Abstracts of the 13th Annual Meeting of the Japanese Society for Alternatives to Animal Experiments

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Forum for Citizen and Scientists
Animal Welfare and Alternative Research

Chairpersons:
Noriho Tanaka; Hatano Research Institute, Food and Drug Safety Center
Atsushige Sato; National Space Development Agency of Japan

F-1. Introduction of Alternatives in the Education (in Japanese)

Makiko Nakano
Student of Veterinary Medicine, Azabu University

F-2. Association Between Citizen Campaigns and Reversion of Animal Administration Law (in Japanese)

Mitsuaki Shiotsubo
Citizens Associaton of Asking Legislations for Animals

F-3. Significance of Experimental Animals in Safety Assessment of Chemicals

Hiroshi Ono
Hatano Research Institute, Food and Drug Safety Center

Usefulness and safety of a chemical should be evaluated and described sufficiently before its marketing providing substantial evidence through standard testings including animal experiments. This is the standard procedure required by the regulatory authority for each chemical usage category, such as pharmaceuticals, food additives, cosmetics and pesticides. This requirement has its origin actually to various disasters caused by chemicals, and seems so far working effectively in preventing the hazards.

Toxicity tests are a major target of the effort to develop alternatives since a large amount of experimental animals are consumed in the testing. The alternatives to toxicity tests should be useful to provide informations equivalent to the animal tests, such as features and potencies of toxicity, or target organs, dose-response relations, toxicokinetics, biotransformation and differences of toxicity by species, strains, age and sex, and moreover the mechanism of toxicological action of a substance.

In vitro testing systems developed by virtue of progress in the cell and tissue culture science have been expected to be likely candidates for the alternatives. Accomplishment of these test, however, is still to be waited, since validation of these tests has not yet completed and insufficient to be accepted by the regulatory authorities and also to be scientists.

F-4. Animal Experimentation is a Prerequisite for Biomedical Research

Tsutomu Miki Kurosawa
Osaka University Medical School

"Animal experimentation is a preriquisite for biomedical research." has been said so long time by researchers. The most of researchers believe that it is not necessary to re-validate this phrase. However some people raise the opinion "Animal experimentation is cruel and should not be conducted." Because I am carrying out animal experimentation and working as a managing director for IEXAS, I would like to ask the citizens to

understand the animal experimentation. I would like to present actual animal experiments in a medical institutions and ask the audience to consider it.
The first example of animal experimentation is a testing of heart valve which has cured so many patients who are sufferred by hear valve failure. In particular, the clinical application of artificial heart valve without animal testing can be accepted or not. The second example is a medical devices general such as an intraocular lens and contact lens which are tested on rabbit eyes but the cataract patients regain the sight by intraocular lens and the people safely use contact lens. Finally chronic renal failure which occurs on thousands patients and we do not know any cure oriented therapy apart from kidney transplantation which are rarely conducted due to the lack of donors. I have developed the model mouse for renal failure as the routine strategy for the solution of incurable diseases in medical field. So far the cause of renal failure was found to be renal tubular fibrosis which may caused by TGF-β. I have found that HGF can be a material which is used for cure oriented treatment for chronic renal failure. However I could not carry on this research if the people ask me to stop animal experimentation. I do not know any other method to find the cure oriented treatment for chronic renal failure. I am working as a scientist at Osaka University Medical School and my main duty is to find a way to help minorities in terms of health care such as patients with chronic renal failure. And I believe that I have found the way to cure these patients with the result of animal experimentation. I would like to ask the audience whether I should stop animal experimentation and ignore the patients who are suffered by incurable illness and are dying without any other help.

F-5. Safety Evaluation and Animal Alternative

Akihiro Kurishita
Research and Development Department, Japan Technical External Relations, Protecr & Gamble Far East Inc.

In the process of safety evaluation, toxicologists judge an acceptable risk associated with newly developed chemicals/technologies in target population. The risk is defined as a measure of the function of probability and severity of health hazard in human, and its measurement is called risk assessment.
Animal experiments is one of positive procedures to generate data for the risk assessment and have been playing a crucial role. Animal alternatives are relatively new procedures developed from animal welfare motives and are expected to replace to animal experiments. An issue that we face today is how we can replace animal experiments to animal alternatives without sacrificing the accuracy of the risk assessment based upon data from animal experiments. The process of the risk assessment can be overall divided into a part of the risk assessment process where we can apply animal alternatives will be discussed.

F-6. Concept of Animal Welfare and Research on the Alternative for Animal Experimentation

Atsushige Sato
Space Utilization Research Programme, National Space Development Agency of Japan

The confrontation between researchers and animal welfare groups has emerged long before, just when the history of animal experimentation began. Recently they have just started to try to reach the concrete solution for that. Researchers also have thought out some steps for their side. As the first, they start to have a new course about Animal Law in universities and to discuss whether animals should have legal rights, which species should have the rights, and which kind of rights they should have. This system came true remarkably contributed by the students of Animal Law Legal Defense Fund in California. The second is to have opportunities to exchange their opinions with the society for the prevention of cruelty, and explain the importance of the animal experiments in the development of life science and medical science research. There are some examples in which people oppose the use of animal change their attitude by knowing how the animal experiments contribute for the development of medical researches. We have kept discussion with the civilians by far, but further efforts should be made to make this society as the permanent organization bounding researchers and people in the movement of prevention

of cruelty to animals.
The third is to think over the necessity of animal experiments in their research. We can find alternatives in some parts of animal experimentation by using today's technology. We should change the methods of the experiment from using animals to human cells. The reconstructed oral epithelium out of human oral epithelial cells and fibroblasts have been developed as alternatives for animals[1]. The fourth is to develop alternative methods for animal experiments and to validate for the practical use. But there still exists some problems, which can be seen from the existance of the neuroscience research under siege[2], and the cancellation of the usage test for ISO 106 just because of the opposition from the animal-right saving activists.

Workshop
Present International Situation of Skin Toxicity Evaluation

Chairpersons:
Hajime Kojima; Nippon Menard Cosmetic Co., Ltd.
Yasuo Ohno; National Institute of Health Sciences

W-1 Is the Human Risk Assessment Possible with Animal Skin Toxicity Testing?

Hajime Kojima.
Research Laboratories, Nippon Menard Cosmetic Co., Ltd.

Test variables such as animal species and strain, numbers application method and period as well as the presence or absence of skin damage have been investigated with regard to a skin toxicity of chemical substances and cosmetics. The Draize primary skin corrosion and irritation test that uses the healthy skin of rabbits or guinea pigs is generally carried out with closed patch application for 4 or 24 hours (n=3).

In evaluation of skin toxicity, differences between animals and humans are the biggest problem. Although I know of no reports of misjudgment regarding corrosive substances, for skin irritation there is not always a good correlation and the rabbit appears to have high sensitivity. From physiological and anatomical viewpoints, the skin of animals markedly differs from that of man, and even skin absorption of chemicals can vary greatly. Considering these specificity, animal data have utilized and play a role for human risk assessment.

It is important that we should thoroughly understand the results of animal experiments if we are to develop, validate and evaluate alternatives to animal testing. After much understanding, in vitro tests should be compared with the animal test approach, aiming for the same level of predictivity with animal test results.

W-2. A New Serum-Free Medium, EpiLife™, Greatly Extends the Lifespan of Cultured Human Keratinocytes Making Large Banks of Highly-Characterized Normal Epithelial Cells Possible

S. Li[1], L.M. Donahue[1], A.K. Shipley[1], K.A. Droms[1], Y. Lin[1], G.D. Shipley[1]. M. Genno[2], S. Niwata[2], R. Yamamoto[2]
[1]*Cascade Biologics, Inc., Portland, Oregon U.S.A.*, [2]*Kurabo Industries, Ltd.*,

Human keratinocytes grown in serum-free medium can be used in two-dimensional or three dimensional culture systems to detect toxic compounds in an aqueous environment. Serum-free media for human keratinocytes developed in the late 70's support their proliferation in vitro without feeder layers. Commercial media developed from these formulas are available, however, a significant drawback their use is the limited lifespan of cells cultured therein. In our experiments, neonatal keratinocytes underwent ~20 population doublings after the primary culture when keratinocyte media from various manufacturers were used. However, the same cells grown in

EpiLife™ medium underwent >50 population doublings (fifteen passages). The ability of EpiLife™ medium to support a greater lifespan is due solely to optimization of the low molecular weight nutrients in the basal medium and not to the macromolecular supplements used. Keratinocytes grown in EpiLife™ medium maintain normal epithelial morphology. EpiLife™ medium may be useful for the creation of large uniform banks of keratinocytes that can be extensively characterized and then used for high-throughput screening purposes such as screening for toxic compounds which may be present in commercial products.

W-3. Alternative Skin Irritation Test Methods Using Living Skin Equivalent

Takuya Ishibashi[1], Hidekazu Takahashi[1], Mikio Nakagawa[2], Keiichi Kawai[2]

[1]Toyobo Co., Ltd., Tsuruga Institute of Biotechnology, [2]Kawai Medical Laboratory for Cutaneous Health

Several in vitro alternatives to animal testing, including the method using living Skin Equivalent (LSE), have recently been proposed to predict the in vivo skin irritancy. The severity of skin irritation is thought to be determined by compound actions of an irritant, for exsample cytotoxicity, permeability and horny layer-disturbing ability. It is necessary for establishing alternatives to the in vivo skin irritation tests to understand and evaluate the features of the actions of an irritant at each stage of irritation process. As many irritants act to directly interact with the stratum corneum on skin irritation, the evaluation of disturbance of the horny layer is thought to be important for predicting skin irritancy. We will introduce the LSE and its potential application to alternative skin irritation test methods, including a method to detect disturbance in the stratum corneum.

