skin irritation test with EPISKIN™

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Objective(s)

EPISKIN™ (SkinEthic, France) is the reconstructed human epidermis model. The skin irritation test used the skin model (EPISKIN™ test) is approved in EU as the official prediction test for skin irritation. However, since this model is manufactured in France, it is difficult to carry out the EPISKIN™ test in Japan as a same condition in EU. The purpose of this study is to validate whether it is able to get same judgment as in EU by using EPISKIN™ transported to Japan. To accomplish this purpose, we confirmed the influence of date of EPISKIN™ after manufacturing. In our testing, it was found out that 10 chemicals tested in Japan were categorized in the same in the results tested in EU. The results indicated that EPISKIN™ test is useful to predict potentials of chemicals on skin irritation in Japan.

Materials and Methods

The skin irritation test was carried out following the standard operation procedure (SOP)[1)] of ECVAM (the European Centre for the Validation of Alternative Methods). The skin irritation was judged following the skin irritation criteria of ECVAM SOP (R38) which are based on both results of a cell survival rate and IL -1 α quantitative value. In the study, we selected chemicals, which were used in the EU validation, as testing samples. The testing protocol was briefly described as follows. After 24 hours cultivation of the skin model (EPISKIN™) with the maintenance medium, a chemical was treated on the skin model for 15 minutes. After washing out a chemical on the skin model, the skin model was cultured with the fresh maintenance medium for 42 hours. Cell survival rate was evaluated by MTT assay. IL -1 α in the 42 hours cultured medium of chemical treated skin model was quantified by the commercially available kit (R & D systems).

Results and Discussion

There is a concern that the extension of culture days during the EPISKIN™ transportation from France to Japan influences the skin irritation judgment. In general, the skin model is built up more tight structure in stratum corneum (SC) depending on a culture period. It is predictable that the structure of SC influences a potential judgment of a sample on skin irritation by alteration of sample permeability into the living cell layer. Thus, there is a possibility that the skin irritation of the chemicals might underestimate. Therefore, in order to clarify influence of differences in SC conditions on the judgment, we carried out the skin irritation test with two kinds of skin models (10 days cultured model (D10 model) and 13 days cultured model (D13 model)). As a result, it was found that judgments of each test chemical with D10 model and D13 model showed completely same results. Then, to expand the validity on judgment of skin irritation in Japan, we carried out further examinations with D10 model using 10 chemicals including the false-negative material (short cultured model). The classification of all chemicals in Japan showed well consistency with results conducted in the EU. From these test results, it was identified that the evaluation with EPISKIN™ for the chemicals was not influenced by the culture days of skin models, and EPISKIN™ method for evaluation of the skin irritation should be available in Japan.

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A Modified Short-Time Exposure (mSTE) Test for Cosmetics: an Alternative to the Draize Eye Irritation Test

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Objective(s)

As economic development of the Chinese market expands, the number of imported cosmetics increases in China. Shanghai is traditionally a strategic port, where cosmetic products from the European Union, United States of America, Japan and other Asian countries are imported into China. Therefore, the Shanghai Entry-Exit Inspection and Quarantine Bureau of China periodically executes sampling inspections of cosmetics for adequate quality control and product regulation.

In an attempt to address recent issues in animal welfare, we innovated an alternative for the Draize eye irritation test, which uses rabbits. The short-time exposure (STE) test is a simple in vitro eye irritation test using a rabbit corneal cell-line (SIRC).

Materials and Methods

We evaluated 19 chemicals, 12 alcohols and ketones using the STE method with modification (mSTE). The results were compared with findings of the Draize test. The sample-concentrations in the mSTE test were 5, 0.5 and 0.05%, while that in the in vivo test was 100%. The results revealed that almost all the false negatives were derived from alkyl alcohols/ketones. To avoid these false negatives, we combined the mSTE with the HET-CAM(the mSTE+HET-CAM).

Results and Discussion

The mSTE test results of the 19 chemicals at concentrations of 5, 0.5 and 0.05% registered sensitivity rates of 75.0, 37.5 and 37.5% with specificity rates of 72.7, 81.8 and 100%, respectively. As for false negatives of the 19 chemicals and 12 alcohols/ketones at the corresponding concentrations, the mSTE+HET-CAM indicated a homogenous sensitivity rate of 100% with specificity rates of 60.0, 66.7 and 80.0%, respectively. When the 23 cosmetics were assessed at corresponding concentrations, the mSTE test indicated sensitivity rates of 92.3, 92.3 and 61.5% with specificity rates of 50.0, 80.0 and 80.0%, respectively. The results of the 31 chemicals (19 chemicals and 12 alcohols/ketones) showed reliable sensitivity and specificity levels at 0.05% concentration, while those of the 23 cosmetics manifested reliable sensitivity and specificity levels at 0.5% concentration. These findings indicate that the mSTE+HET-CAM may serve as a reliable safety assessment assay for eye irritancy of chemicals, and the mSTE may be adequate for assessing eye irritancy of cosmetics.