W-4. The Present Researches of Cultured Skin Model: Impression of Third World Congress on Alternatives and Animal Use in the Life Sciences

Katsuyasu Morota

Research and Development Division, GUNZE Ltd.

I took part in Third World Congress on Alternatives and Animal Use in the Life Sciences in Bologna, Italy. Many presentations about development of cultured skin model ware announced. They can divide three patterns.

1, Analysis, comparison and validation of ordinal skin models.
2, New application of ordinal skin model.
3, Development of new variation models.

I try to report the present researches of cultured skin model and my impression of the world congress.

W-5. Present Situation of Skin Toxicity Evaluation in Europe

Tasuku Takamatsu

Shiseido Life Science Research Center

Present situation of *in vitro* skin toxicity evaluation in Europe is discussed in connenction with the sixth amendment of Cosmetic Directive and the following decision which proposed a ban on marketing of cosmetic products containing ingredients that were tested using animals. Approaching to the deadline, June 30, 2000, for such ban, the seventh amendment of the cosmetic directive is being under consideration. The key word for the seventh amendment seems to be a coexistence of consumer safety and animal right, where regulatory acceptance and international harmonization are to be seriously discussed in addition to the scientific validation. And it is a time now to fully participate in an international information exchange at many different levels.

W-6. Present Situation of Skin Toxicity Evaluation in US and in Japan

Yasuo Ohno,
Divisoin of Pharmacol., BSRC, Nat. Inst. Health Sci

US established interagency committee (ICCVAM) for the evaluation of new toxicity test in 1994. After the discussion on the criteria for the evaluation of alternative test method, ICCVAM started to evaluate several test methods. COROSITOX was the first to be reported. ICCVAM considered that it is useful as a test method for the evaluation of skin corrosiveness. In Japan, there were no regulatory institute or laboratory, which is specified for the evaluation of alternative method. Instead, MHW organized or supported research group. Alternatives to Draize eye irritiation tests were evaluated by the collaborations of the MHW-supported research group and Japanese Cosmetic Industry Association. They prepared "Guidance on alternative appraisal methods for determining the eye irriation potential of cosmetic raw materials (draft)" and submitted to MHW this spring. The researh group and Japanese Society of Alternatives to Animal Experiments were discussing what toxicity tests should be evaluated and how. Skin toxicity test is one of the candidates.

Plenary Lecture

Chairperson: Hitoshi Endou; Kyorin University School of Medicine

PL. Conjugate-Dependent Toxicity: Alternatives to Animal Studies

M. W. Anders
Department of Pharmacology and Physiology, University of Rochester Medical Center, 601 Elmwood Avenue, Box 711, Rochester, New York 14534.

Studies on the mechanisms of conjugate-dependent toxicity are a major focus of the research in our laboratory. These studies are particularly concerned with mechanisms of glutathione-dependent toxicity. The cysteine conjugate b-lyase (b-lyase) pathway is a major mechanism for the bioactivation of nephrotoxic haloalkenes. The initial step in this pathway is the hepatic glutathione transferase (GST)-catalyzed reaction of haloalkenes with glutathione to form glutathione S-conjugates. The glutathione S-conjugates are excreted in the bile and undergo g-glutamyltransferase- and dipeptidase-catalyzed hydrolysis to the corresponding cysteine S-conjugates, which are transported to the kidney where they are taken up by amino acid transporters and undergo b-lyase-catalyzed bioactivation to toxic metabolites. The amino acid transport systems and the presence of b-lyase in the kidney are the major determinants of the kidney-selective toxicity of many haloalkenes.

Because the bioactivation of nephrotoxic haloalkenes requires the participation of both the liver and kidney, some studies in intact laboratory animals are required. We are committed, both by personal philosophy and governmental regulations, to reduce the number of intact animals used in biomedical research and to limit pain and discomfort. Accordingly, we have used a number of alternatives to intact animal studies in our research on the b-lyase pathway. These alternatives included the use of freshly isolated cells, cultured cells, isolated subcellular fractions, purified enzymes, and enzyme model systems. Although many of these preparations are derived from intact animals, their use allows a reduction in the number of animals used. Finally, we have used Fourier-transform ion cyclotron resonance mass spectrometry (FT-ICR MS) to investigate reactive intermediate formation and have recently begun computational chemistry studies on glutathione S-conjugate formation. In my presentation, I willdiscuss our work and that of others on in vitro and model systems that we have employed in our studies on the b-lyase pathway.

The first step in the b-lyase pathway is the formation of glutathione S-conjugates of haloalkenes in the liver. Rat and human hepatocytes have been used in our laboratory to investigate glutathione S-conjugate formation. In one series of experiments, we investigated the reaction of glutathione with chlorotrifluoroethylene. Previous

chemical and enzymatic studies had shown that glutathione is added stereoselectively to chlorotrifluoroethylene. Accordingly, we exploited the stereochemistry of the reaction and rat hepatocytes and liver subcellular fractions toinvestigate this reaction. We found that the microsomal GST was responsible for about 85% of the S-(2-chloro-1,1,2-trifluoroethyl)glutathione formed[1].

These experiments could not have been conducted in intact animals, and the use of the alternative system allowed us to investigate this point. In another experiment, we used rat and human hepatocytes to explore the biotransformation of chlorotrifluoroethene to S-(2-chloro-1,1,2-trifluoroethyl)glutathione[2]. These studies showed that the rate of conjugate formation is similar in both rat and human hepatocytes. Because conjugate formation could not be studied in human subjects, the use of an alternative system allowed the conduct of experiments that provided useful comparative data.

The renal proximal tubule is the major site for the bioactivation of haloalkene-derived cysteine S-conjugates. Accordingly, we have used isolated rat renal proximal tubular cells and cultured kidney cells (LLC-PK1 cells) to study cysteine S-conjugate bioactivation. Isolated renal proximal tubular cells probably reflect the in vivo situation but are viable for only a few hours, whereas cultured cells undergo significant changes during immortalization but allow experiments to be conducted over long times. Specific inhibitor of some steps in the b-lyase pathway can be used in both freshly isolated and cultured cells, but their toxicity limits their use in intact animals. Studies in isolated rat renal proximal tubular cells demonstrated that biotransformation of haloalkene-derived glutathione S-conjugates to the corresponding cysteine S-conjugates was necessary for expression of toxicity, that cysteine S-conjugates are transported by amino acid and organic anion transporters, and that b-lyase is essential for the expression of toxicity[3,4]. Recent studies with LLC-PK1 cells allowed comparison of the cytotoxicity and genotoxicity of a panel of bromine-containing nephrotoxic cysteine S-conjugates; comparable in vivo experiments would have required the use of a large number of animals [5].

The two cytosolic and two mitochondrial renal b-lyases are pyridoxal phosphate-dependent enzymes. The catalytic cycle of pyridoxal phosphate-dependent enzymes has been well studied, which has lead to the development of model systems that mimic the action of the enzymes. We have used N-dodecylpyridoxal in cetyltrimethylammonium micelles[6] and a Cu^{2+}/pyridoxal 5'-phosphate model[7] to mimic enzymatic reactions, thereby completely avoiding the use of animals. Recently, we have used FT-ICR MS to study the bioactivation of cysteine S-conjugates in the gas phase, which reflects results obtained in condensed phases. Finally, computational chemicals studies offer much promise to predict enzymatic reactions. Recent studies have modeled the reaction of glutathione with chloroalkenes and the b-lyase-dependent bioactivation of cysteine S-conjugates[9,10].

Our work shows that alternatives to animals can be used to study the fate and toxicity of haloalkene-derived S-conjugates. Future work will likely provide additional methods that allow the reduction of the use of animals in drug metabolism and toxicity studies. (Work in our laboratory was supported by NIEHS grant ES03127).

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AATEX Excellent Paper Award Lecture

Chairpersons:
Tadao Ohno; RIKEN
Atsushige Sato; National Space Development Agency of Japan

AW-1. Validation Study on Five Cytotoxicity Assays by JSAAE II. Statistical Analysis

T. Omori[1], K. Saijo[2], M. Kato[2], M. Hayashi[3], H. Itagaki[4], S. Miyazaki[5], T. Ohno[2], H. Sugawara[5], N. Teramoto[2], N. Tanaka[6], S. Wakuri[6], and I. Yoshimura[1]
[1] Science University of Tokyo, [2] RIKEN Cell Bank, [3] National Institute of Health Sciences, [4] Shiseido Safety Analytical Research Center, [5] National Institute of Genetics, [6] Food and Drug Safety Center

The validation study organized by JSAAE (JSAAE study) brought to light about three statistical problems, namely, (1) how to obtain a reliable estimate of ED50 from each experiment, (2) how to check the availability of the ED50 estimate obtained from each experiment, and (3) how to evaluate the feasibility of assays included in the JSAAE study.

To resolve the first problem, the authors devised a computer program (LAP-JSAAE) on SAS which incorporated a non-linear least squares method to obtain estimates of ED50 based on a logistic model for raw measurements. To resolve the second problem, the authors set several criteria for checking data which had previously been treated with manual adjustments for trivial errors such as descriptions out of format. They are related to detecting extraordinary large observations, inspecting excessively wide confidence intervals obtained by LAP-JSAAE, checking whether observed responses, in at least one dose, are within the range of 20% and 80%, assessing the lack of fit to the logistic model, and so on. To resolve the last problem, the authors devised the "power-for-distinction" (PFD) which was defined as the ratio of the range of medians to the mean value of the hinge-spreads for log(ED50), where medians and hinge-spreads are among laboratories and the range and the mean are among chemicals.

After establishing the methods, the authors performed data analysis for the JSAAE study and concluded that the crystal-violet staining assay (CV) with HeLa.S3(sc) cells and the colony formation assay (CF) with HeLa.S3(sc) cells are reliable in the sense that they give high values of the PFD.

AW-2. Evaluation of Skin Irritation in a Reconstituted Human Dermal Model (3-D Model) Using Water Insoluble Fatty Acid, Fatty Alchols and Hydrocarbons

Hajime Kojima, Atsushi Sato, Asao Hanamura, Tomonori Katada and Hiroaki Konishi
Research Laboratories, Nippon Menard Cosmetic Co., Ltd.

With the aim of improved prediction of human skin irritation, cytotoxicity tests were conducted using a reconstituted human dermal model (Skin^{2TM} cultured 3-Dimensional Skin Model, ZK1300 : 3-D model). A range of water- insoluble substances, namely fatty acids, fatty alcohols and hydrocarbons, were tested with MTT(3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyl tetrazolium bromide) reduction, LDH (lactate dehydrogenase) release and prostaglandin E_2 (PGE_2) release using as cytotoxicity markers. Results for these parameters were

compared with those obtained from a cytotoxicity test (MTT reduction assay) using cultured monolayers of human dermal fibroblasts (NB1RGB), from a rabbit primary skin irritation test and from human patch testing.

The MTT reduction assay with the 3-D model proved able to predict human primary and cumulative skin irritation of the test substances, though the match to fibroblasts results and rabbit primary skin irritation score was not perfect. Our finding also showed little variation with the different cytotoxicity markers applied in the 3-D model for the present insoluble test substances.

We therefore consider that the MTT reduction assay in the 3-D model is a reliable method to evaluate the human skin irritation potential of new chemicals.

AW-3. Experience of 1st Validation Study on Five Different Cytotoxicity Assays

Tadao Ohno

RIKEN (The Institute of Physical and Chemical Research)

JSAAE, together with 47 laboratories, organized the first step inter-laboratory validation study of five cytotoxicity assays in two cell lines each: colony formation assay (CF), crystal-violet staining assay (CV), lactate dehydrogenase release assay (LDH), neutral red uptake assay (NR), and MTT assay. From 3-year long mining of the huge amount of raw data, we concluded that CF, CV, MTT, and NR may be recommended from the view point of performance of the assays and considering the time required, simplicity, precision, and effect of using different cell lines in each assay, we recommend CV as being the most practical cytotoxicity assay. Our experience of the long-term data mining led us that, we must plan the validation study from the following view points; Thorough planning, especially on the statistical analysis and SOP, is important; Do not simply believe technical levels of the laboratories; Any processing steps should be organized under an unified control system; Budget for man-power and data mining should be prepared separately (It is big); So many human errors will occur. We do hope our experience will contribute for the further validation studies.

Symposium: Potential and Problems of Alternative Research -Alternatives, Biosafety and Ethics in Laboratories with Human Cells-

Chairpersons:
Tohru Masui; National Institute of Health Sciences
Tetsuya Kamataki; Hokkaido University

S-1. Framework of the Use of Human Materials in Pharmacogenetics: the Aim of This Symposium.

Tohru Masui

National Institute for Health Sciences, Division of Mutation and Genetics, Cell Bank

Public demand on the use of human tissue for research and development comes on the stage of action. The recent report from Minstery of Health and Welfare (Kurokawa report) publicly announced the guideline on the use of human tissue from surgical operations in pharmacological studies. The report enables us to plan the actual process in efficient and authorized use of human materials in Japan. On the other hand, the growing information on human genome leads us to combine the genomic information with pharmacological studies. In Japan pharmacogenetic studies using human materials requires 3 essential elements, i.e., discussion and establishment of ethical and legal issues, scientific studies supporting the pharmacogenetics, and public domain (banking system) that provides us human materials. In this symposium leading scientists will talk and discuss about these issues.

In the scientific section, Dr. Kamataki will present alternative expression systems of human drug metabolizing enzymes, Dr. Hosokawa will talk about the use of human cells in pharmacogenetics, and Dr. Ozawa will summarize the polymorphisms of human drug metabolizing enzymes in pharmacogenetic studies.

In the ethical section, Dr. Takebe will comment on sampling and handing of human material in genome sciences, Dr. Matsumura will summarize the effort of constructing ethical standard of handing human tissues and cells in The Committee on Ethical Affairs of The Japanese Tissue Culture Association, and Dr. Hirai also a member of the committee will present biosafety issues of human materials.

We hope these required area together will coordinately promote the pharmocogenetic studies in Japan.

S-2. Genetically Engineered Mammarian Cells, Bacteria And Transgenic Animals Carrying Human Drug Metabolizing Enzymes: Perspcitives

Tetsuya Kamataki

Division of Drug Metabolism, Graduate School of Pharmaceutical Sciences, Hokkaido University

The catalytic properties of enzymes involved in the metabolism of drugs vary depending on the animal species, causing species differences in the responses of animals to drugs. Thus, it is necessary to develop alternative methods to allow the prediction of drug metabolism in humans. Applying gene technology, we could establish genetically engineered mammalian cells, bacteria and animals carrying human genes, as humanized cells or animals. The advantages and disadvantages of the cells and animals and the future possibilities will be discussed.

S-3. Functional Expression of Drug Metabolizing Enzymes in Novel Established Human Hepatoma Cell Lines and cDNA Epressed in Mammlian Cell Lines

Masakiyo Hosokawa[1], Hiroko Shinohara[1], Yoko Tanaka[1], Eiko Tsukada[1], Mieko Mori[1], Maki Fukumoto[1], Yuko Ogasawara[1], Seishi Nagamori[2], Kan Chiba[1]

[1] *Faculty of Pharmaceutical Sciences, Chiba University*

[2] *Department of Internal Medicine, The Jikei University School of Medicine*

The functional expression of cytochrome P450 (CYP) and carboxylesterase (CES) in FLC4, FLC5 and FLC7, which cell lines were recently established from human hepatoma cells, were investigated. And we also compared the inducibility of CYP1A1 in these cell lines by rifanpicin(RIF), b-naphthoflavone (BNF) and proton pump inhibitors (PPIs), and the CYP1A1 transcriptional activation was studied in FLC4. Furthermore, cDNA cloning, characterization and expression of CES isozymes from human liver, brain and small intestine were investigated. When BNFwas treated with FLC cell lines, 7-ethoxyresorufin O deethylase activity was increased in dose dependent manner in all cell lines. Moreover, treatment with 10μM BNF enhanced induction of CYP1A1 protein and mRNA, but not CYP1A2. As measured by the same analyses, we studied CYP1A1 induction by PPIs. Omeprazole and lansoprazole induced CYP1A1 in any cell lines. CYP1A1 induction of pantoprazole was lower than those of other PPIs. These results indicate that CYP1A1 induction differs among these PPIs. The presence of the activated aryl-hydrocarbon receptor in the nuclei of FLC-4 exposed to BNF was clearly demonstrated by gel mobility shift assay. This result suggests that induction of CYP1A1 in FLC-4 was mediated aryl-hydrocarbon receptor. The hydrolase activity towards CPT-11 and temocapril in human liver, brain and intestine CES isozymes expressed in V79 cells were investigated. These therapeutic prodrug were metabolically activated by human CES isozymes, although the capability of metabolic activation was difference in each isozymes.

S-4. High Throughput Monitoring of Hepatic Drug Metabolizing Enzymes Using Human Blood Tissues

Shogo Ozawa
Division of Pharmacology, National Institute of Health Sciences

Activities of drug metabolizing enzymes (DMEs) should often be estimated with individual patients in clinical aspects. A great number of mutated alleles that result in total deficiency of enzymatic activities are known with CYP2D6, CYP2C19 and arylamine N-acetyltransferase 2 (NAT2) genes (Table 1).

Table 1. Major mutated alleles of CYP2D6, CYP2C19 and NAT2 genes found in Japanese population resulting in deficiency of enzymatic activities

Enzymes	Examples of substrates	Mutated alleles
CYP2D6	Debrisoquin, Dextromethorphan, etc.	CYP2D6*2, *4, *5, *10, *21
CYP2C19	Mephenytoin, Omeprazole, etc.	CYP2C19*2, *3
NAT2	Isoniazid, Sulfamethazine, carcinogenic aromatic amines, etc.	NAT2*5, *6, *7

An allozyme encoded by CYP2D6*10 shows decreased enzymatic activity.
These mutated alleles are determined using genomic DNA extracted from peripheral blood lymphocytes by a method of polymerase chain reaction-restriction fragment length polymorphism. Genotyping methods for CYP3A are not available thus far, though CYP3A forms metabolize many clinical drugs.