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International Validation study of the in Vitro Alkaline Comet Assay

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The Comet assay is widely used for detecting initial DNA damage in individual cells. The *in vitro* alkaline Comet assay, especially, might serve as a simple and sensitive test for identifying the genotoxic hazard of chemicals. The performance of this assay, however, depends on the procedures used and published results for the same chemical greatly vary. To develop a standardized protocol for the *in vitro* alkaline Comet assay and to evaluate assay reliability and relevance, JaCVAM is supporting an international validation study. In this study, TK6 human lymphoblastoid cells are treated with a chemical for 4 h with or without metabolic activation (i.e., S9 mix). As cytotoxicity parameters, trypan blue dye exclusion (TBDE), non-detectable cell nuclei (NDCN; hedgehog), and cell growth after the treatment are measured. The recommended top concentration is one with 80% TBDE, 20% NDCN, or no cell growth. To date, 5 laboratories have examined 11 genotoxic or non-genotoxic chemicals using a standardized protocol. In the absence of S9, appropriate positive responses for ethylmethanesulfonate, 9-aminoacridine, camptothecin, and etoposide were obtained, while a significant increase in DNA migration was not detected for mitomycin C, a cross-linking agent. Also, the non-genotoxic chemicals (cycloheximide, triton-X, mannitol) were appropriately negative. However, the results from S9 studies with genotoxic chemicals requiring metabolic activation (2-aminoanthracene, cyclophosphamide, diethylnitrosamine) were inconsistent, indicating that additional optimization of the protocol is needed. The results from the completed phases and the overall design of the validation study are presented. According to the validation study, we would like to propose a standard protocol, criteria for data acceptance, and a statistical method to judge the results in the *in vitro* Comet assay for regulatory use.

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Whole embryo culture with minivial on rat embryo of day 9.5 .

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The conceptuses were transferred into 25ml-bottles or 15ml-bottles containing immediatery centrifuged and heat-inactivated male rat serum (5ml or 2ml). Then, the bottles were rotated at 20 rev. / min in an incubator at 38℃. At the end of the culture period, the conceptuses were transferred to Tyrode solution and examined under a stereoscopic microscope. The degree of differentiation and development of each conceptus was evaluated by a morphological scoring system. Rat embryos of day 9.5 of gestation were cultured for 48 hours. The embryo toxic effects were not observed on cultured rat embryos.

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In vitro phototoxicity assessment of tattoo pigments using 3T3 fibroblast and reconstructed human skin model

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Objective(s)
Phototoxicity is an acute toxic response induced by skin irritation after the systemic or local administration of a chemical and subsequent exposure to light. In vitro 3T3 NRU PT is widely used to predict phototoxicity of chemicals. However, this method has several limitations such as chemical solubility and application pathway. So, it is necessary to adequate for phototoxicity assessment with accurancy. In this study, we performed in vitro phototoxicity test using 3T3 fibroblast cell lines and reconstructed human skin model (H3D) with chemical of low solubility, and compared with results between two tests.

Materials and Methods
In vitro 3T3 neutral red uptake phototoxicity test The test was conducted according to OECD TG 432[1] using 3T3 Balb/c fibroblast. Phototoxicity was identified by the photoirritation factor (PIF) and the mean photo effect (MPE) values. PIF was calculated at the ratio of cytotoxicity for test chemical with and without UV light[2]. Also, MPE was calculated based on comparison of the complete concentration response curve[3].
EpiDerm^{TM} phototoxicity test The test was performed according to Liebsch et al.[4]. EpiDerm^{TM} Epi-200 tissues were exposed to the test chemicals for 24 h. One set of tissues was irradiated with 6 J/cm^2 UVA. Phototoxic chemicals were predicted to have decrease viability exceeding 30% in +UVA part compared with identical concentration of the –UVA part.

Results and Discussion
We performed in vitro 3T3 NRU PT with 7 tattoo pigment following to OECD TG 432. As a result, we identified the 5 tattoo pigments (cadium selenide, mecury(Ⅱ) sulfide, chromium oxide, cobalt aluminate) were non-phototoxic chemicals and 2 tattoo pigments (cadium sulfide and carbazole) were phototoxic chemicals. Although in vitro 3T3 NRU PT were well-defined, it is difficult to predict phototoxicity of low soluble or insoluble and topical application chemicals. So, phototoxicity test using H3D is suggest as an adequate method to overcome limitation of in vitro 3T3 NRU PT. Tattoo pigments have low solubility. Thus, we conducted phototoxicity test using H3D. The results of H3D PT were identical with those of in vitro 3T3 NRU PT except one chemical, cadium sulfide. Contrast with in vitro 3T3 NRU PT, cadium sulfide was predicted as non-phototoxic chemical in phototoxicity test using H3D.