Table 2. Clinical drugs that are metabolized by various forms of human cytochrome P450s

Enzymes	Clinical drugs metabolized
CYP3A	Nifedipine, Erythromycin, Midazolam, Cyclosporin A, Testosterone, Cortisol, Carbamazepine, Lidocaine, Ethylmorphine, etc.
CYP2D6	Debrisoquin, Sparteine, Dextromethorphan, Codeine, etc.
CYP2C9	Phenytoin, Tolbutamide, etc.
CYP2C19	Mephenytoin, Omeprazole, etc.
CYP1A2	Theophylline, Antipyrine, etc.

Chiba, K. Xenobiotic Metabolism and Disposition 10: 391 (1995)

Hepatic DME activities have often been estimated by using probe drugs and enzymatic activities or mRNA levels in easily accessible tissues.

We have shown that ST1A3, a major hepatic form of human phenol sulfotransferase, catalyzes the metabolic activation of N-hydroxylated carcinogenic aromatic amines. Polymorphic appearance of sulfation has been shown in human livers, small intestines and platelets with p-nitrophenol. Therefore, the phenol-sulfating activity might be a determinant of individual susceptibility to environmental carcinogens. We then attempted to develop a high throughput screening method for phenol-sulfating activities by a simple colorimetric assay. We also found out variant mRNAs in livers of American-Caucasians that encoded Arg/His and Met/Val at codons 213 and 223, respectively. Platelets from individuals with homozygous 213His were shown to possess enzymes with both low activities (7-fold lower than 213Arg homozygotes) and low thermal stability (Raftogianis, et al. BBRC (1997)). We expressed two cDNAs encoding 213Arg- and 213His-type ST1A3 in Escherichia coli. The

expressed 213His-type ST1A3 was heat unstable as compared to the 213Arg-type, which was in consistent with the results by Raftogianis et al. 213His-type ST1A3 showed p-nitrophenol sulfating activity that was about 70% of that by 213Arg-type ST1A3 (Ozawa et al. J. Biochem (Tokyo) (1999); Table 3).

Table 3. Relationship between platelet ST1A3 activity and genotypes, and properties of *E. coli*-expressed 213Arg- and 213His-type ST1A3

Platelet			Expressed enzymes		
Genotype	Stability	Enzyme activity	Expressed enzyme	Stability	Enzyme activity
213Arg/Arg	0.62	1.1	213Arg	0.67	10.2
213Arg/His	0.53	0.9			
213His/His	0.09	0.1	213His	0.05	7.5

Hepatic activities from 213His/His homozygotes did not seem to associate very low enzymatic activities, suggesting other mechanisms involved in liver sulfation polymorphisms. Thus, caution should be taken to assess hepatic DMEs based on those in peripheral blood lymphocytes, though these assays are very useful for those whose deficiency could not be known by available genotyping methods.

S-5. Ethical Considerations on the Use of Human Tissues and Cells

Toshiharu Matsumura
Meiji Cell Technology Center, Meiji Milk Products, Co. Ltd.

Recent discussions on the handling of human tissues and cells in Committee on Ethical Affairs, Japanese Tissue Culture Association (JTCA members; Tohru Hasumura, Hiroki Hata, Reiko Hirai, Tohru Masui, Keiki Sato, Motoko Shibanuma, Noriho Tanaka, Makoto Umeda, non-JTCA member; Shin Utsugi, and chairperson; Toshiharu Matsumura) have been publicized in Tissue Culture Research Communications 17:171-171(1998) under the title of "On the handling of human tissues and cells in non-clinical field". Essential part of the paper will be summarized and discussed as follows:

1. Basic points under consideration

a. Difference from other materials: Although separated from the body, human tissues and cells are still bound to their donors in various ways including their donor's wishes or of the wishes of their families. In many cases, detailed procedures for handling have not been legally specified. Therefore, it is important to consider the fundamental laws and ethics.

b. Those in scientific and technological professions should recognize others from different professions as equal partners in ethical considerations.

c. It should be recognized that people have diverse beliefs concerning the relationship between the physical and mental aspects of the human body.

d. To injure a human body is often considered a crime. Therefore, when taking tissue and cell samples is exempted from criminal law, this should best be done by passing specific laws to that effect. Also, it should be recognized that there is currently an almost complete absence of such laws in non-clinical area.

e. On the matter that law does not regulate, people have the freedom to act. Such action should carefully be preceeded by efforts for the society to accept what is being done.

f. Safe handling: Human tissues and cells are best treated with the understanding that they are contaminated with transmissible elements.

2. Basic points to follow:

a. Practice of peer review: Handling human tissues and cells without a prior review should be avoided. Consultation and advice of those who are in independent positions, preferably forming an institutional review board, are

essential.
b. Keeping records: Record keeping should be carefully practiced. If any doubts arise, precise records should allow for the quickest possible resolution.
c. Distinction between keeping secrecy and publicizing information : Privacy of donors and secrecy regarding intellectual properties should be protected. However, this protection should be carefully balanced with the right of the public to be informed of the use of human tissues and cells.
d. Adherence to the law and application of self-regulatory rules : International recommendations such as the Helsinki Declaration should be respected, and the domestic laws adhered to. As to matters not specifically described in the law, self regulatory rules should be established in each institution, and these rules should be publicized. Such regulatory rules should not only be compatible with the existing
domestic laws, but be in harmony with social beliefs of local region, in this case, Japan.
(1) Tissues, cells, and cultured cells lines obtained during the course of scientific researches should be provided to users without monetary compensation, except for expenses such as handling fees. Exceptions may be applied to those cell lines which are intellectual property.
(2) While asking for free provision of tissues, cells and cell lines to donors and scientists, those who receive such tissues, cells, and cell lines with industrial and/or economic intentions should establish their own rules for sharing a part of their profit, once obtained, with the donor or the providing entity in socially responsible indirect ways.
(3) It should not be prohibited for inventors or institutions to obtain intellectual property right on tissue, cells and cell lines which they possess.
3. Current position of the committee
a. The review process: Except for reviewing process in GCP, no general governmental regulation has been established in Japan to regulate reviewing bodies or the review process. Recognizing this situation, the committee recommends that each institution establish their own rules and their own review committees, and that these rules be made open to the public.
b. The donation process: The primary procedure for donation of tissues and cells is by written informed consent. Nevertheless, other processes may not be excluded. The right to refuse donation must be preserved, but tissue and cells properly obtained may in certain cases be used for specified purposes without having to obtain prior concent. The right to freely use tissues and cells that have been discarded may also be
allowed under special conditions. In any case, the committee considers it important that the process of handling tissues and cells be reviewed and accepted as part of an overall review process within the responsible institution.

S-6. Ethics of Human DNA Sampling

Hiraku Takebe
Atomic Energy Research Institute, Kinki University

In obtaining human materials for DNA analysis, standard informed consent and ethical consideration must accompany the obtaining procedure. In Japan, where genetic counseling is still scarce, detailed and practical guidelines should be given to the researchers and doctors.

S-7. Biosafety in Laboratories Working with Human Materials

Reiko Hirai
The Tokyo Metropolitan Institute of Medical Science
A Member of The Committee for Ethical Matters in The Japanese Tissue Culture Association

We recommend that all human materials should be handled using Biosafety Level-2 (defined by WHO) practices and containment, after testing for dangerous pathogens such as HBV, HCV, and HIV.

S-8. Human Materials Resource in Japan: Present and Future Perspectives

Tetsuo Satoh[1], Satoshi Suzuki[1], Norimitsu Kurata[2], Yuki Noshimura[2], Mariko Iwase[2], Hua Li[2], Youji Ito[3], Mitsuo Kusano[3], Yasuo Ohno[4], Eiji Uchida[2], Hajime Yasuhara[2]
[1] Biomedical Research Institute, [2] Department of Pharmacology, School of Medicine, Showa University, [3] Department of Surgery, School of Medicine, Showa University, [4]National Institute of Health Sciences

Human biomaterials have been available for research purpose in the U.S.A. since 1980s, however, it has been used for only medical examinations in Japan. In 1998, Health Science Council, Japanese Government, has sent the report to the Minister of Health and Welfare, indicating that a portion of the surgical tissues can be used for drug development. This must be extremely beneficial for future development of new drugs in Japan. Prior to the regular use of surgical tissues for drug development in the near future, we have tried to know the recent situation on the use of human materials by distributing the survey to the Department of Gastroenterological Surgery, Medical schools of the Universities and some National Cancer Centers in Japan. In this symposium, I will report the results of the survey on this issue, and the research results using surgical tissues on the studies of the metabolism and safety evaluation of the drugs in our laboratories are also presented.

S-9. Public Research Resources: Human cell

Hiroshi Mizusawa
JCRB cell bank, National Institute of Health Sciences

The JCRB Cell bank of the Ministry of Health and Welfare (MHW) are providing many cultured cells to medical and pharmaceutical research communities and support many researches including understanding human diseases and developments of methods of diagnostics and treatment.
Recently, requirements to use human tissues adding to human cells for this research field have become increased quickly. Along this situation it becomes quite important to consider ethical issues to obtain human tissues. Since ethical issues have not been discussed seriously before in Japan, many people may be looking at medical doctors and scientists suspiciously. It is important now to open information thoroughly and to discuss with people to get their understanding. Once people understand, it may not be difficult to get consent to use human tissues from public community.
We are now starting to discuss to establish ethical rules that are required to operate cell bank. Important points of the rules possibly are unlinking of samples from individual identification data. These information including process of discussions and methods of handling human tissue materials are now representing in the Web server of the JCRB cell bank.