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Integration of Micronucleus Assay into General Toxicity Testing for 3Rs

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Objective(s)

In general, *in vivo* rodent micronucleus(MN) test is widely used in regulatory safety assessment to evaluate the potential for induction of chromosomal damage. Recent data suggest that MN studies using peripheral blood in the rat can substitute for bone marrow-based MN test. Based on this evidence, we tried the study on integration of genotoxic endpoints into repeated dose 13-week toxicity test to avoid duplicating animal use.

Materials and Methods

First of all, we established the method for the detection of MN-reticulocytes using flow cytometry in rats treated with four genotoxicants(cyclophosphamide, EMS, vinblastine and cisplatin) and three nongenotoxic substances(clofibrate, ethionine, dioxane). And then we measured MN-reticulocyte frequency in periperal blood of F344 rats which were treated with four kinds of test substances for 3 days. In addition, the results of MN-reticulocytes(%) by FCM analysis were compared to the data of microscopy-based scoring of MN in mouse bone marrow treated with same test compounds.

Results and Discussion

Flow cytometry analyses of micronuclei based on anti-CD71 staining of immature reticulocytes can distinguish genotoxicity from nongenotoxicity. Furthermore, flow cytometry analysis of micronucleated reticulocytes in the peripheral blood integrated into general toxicity testing has several advantages such as less labor-intensive work, high-throughput analysis, more efficiency and animal welfare etc. There is no positive control experiment in general toxicity testing, the accumulated historical background data in regard to MN are required for the evaluation and interpretation of results with accuracy.

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Reduced numbers of fish used in acute toxicity testing

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Objective(s)

To find out if the threshold test can be used to determine acute toxicity test rather than a full toxicity study (OECD 203). This would reduce the number of fish used for testing.

Materials and Methods

The study was a retrospective (theoretical) investigation based on Hutchinson *et al.* (2003) with modifications. The threshold test is based on the experience that *Daphnia* and algae are more sensitive than fish in many cases. Five fish are exposed to the lowest EC50 value from testing with algae and *Daphnia* in a limit test: if no toxicity (≤1 death) is observed, no further testing is needed. In this case, risk assessment and classification and labeling can be based on the lowest EC50 value from testing with algae and *Daphnia*. If toxicity is observed (>1 fish dies), the test concentration is lowered and another limit test is performed. This is repeated until no toxicity (≤1 death) is observed. The retrospective (blinded) investigation used data on 507 chemicals tested at NOTOX. The number of chemicals for which fish were not the most sensitive taxonomic group was determined, and the number of fish that would have been needed using the threshold test was calculated. This was compared to the number of fish needed in full toxicity tests (OECD 203).

Results and Discussion

The number of fish needed using the threshold approach was calculated to be much lower than that using the full toxicity study. For agrochemicals, industrial chemicals, pharmaceuticals and chemicals of unknown origin, the number of fish needed for testing could be reduced by 85, 89, 89 and 86%, respectively, using the threshold test instead of the traditional OECD 203 acute toxicity test.
The OECD 203 test guideline is currently being updated. The threshold approach is recommended, but the OECD proposes one deviation from the approach used in our study: if ≥1 fish dies, a full study should be used.

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Real-time evaluation of hemocompatible materials by substandard human blood for transplantation by blood bank

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Introduction

To establish alternative methods to animal testing and experimentation for hemocompatible materials, we had been developed an apparatus consisting of a modified cone and plate-type rheometer combined with an upright epi-fluorescence microscope. Through this apparatus, we could conduct real-time evaluation of platelet-material interactions, the initial event of thrombus formation, under shear flow conditions using small platelet suspension volumes (7.5~500 l per material). The use of human blood from a blood bank allows easy access to large amounts of human blood and does not require medical doctors and volunteer donors to draw the blood. Therefore, to test the hemocompatibility of materials, we used human blood from a blood bank.

Methods

Blood was obtained from volunteer donors and blood bank. Platelets were prepared by centrifugation of the PRP at 2,200g for 10 min. 5- or 6 - (N-succinimidyloxycarbonyl) - 3', 6 '-O, O' - diacethyl-fluorescein was used to label the platelets. For cell labeling, the CFSE working solution was prepared by diluting the stock solution 1:300 in PBS. The cells were then suspended in the CFSE working solution and incubated for 30 min and resuspended in platelet-free plasma at a near physiologic concentration of $1.0x10^5$cells/μl. The number of platelet adhesions onto material surfaces was measured for 15 min under shear flow conditions 1, $50s^{-1}$.

Results and Discussion

In order to compare the properties of platelets between fresh and bank blood, the number of adhering platelets, the trigger of the thrombus formation, on two test materials (AC and PE) was counted under shear flow conditions of $1s^{-1}$ and $50s^{-1}$. Results showed that there was no difference in the number of adhering platelets to the two material surfaces between the fresh and bank blood under shear flow. However, increase in shear flow decreased the number of adhering platelets. In addition, shear stress changed the rank of the materials. In conclusion, it was suggested that our cone and plate-type rheometer system with human blood from a blood bank is a useful research tool for testing and screening the hemocompatibility of materials instead of animal experiments.

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