Poster Presentation:
Reproductive/Developmental Toxicity 1

Chairperson: Yukiaki Kuroda; National Institute of Genetics

P-1. Developmental Toxicity of 2,3,7,8-Tetrachlorodibenzo-p-Dioxin(TCDD) and Some of Its Relaed Compounds in Xenopus Laevis

Shin Mima[1], Michiko Sakamoto[2] and Takashi Tanimura[3].
[1]Osaka Women's Junior College, [2]Department of Anatomy, School of Medicine, [3]Life Science Research Institute, Kinki University

Developmental toxicity of 2,3,7,8-TCDD and its related compounds humans is a great concern in the modern society. We previously reported 2,3,7,8-TCDD cause death, anomalies (mainly edema) and growth retardation in Xenopus larvae when exposed shortly after fertilization at and over 100 ppb. The present study was to make a comparison of 2,3,7,8-TCDD and some of its related compounds in the developmental toxicity.

Xenopus embryos were continuously exposed to 2,3,7,8-TCDD and other 5 chemicals for 4 days from day 0-4(early exposure) or 4-8(late exposure) after fertilization. Each group received 2,3,7,8-TCDD(200 ppb), 1,2,3,4-TCDD(200 and 1000 ppb), 1,3,6,8-/1,3,7,9-TCDD(200 and 1000 ppb), 1,2,3,7,8-PCDD(25, 50 and 100 ppb), or 2,3,7,8-TCDF(100, 200 and 400 ppb) dissolved in 0.25% DMSO(controls). Death, external anomalies and growth of larvae were examined. 2,3,7,8-TCDD at 200 ppb induced death, edema and growth retardation after exposure. The incidences of the death and edema were higher on late exposure than early one in the case of 2,3,7,8-TCDD. All of the larvae died at 25 or higher ppb of1,2,3,7,8-PCDD after early or late exposure. 2,3,7,8-TCDF caused death and edema of larvae at 100 ppb after either exposure. No effects were observed after either exposure of 1,2,3,4-TCDD and 1,3,6,8- /1,3,7,9-TCDD.

The order of developmentally toxic potency in Xenopus laevis is estimated to be 1,2,3,7,8-PCDD > 2,3,7,8-TCDF > 2,3,7,8-TCDD >1.2,3,4-TCDD = 1,3,6,8-/1,3,7,9-TCDD.

P-2. Pharmacological and Toxicological Studies Using Chick Embryos (14) Diabetes Model in Chick Embryos Treated with Streptozotocin

Takashi Sugiyama[1], Miyuki Itoh[1], Mariko Fukumoto[2], Hideyo Shimada[1,3] and Kanji Tsuchimoto[1,3].

[1]*Division of Pathophysiology,* [2]*Division of Toxicology, Center for Clinical Pharmacy and Clinical Sciences, School of Pharmaceutical Sciences, Kitasato University,* [3]*Kitasato Institute Hospital*

In an attempt to reduce the number of mammals used in drug research, we have been examining the use of chick embryos and found that they may be superior for predicting the effects of drugs. In this study, we tried to produce a model of diabetes mellitus in chick embryo by treatment of streptozotocin (STZ), which has been widely used for induction of insulin-dependent diabetes in mammals.

Materials and Methods : Exp.1. *Blood glucose and insulin levels in developing chick embryos.* Blood was each collected from vitelline arteries of fertile eggs on the 9, 11, 12 14, 16 or 18th day of incubation. Levels of glucose in whole blood and insulin in serum were determined by enzyme-electrode method (Arkray factory) and by ELISA Insulin kit (Seikagaku Co.), respectively. In addition, weights of the body and pancreas were measured in each developing stage. **Exp. 2.** *Effects of STZ in chick embryos.* STZ (1000 μg/egg) was injected into the albumen on the 14 day of incubation and blood was collected on the 18th day of incubation. The levels of blood glucose and serum insulin were determined in same methods.

Results : Exp.1. Weights of body and pancreas, and level of blood glucose increased with developing stages. Small amount of insulin was shown in the 14th day-embryos, but thereafter insulin level increased. Levels of blood glucose and serum insulin were 178±12 mg/dl and 210±48pg/dl in the 18 day-embryos, respectively. **Exp.2.** Blood glucose level in the 18th day-embryos treated with STZ was significantly higher than that in the control. Conversely, serum insulin level was lower than that in the control.

Conclusion: These results suggest that streptozotocin may be used as an agent to induce a model of insulin-dependent diabetes mellitus in chick embryo.

P-3. *In Vitro* Embryotoxicity Testing of Metals for Dental Use by Differentiation of Embryonic Stem cells into Contracting Cardiac Myocytes

Koichi Imai[1], Horst Spielmann[2], Gabriele Scholz[2], Ingeborg Pohl[2], Masaaki Nakamura[1]

[1]*Dept.of Biomaterials, Osaka Dental Univ.,* [2]*ZEBET, BgVV, Germany*

The effects of metals used in dental practice on the capability of mouse embryonic stem cells (ES) cells (cell-

line D3) to differentiate into contracting cardiac myocytes and on cytotoxicity to differentiated mouse 3T3 fibroblasts were examined. pluripotent ES cells can be maintained in the undifferentiated state in the presence of LIF (Leukemia Inhibitory Factor). Upon withdrawal of LIF, those cells differentiate into the various cell types under appropriate conditions. Metal powder of silver, cobalt, chromium, nickel and palladium was dissolved in tissue culture media, with which cells were cultured. The embryotoxic potential was obtained from a biostatistically based prediction model with three endpoints, ID50, IC50ES and IC503T3. It is considered that silver might be weakly embryotoxic, as it was classified as a "weak embryotoxic" by the model, but other metals were not. Further accumulation of data is needed to apply the testing method utilizing ES cells to dental biomaterials.

P-4. Evaluation of Embryotoxicity of Estradiol Benzoate and Diethylstilbestrol Using Whole Embryo Culture

Makiko Kuwagata[1], Chiaki Watanabe[1], Hiromasa Takashima[1], Hiroaki Aoyama[2], Toshiaki Watanabe[3] and Hiroshi Ono[1]

[1]Hatano Research Institute Food and Drug Safety Center, [2]Institute of Environmental Toxicology, [3]Yamagata University School of Medicine

It has been reported that some estrogenic or antiestrogenic chemicals have the adverse effects on the neurobehavioral development and reproductive function in offspring, when administered duringembryogenesis. These defects appear to occur through the interaction of chemicals with a steroid hormone receptor, such as the estrogen receptor (ER). However, the underlying mechanisms to explain these phenomena are not understood. Therefore, the objectives of this study are to examine the stage of ER expression in the development and to evaluate the utility of whole embryo culture for detecting and characterizing estrogenic chemicals.

The western blotting analysis was used to determine the expression of ER in the untreated rat embryos on ED12 and ED13. Day 11 rat embryos (ED11) were exposed to two potential estrogenic chemicals, 0.01-100 g/mL of estradiol benzoate (EB) or 0.05-100μg/mL of diethylstilbestrol (DES), and were cultured for 48 hours.

Estrogen receptor β (ER β) was detected by the western blotting in extracts of whole rat embryos both on ED12 and ED13. The cultured embryos exposed to EB or DES showed dysmorphogenesis on the cardiovascular system, such as edema around umbilical vessels (EB and DES groups), enlargement of heart (EB group) and hemorrhage of head area (DES group).

These results suggest that the embryotoxicity of EDCs may be induced by the interaction between the EDCs and ER β at the embryonic stage in rats, and it is possible to evaluate the embryotoxic potential of EDCs by the binding ER assays in the whole embryo culture system.

(This study was supported by Japanese Society for Alternatives to Animal Experiments.)

P-5. A New Differentiation Marker in Cultured Whole Embryos Neuronal Connections with Sensory Organs, in Special Reference to Vibrissa Rudiments

Makoto Yamakado[1], Masaharu Akita[2], Atsushi Yokoyama[2] and Yukiaki Kuroda[3]

[1]Jichi Medical School, [2]Kamakura Women's College, [3]National Institute of Genetics

In whole embryo cultures, it is extremely important that what markers adopt for growth and differentiation of cultured embryos. At present, we used the heart beating rate, the number of somites and the crown-rump length of cultured embryos for development and differentiation markers, but it is necessary to establish another marker further. In the present study, we found the possibility of the use of neuronal connections with sensory organs, in special reference to vibrissa rudiments as a new differentiation marker in cultured whole embryos.

The ICR mouse embryos at 11.5 days of gestation was cultured in a rotated bottle, using 100% rat serum as cultured medium. After culture for 24 hours, DiI tracing method in the embryos fixed with 4% paraformaldehyde evaluated the development of neural connections with vibrissa rudiments. The growth of embryos was assessed to determine the crown-rump length and total number of somites.

In mouse embryos after cultivation for 24 hours, the crown-lump length increased from 7.0 to 9.1mm and the total number of somites increased from 55 to 63. Furthermore, 2.3% decrease was shown in the crown-lump length in mouse embryos cultured for 24 hours compared with the normal embryos at 12.5 days of gestation in vivo, but no difference was shown in the total number of somites.

On the other hand, vibrissa rudiments developed in the normal way in culture up to six or ten in number, and then nerve fascicles extended new branches to connect with vibrissa rudiments as same as those in embryos grown of in vivo.

As the above results, it was suggested that neuronal connections with sensory organs, in special reference to vibrissa rudiments could be used for a new differentiation marker in cultured embryos.

Reproductive/Developmental Toxicity 2

Chairperson: Masaharu Akita; Kamakura Women's College

P-6. The Histological Study of Whole Rat Embryos Cultured in Rat I.C. Serum Produced by Imamichi Institution of Animal Reproduction

Masaharu Akita[1], Atsushi Yokoyama[1], Shigekazu Shimizu[2], Hiroo Shikuma[2], Yoshihiro Nozaki[2] and Yukiaki Kuroda[3]

[1]Kamakura Women's College, [2]Fuji Biomedix Co. Lim., [3]National Institute of Genetics

Since 1997, we presented at Annual Meeting of this Society the possibility of the use of the rat I.C. (immediately centrifuged) serum, which is manufactured and marketed by IIAR, as the culture medium used for whole embryo cultures. In the present study, we report examine the histological features of culturing rat embryos at 11.5 days cultured for 48 hours.

Wistar-Imamichi rat embryos at the stage of 11.5 days were cultured for 48 hours using the rotator apparatus, which permits continuous gassing in culture bottles during rotation. By using a rat I.C. serum (D.I.C.) purchased from IIAR as the culture medium, rat embryos cultured for 48 hours were immediately fixed in bouin solution, routinely processed for embedding in paraffin, and sections were stained with Hematoxylin and Eosin (H&E) to examine the histological features of organs and tissues. The sections were compared with those of embryos cultured in rat I.C. serum collected the present laboratory as the control medium.

After cultivation for 24 hours, embryos in control medium showed the crown-lump length of 5.3 ± 0.2 mm and the total number of somites of 43 ± 1. Embryos cultured in D.I.C. serum showed the crown-lump length of 5.3 ± 0.1 mm and the total number of somites of 44 ± 1, indicating no significant difference between embryos cultured in both sera. In the embryos cultured for 48 hours no difference were shown in the total number of somites in the D.I.C. serum group compared with the control serum group.

On the other hand, in histological observation, no difference was found in the epithelium and the main organs and tissues between the embryos cultured in the control serum and those cultured in D.I.C. serum.

As the above results, it was suggested that the D.I.C. could be used for cultivation of rat embryos at 11.5 days of gestation.

P-7. Effects of Trichlorobenzene as Endocrine Disrupting Chemical on Cultured Rat Embryos

Atsushi Yokoyama[1], Masaharu Akita[1] and Yukiaki Kuroda[2]

[1]Kamakura Women's College, [2]National Institute of Genetics

In the present study, we conducted on embryo culture with trichlorobenzene to examine teratogenicity. Rat embryos on day 11.5 of gestation (plug day = 0) were cultured for 48 hours. As a drug, trichlorobenzene was

added to the medium at a concentration of 1, 5 or 10 μg/ml to examine its effect.

Although embryos after 48 hours culture showed a heart rate was 176 ± 10 beats/min., total number of somites 54 ± 2 and crown-rump length 8.4 ± 0.2 mm in the control group, their treated group (5, 10μg/ml) showed no change in heart rate, but the total number of somites was 10% smaller, and crown-rump length was 10% shorter, showing inhibition of systemic growth. A no change in blood circulation was also observed. From the results, trichlorobenzene significantly inhibited systemic growth in cultured rat embryos. On the other hand, trichlorobenzene-induced teratogenic effects were observed the edema (48%), cleft lip (48%), defect of brain development (50%) or toxicity of hand development (60%). The dose (10 μg/ml) of trichlorobenzene was caused major malformation.

P-8. Effects of Monochlorobenzene as an Endocrine Disrupting Chemical on Cultured Rat Embryos

Atsushi Yokoyama[1], Masaharu Akita[1] and Yukiaki Kuroda[2]
[1]Kamakura Women's College, [2]National Institute of Genetics

In the present study, we conducted to examine the teratogenicity of MONOCHLOROBENZENE on embryos in whole embryo culture. Rat embryos on day 11.5 of gestation (plug day = 0) were cultured for 48 hours. As a drug, monochlorobenzene was added to the medium at a concentration of 1, 5 or 10 μg/ml.

Although embryos after cultivation for 48 hours showed the heart beating rate of 168 ± 4 beats/min., total number of somites of 56 ± 2 and crown-rump length of 8.2 ± 0.2 mm in the control group, embryos treated with (5, 10 μg/ml) of monochlorobenzene showed no change in heart rate, but the total number of somites was 10% smaller, and crown-rump length was 10% shorter, indicating the inhibition of systemic growth. No change in blood circulation was observed. As the results, monochlorobenzene significantly inhibited systemic growth of cultured rat embryos. On the other hand, monochlorobenzene-induced teratogenic effects were observed on the edema (48%), cleft lip (48%), defect of brain development (20%) or inhibition of hand development (2%). Monochlorobenzene of a dose of 10 μg/ml caused major malformation.

P-9. Effects of Bisphenol A on Cultured Rat Embryos

Masaharu Akita[1], Atsushi Yokoyama[1] and Yukiaki Kuroda[2]
[1]Kamakura Women's College, [2]National Institute of Genetics

In the present study, we conducted to examine teratogenicity of bisphenol A on embryos cultured in whole embryo cultures. Rat embryos on day 11.5 of gestation (plug day = 0) were cultured for 48 hours. As a drug, bisphenol A was added to the medium at a concentration of 1 or 100 ppm. Although embryos cultured for 48 hours culture showed a heart beating rate of 180 ± 10 beats/min., total number of somites of 48 ± 2 and crown-rump length of 8.2 ± 2 mm in the control group, embryos treated on the concentration of 1, 100 ppm showed a decrease in heart rate, and the total number of somites in embryos treated at a concentration of 100 ppm was 10% smaller. The crown-rump lengths showed no change in all groups and culture time. The blood circulation in yolk sac and embryos decreased down from control level. As the results, bisphenol A significantly inhibited in the heart beating rate, total number of somites or blood circulation in cultured rat embryos. On the other hand, bisphenol A-induced malformations showed the edema, defect of brain development or inhibition of hand development. Bisphenol A at a concentration of 100ppm caused major malformation.

New Cytotoxicity Text

Chairperson: Takashi Sugiyama; Kitasato University

P-10. Establishment of Downs Syndrome Cells to Study Cytotoxicity of Dental Materials

Yoshitomo Ogawa[1], Mikio Kato[1], Daito Michiharu[1] and Koichi Imai[2]

[1]Department of Pediatric Dentistry,Osaka Dental University, [2]Department of Biomaterials,Osaka dental University

Downs syndrome is one of the chromosome abnormality that occurs with high frequency. And there is a report indicating that the susceptibility of these cells to medicine may differ. There is also possibility of a different toxic level but there are not basic data to guide the selection of dental materials. Therefore, we compared the IC50 of fibroblast-like Down syndrome skin cells (Detroit529) and fibroblast-like normal skin cells (HUC-F) using 10 dental materials. As a result, there was a difference in IC50 between the two types of cells. Especially, IC50 of Detroit529 declined mainly at the a root canal filling material and a resin modified glassionomer cement. In our opinion all of the chemical components of these materials must be analyzed. The average lifetime of Down syndrome patients has improved to about 50 years old. Using this method, there is possibility to reduce the number of experimental animals and used many Down syndrome patients will have an increased QOL.

P-11. Inhibitory Effect of Enviromental Endocrine Disruptors on Thymosin $\alpha 1$ Production in Thymus Epithelial Cell Culture

Kou Sakabe[1], Takahiko Yoshida[2], Hiroyuki Aikawa[2], Shun-ichiro Izumi[3], Yoshiyuki Osamura[4] and Fujio Kayama[5]

[1]Department of Morphology, [2]Department of Environmental Health, [3]Department of Obstetrics and Gynecology, [4]Department of Pathology, Tokai University School of Medicine, [5]Department of Environmental Health, Jichi Medical School

Inrtoduction

Natural estrogens such as 17 β-estradiol, are well known to modulate immune responses in various animals including humans. For example, changes in circulating estrogen levels during pregnancy deeply influence immune functions such as mitogen responses and skin graft rejection times. Fluctuations in circulating estrogen concentrations also affect thymic T-cell subpopulations. In addition, thymus activity in secreting its hormones, such as thymosins, thymulin, and thymostimulin, is markedly influenced by circulating estrogen levels.

Although this family of natural estrogens are steroidal in structure, a variety of exogenous non-steroids has been found to act like estrogens. In fact, estrogenic chemicals (i.e., environmental estrogens), which are one of the endocrine disruptors, have been implicated in a number of human health disorders. These substances are derived from a number of relatively common and abundant sources such as plants, plastics, agricultural products. However, little is known about the pharmacological and/or toxicological effects on immunocompetent cells of exposure to these substances especially in reference to the cause of animal and human immune disorders. To address this issue, the present in vitro study focuses on the effects of natural and environmental estrogens on the production of thymic hormone by thymus epithelial cells, a type of immunocompetent cells, at its peptide levels.

Experimental

Thymus epithelial cells derived from rat thymus (TECs; IT-45R1) originally established by Itoh et al. (Biomed. Res., 2: 11-19, 1981) was used. The cell line was maintained in DMEM, supplemented with 10% fetal calf serum pretreated with dextran-charcoal solution (CDFCS) to remove endogenous estro-

gens, and then used in the experiments. To determine the effect of the natural and environmental estrogens on thymosin-α 1 production, TECs were seeded in DMEM (phenol red-free) supplemented with 10% CDFCS in 250 cm3 flasks. After two days of incubation, $3\times10^{-12}\sim3\times10^{-4}$M 17$\beta$-estradiol or various estrogenic chemicals were added to the culture medium, and incubation was continued for 7 days. After seven days of incubation, fresh DMEM transferred from each flask was mixed thoroughly with five volumes of 1N acetic acid, and then boiled in a hot-water bath. After thoroughly mixing with petroleum ether for 10 min, the mixture was centrifuged at 800 x g for 20 min. The supernatant was filtered through Whatman No.1 filter paper, and the filtrate evaporated on a hot-water bath After adding Tris-HCl solution, the sample solution was applied to an Amicon molecular sieve column. Fractions constituting the void volume were lyophilized, dissolved in 10 mM HCl and chromatographed on a SynProPep C18 column in a Hitachi L-6210 HPLC system. The linear gradient was programmed over 30 min from 100% solvent A (10 mM HCl) to 100% solvent B (30% acetonitrile/70% pure water). The peak flow was detected with a Hitachi L-400 UV Detector at 215 nm, and the concentration of thymosin-α 1 determined from the standard curves which run simultaneously with the sample specimens.

Results and Discussion

The present study was carried out to assess the direct effect of natural estrogen and environmental estrogen on TECs production of the thymic hormone thymosin-α 1 by using the technique of quantitative high-performance liquid chromatography. Its concentration significantly decreased with increasing amounts of natural and environmental estrogens added to the culture media. In addition, the inhibition of thymosin-α 1 production was dose-dependent, a significant inhibition being obtained at 17β-estradiol doses over 3×10^{-11}M, genistein (plant) doses over 3×10^{-9}M, coumestrol (plant) doses over 3×10^{-9} M, α-zearalanol (livestock anabolic) doses over 3×10^{-7}M and bisphenol-A (plastic) doses over 3×10^{-6} M, respectively. No concentration of progesterone and cholesterol had a significant effect on the production of thymosin-α 1.

In conclusion, the present results showed that natural and environmental estrogens suppress the production of thymic hormone, and that all environmental estrogens derived from plants, plastic and livestock anabolic have this action although to varying degrees. Furthermore, our finding may be useful for evaluating biological effects of chemicals with estrogenic activity.

Acknowledgments
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P-12. Carcinogen Detection Potential of the BALB/3T3 Cell Transformation Assay by Using a Medium Supplemented with Low Concentration of Serum and Some Growth Factors (Improved Assay Method)

Yoshitsugu Kajiwara and Syozo Ajimi
Chemicals Inspection & Testing Institute

[Introduction] Cell transformation has been recognized as being directly rerevant to carcinogenesis and an important method for the screening of potential carcinogens. However, this assay has not been fully employed, since some difficulties such as low sensitivity, low reproducibility and long test period,still exist in employing it as a routine procedure. Hence, we scrutinized an improved assay method using a medium containing a low concentration of serum and some growth factors. Thereafter, we examined some geno-and non-genotoxic carcinogens, and some genotoxic non-carcinogens to confirm the availability of this method for predicting potential carcinogens.

[Materials and methods] BALB/3T3 cells were plated at 5x105 per 10-cm plastic dish containing 10ml of

10%FCS/MEM, incubated for 1 day, treated with test chemicals for 4~24 h, and then replated in 5ml of DME · F12 medium supplement with 1%ITES and 2%FCS at 5x104 per 6-cm dish. The control cells were replated in the same medium at 2.5x104 per 6-cm dish. The cells were cultured for 20~28 days changing the medium twice a week. The cells were then fixed with ethanol and stained with Giemsa solution.Transformed foci were scored referring to the criteria

[Results] We examined eight genotoxic carcinogens, six non-genotoxic carcinogens,three genotoxic non-carcinogens and four inorganic carcinogens. Every carcinogen, genotoxic,non-genotoxic and inorganic, were positive results,i.e.sensitivity was 100%. On the other hand, two out of three genotoxic non-carcinogens were negative, although one was positive,i.e.specificity was 66.7%.

[Discussion] Matthews et. al. showed that the transformation assay could not precisely discriminate mutagenic non-carcinogens from mutagenic carcinogens.The improved assay method could not detect mutagenic non-carcinogens with high probability. However,this method could precisely identify not only genotoxic carcinogens but also non-genotoxic carcinogens. Especialy, it is noteworthy that the improved assay method could identify pheonobarbital and clofibrate as carcinogens,since it was difficult to detect them by the original method. In conclusion, it is considered that the improved assay method is extremely useful for screening potential carcinogens because it can detect various carcinogens with high sensitivity within a short duration.

P-13. Kinetic Analysis of Cell Killing Effect Induced by Busulfan in Chinese Hamster Cells

Mariko Fukumoto[1], Takashi Sugiyama[2], Eriko Mitsuoka[1], Akira Ogamo[1] and Kanji Tsuchimoto[1]

[1]Division of Toxicology, [2]Division of Pathophysiology, Center for Clinical Pharmacy and Clinical Sciences, School of Pharmaceutical Sciences, Kitasato University

Busulfan as a high-dose therapy is an important component of many of the myeloabalative regimens for BMT in adults and children. Its dose limiting toxicity is the hepatic veno-oclusive disease(VOD). In adults, pharmacodynamic studies have shown a positive correlation between AUC of the first dose and the occurrence of VOD. Based on the previous finding that cell killing effects of cell cycle phase-nonspecific agents depend on the concentration-time product (C x T) or the area under the curve (AUC), we investigated whether if in vitro cytotoxic effects of busulfan depend on the C x T value or AUC.

MATERIALS AND METHODS

Chemical: Buslufan. Cell Culture: Chinese hamster V79 cells were grown in Eagle's MEM medium supplemented with 10% fetal bovine serum. Cell line was cultured in the presence of amphotericin B at 37℃ in a humidified atmosphere of 5% CO_2 and 95% air. Estimation of Cell Killing Effect of Drugs: Colony formation assay; After cell number was determined, V79 cells were seeded at a cell density of 100-200 cells into 60-mm dishes containing 4.0 ml of culture medium. Twenty μl of drug solution was added to each dish on the next day after seeding, and the cultures were then incubated for various periods. All assays were done in quadruplicates. At the end of the drug exposure, the plate was washed twice with 3 ml of phosphate-buffered saline (PBS) and again incubated with culture medium. On the seventh day after seeding, the dishes were washed once with PBS, fixed with 10% formalin, and stained with 0.04% crystal violet. The colonies comprising more than 50 cells were counted. IC_{50} values were calculated from the colony formation frequency (relative percentage of the control). Other assay; MTT assay.

RESULTS AND DISCUSSION

We analyzed cell kill kinetics for busulfan and showed C x T dependence of cell killing action of the drug using Chinese hamster V79 cells. These results indicate the good correlation between the in vitro and clinical response rates.

Irritation Reactivity

Chairperson: Yoshio Katsumura; Shiseido Co., Ltd.

P-14. Preparation and Evaluation of Artificial Membrane for Prediciting in Vitro Human Skin Absorption of Drugs

Aki Matsuda[1], Sachihiko Numajiri[1], Daisuke Kobayashi[1], Hideshi Natsume[1], Masahiko Ogihara[1], Yasunori Morimoto[1], Ken Ichikawa[2], Kurao Sudo[2] and Hiromi Kitano[2]
[1]*Faculty of Pharmaceutical Sciences, Josai University*, [2]*Faculty of Engineering, Toyama University*

Crosslinked polymeric membranes were prepared consisting of various ratio of methyl methacrylate, glycidyl methacrylate and VPE-0210 copolymer and drug permeability through these membrane for predicting in vitro human skin absorption of drugs were evaluated.

From permeation studies of the aqueous solution or suspensions of various lipophilic and hydrophilic drugs, a membrane had a very similar drug permeability to human skin for lipophilic drugs. Permeability of hydrophilic drug, antipyrine, however, was 2 order lower than that of the value from human skin permeation data. It seemed that hydrophilic domains (aqueous pores), which can easily permeate hydrophilic drugs, did not form in the prepared polymeric membrane.

It is concluded that the synthetic polymer membrane may be utilized as an alternative tool to predict human skin permeability of various lipophilic drugs.

P-15. Validation of the Neutral Red Uptake Phototoxicity Method Using Three Cell Lines

Mariko Sugiyama, Hiroshi Itagaki, Yoshio Katsumura and Shinobu Kato
Shiseido Life Science Research Center

Many in vitro methods to predict phototoxicity have been reported. EU/COLIPA project on in vitro phototoxicity testing had validated several of them. Based on the results of this validation study, the OECD draft guideline recommends the 3T3 mouse fibroblast neutral red uptake phototoxicity test (3T3 NRU PT) as a promising alternative.

In this study, we reported NHEK (normal human epidermal keratinocytes) and NB1RGB (normal human skin fibroblasts) as cells for in vitro phototoxicity test using essentially the same protocol described in the OECD guideline to compare the results with those using Balb/c 3T3 fibroblasts. Twenty-three chemicals (of which 9 were phototoxic and 14 were non-phototoxic in vivo test using guinea pigs) were tested and phototoxic potential of test chemicals were assessed by determining the Photo-Irritation-Factor (PIF) using a cut-off value of 5. Seventeen (74%) chemicals fell into the same criteria in terms of phototoxicity in 3 cell lines. The sensitivity, specificity, positive predicting value, negative predicting value and equivalence for the assay in 3 cell lines were 67-78 %, 64-79 %, 58-67 %, 79-82 % and 67-72 %, respectively.

The correlation between the in vitro and the in vivo studies were relatively high in any types of cell lines, but false negative result was observed in 2 or 3 chemicals in common. These results suggest that there is not much difference as far as safety assessment is concerned when the neutral red uptake phototoxicity test is employed using 3 cell lines reported here.

P-16. Evaluation of Cell Recovery Test Using a 3-Dimensional Human Cell Epidermis Model and Closed Patch Test for Skin Irritation Test

Matsumi Terasawa and Noriko Nakagawa
IVY COSMETICS Co.,LTD.

24hrs and 48hrs closed patch tests are popularly used for detecting irritants. The latter is preferred to detect very weak irritants. The animal experiments are considered screening tests of human patch tests. Recently, various methods alternative to animal experiments have been developed.

We are evaluating the cell recovery test using a 3-dimensional human cell epidermis as an alternative to animal experiments.

We found the cell recovery test using a 3-D could alternate to the 48hr closed patch tests for detecting very weak irritants.

P-17. Potential of LSE-High (Living Skin Equivlent) as a Model Membrane to Evaluate Skin Permeation and Skin Irritation of Drugs

Tetsuya Watanabe[1], Tetsuya Hasegawa[1], Hidekazu Takahashi[2], Takuya Ishibashi[2], Masakazu Kiuchi[3], Kozo Takayama[1] and Kenji Sugibayashi[3]

[1]Josai University, [2]Toyobo Bio Laboratory, [3]Hoshi University

LSE-high was evaluated as a model membrane to predict skin permeation and skin irritation of drugs. LSE-high or excised hairless rat skin piece was mounted in a two-chamber diffusion cell (èffective diffusion area : 0.95cm2). A drug suspension in 0.9 % NaCl (2.5 mL) was applied to the stratum corneum side, and the same volume of 0.9 % NaCl (for hydrophilic drugs) or 40 % PEG (for lipophilic drugs) was to the dermis side. Skin permeation of drugs from enhancer solutions was also investigated to compare the enhancement effect among the two membranes. Skin permeation of each drug was followed by periodical sampling from the receiver solution and assay by HPLC. The cumulative amount of each drug permeated through LSE-high over 30 min was comparable to that through rat skin over 8 hr, suggesting that LSE-high permeation is an index for rat skin permeation. Every enhancer also showed the penetration? promoting effect on the LSE-highpermeation. And the rank order was almost the same to that for rat skin. The skin irritation induced after exposure of different concentrations of sodium dodecyl sulfate (SDS) was also quantified using the MTT assay and fractal analysis of LSE-high. The obtained irritation was dependent on the concentration and applied period of SDS. In conclusion, LSE-high is a useful tool as a model skin to test the skin permeation and local irritation.

P-18. Evaluation of Cosmetic Ingredients with Cultured Human Skin Model

Tamie Suzuki, Masaki Uda, Itaru Miyamoto and Takao Ishida

Central Research Laboratory , FANCL Corporation

Cultured human skin model LSE-high(Living Skin Equivalent TOYOBO CO., LTD) have been expected for obtaining close results to thoses of in vivo skin irriation test, since LSE-high is composed of the living dermal model and corneal model.

We examined some kinds of cosmetic ingredients (e.g., fatty acids and surfactants) and cosmetic products (cleansing products) with this model. The appropriateness of this model was evaluated as an alternative to the skin irritation test with animals. The cytotoxicity of cosmetic ingredients or products was estimated by MTT assay on LSE-high and the effective concentration (EC50) was calculated 50% inhibition of cell growth. The EC50 values obtained from in vitro test were compared with the results of in vivo skin irritation test.

Those values of fatty acid and aliphatic alcohol corresponded to the results of in vivo test. Furthermore, a good correlation was shown between the length of the alkyl chain of aliphatic alcohol and their cytotoxicity, which was coincident with the results of the human patch tests. The EC50 values of other cosmetic ingredients also corresponded to the results of in vivo tests except for a few surfactants. The results of cleansing products, moreover, showed the good correlation with those of the human patch test. From these results, it is considered that the test using LSE-high model is rliable as alternative to in vivo skin irritation tests and applicable for the evaluation of the cosmetic products.

Drug Transport Evaluation System

Chairperson: Yasuyuki Sakai; Institute of Industrial Science, University of Tokyo

P-19. In Vitro Systems for the Quantitative Predicton of Drug Interactions via the Hepatobiliary Transporers

Yukio Kato[1,2], Kanji Komatsu[1], Kaoru Ueda[1], Hiroshi Suzuki[1,2], and Yuichi Sugiyama[1,2]
[1]*Graduate School of Pharmaceutical Sciences, University of Tokyo,* [2]*CREST, Japan*

It would be desirable to predict drug interactions involving the detoxification process such as enzymatic metabolism and biliary excretion. Although in vitro metabolic studies using microsomes, as well as CYP enzyme expression systems, have been demonstrated to be used to make for such predictions for P450-mediated reactions, there is little information available about how to make predictions involving biliary excretion. The purpose of the present study is to propose a method for predicting drug interactions via the hepatobiliary transporters. The pharmacokinetic interaction between methotrexate (MTX) and probenecid (PBD) was taken as a model case. The degree of the reduction in intrinsic clearance of MTX (% of control) under linear pharmacokinetic conditions should be $1/(1+I/Ki)$ where I and Ki represent the PBD concentration in vivo and inhibition constant obtained in vitro, respectively. The predicted values for the hepatic uptake clearance and biliary excretion clearance with respect to the hepatic concentration of MTX were comparable with the observed values. The methodology has been proposed which does not "underestimate" the degree of reduction in the predictiction of the inhibition of the net biliary excretion from circulating plasma to the bile. .

P-20. Cryopreserved Human Hepatocytes as a Tool for the prediction of in vivo Drug Transport and Transporter-Mediated Drug-Drug Interactions

Yoshihisa Shitara[1], Chuang Lu[2], Albert P. Li[2], Yukio Kato[3], Hiroshi Suzuki[3], Kiyomi Ito[1], Tomoo Itoh[1], Yuichi Sugiyama[3]
[1]*Kitasato University,* [2]*In Vitro Technologies, Baltimore, USA,*
[3]*The University of Tokyo*

We have compared the drug uptake activity of cryopreserved human hepatocytes with fresh ones and evaluated the potential of them as a tool for predicting in vivo drug transport and transporter-mediated drug-drug interactions. While the drug uptake activity was maintained, even after cryopreservation, in 2 lots of hepatocytes, in another 2 lots, it was reduced to a large extent. Although there are some lots, which lacked drug uptake activity, we were able to perform drug uptake studies by using lots, which were found to maintain high drug-uptake activity. Cryopreserved human hepatocytes are a useful tool for the prediction of in vivo drug uptake and the transporter-mediated drug-drug interactions.

P-21. Development of a simple Multi-Compartment Perfusion-Culture System for Toxicity Testing Including Absorption and Biotransformation Processes

Motoyuki Suzuki, Terumasa Ohiso, Yasuyuki Sakai and Akiyoshi Sakoda
Institute of Industrial Science, University of Tokyo

To mimic the actual metabolic processes for toxicants taken into humans, we tried to develop a toxicity testing system that consisted small intestine barrier, liver tissue and blood vessels. Caco-2 (human colon carcinoma cells) and Hep G2 (human hepatoma cells) were employed as representatives for small intestinal cells and liver

cells as the target organ, respectively. Caco-2 were cultured in a layer using commercially-available membrane culture inserts. Hep G2 were cultured in 25cm2 cell culture flasks. They were connected to each other in physiologically-relevant circuit with silicon tubes for perfusion culture. In control experiment, the increase of albumin concentration in the culture medium was almost in a linear fashion, on the contrary, after exposure to Paraquat from the apical side of the Caco-2 cell layer, the increase began to decline from 12h. However, it is doubtful whether or not the declination resulted from Paraquat through the Caco-2 layer, because the layer seemed to be damaged after 12h due to extraordinary high dose of Paraquat based on remarkable difference in cell number to liquid volume ratio between in humans and this system. We are now setting some experiments on a small intestine-liver-lung system using nonwoven fabrics or microcarriers for cell support materials. This kind of system is likely to be useful in the quantitative evaluation of the toxicity expressions resulting from organ-to-organ interactions.

P-22. The Establishment and Characterizaton of Kidney-Derived Cell Lines Expressing Human Organic Anion Transporter 1 and 3 (hOAT1 and hOAT3)

Shin-ichi Narikawa, Takashi Sekine, Seok Ho Cha, Makoto Hosoyamada,
Michio Takeda and Hitoshi Endou

Department of Pharmacology and Toxicology, Kyorin University School of Medicine

The organic anion transporters play an important role in the elimination of drugs and toxic compounds from the body. In this study, we established the cell lines stably-expressing human OAT1 and OAT3. The substrate selectivity of hOAT1 and hOAT3 are overlapping, and both mediate the transport of several anionic compounds such as *p*-aminohippurate and ochratoxin A. However, the affinity and transport capacity of these substrate for hOAT1 and hOAT3 were revealed to be different. The present study demonstrated the distinct properties between hOAT1- and hOAT3-mediated transport of organic anions. Furthermore, the cell lines expressing the human organic anion transporters will provide an useful *in vitro* assay system to predict and analyze the pharmacokinetics of anionic drugs.

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