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Current Progress of Next Generation Battery of Toxicology-Cellular and Molecular Toxicology

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Abstract

The detection and the regulation of man-made synthetic chemicals and the establishment of toxicity that may pose a genetic hazard in our environment are subjects of great concern because of its close correlation between environmental contamination and human health. Since the tens of thousands of man-made chemicals that have been introduced into the environment in the last few decades must also be tested for their damaging effect on DNA, the agents that cause this damage must be identified.

Keywords: environment, toxicology, chemicals, DNA, damage

Toxicology has been defined as the qualitative and quantitative study of the adverse effects of xenobiotics on living organisms. Moreover, modern toxicology goes beyond the study of the adverse effects of exogenous agents to the study of cellular and molecular effects of toxicants using molecular biological tools. Toxicology is a multidisciplinary field, and an important science that impacts both environmental health regulation and the development and practice of medicine[1]. Classical toxicological tools require hundreds of animals and provide little information with respect to mechanism (s). For example, descriptive studies in genetically inbred animals do not explain genetic and biological differences in the human population that influence individual response to drugs and environmental xenobiotics.

Since the remarkable progress in cell biology and biochemistry, the use of cell lines, enzymes, construction of genes, etc. became easier to handle in the laboratories. In this respect, classical toxicology moved to cellular and molecular toxicology at present, and will move to toxicogenomics[2] in the near future. For example, genetic toxicology has great changes in methodology and sensitivity. Generally, the carcinogenicity including genotoxicity is one of the potential toxicity that may consider for the human health. It has been widely assumed that mutation represents at least one step in carcinogenesis. The evidence supporting this idea is that the majority of mutagens are carcinogens[3] and, for at least some compounds, mutagenic potency is closely correlated with carcinogenic potency[4]. Moreover, mutagens and certain non-mutagenic carcinogens have also been found to induce chromosomal rearrangement[5] which may affect carcinogenesis by altering gene expression, perhaps by allowing the activation or inactivation of cellular cancer genes[6]. Several classical genotoxicity assay systems with rapidity and reliability have been introduced and practically applied[7-9] for predicting the carcinogenicity of chemicals and also been introduced to the evaluation of genotoxicity[6,11-16] and of antimutagenicity[14,17,18]. Cytogenetic studies on mammalian cells *in vivo*[11,12,19] as well as *in vitro*[10] have also been widely used as a screening method for DNA-attacking substances. However, these kinds of toxicity evaluation tools cannot elucidate the mode and/or mechanism of actions of chemicals, especially carcinogens and mutagens. Moreover, many scientists try to develop more precise, convenient and sensitive techniques for the detection of DNA damages as an index of carcinogenicity.

Recently, several new methods for the detection of genetic damages *in vitro* and *in vivo* were introduced according to the rapid progress in toxicology combined with cellular and molecular biology[20-22]. Among these methods, the single cell gel electrophoresis (comet assay) which can be detected DNA damages in cell level[15,23-27], mouse lymphoma thymi-dine kinase (*tk*) gene assay[16,28], FISH (fluorescence *in situ* hybridization)[12], PRINS (primed *in situ* hybridization)[29] and transgenic animal and cell line model as a parameter of *lac I* (Big Blue)[30-35] or lac Z (Muta Mouse)[36] gene mutation are newly introduced based on cellular and molecular toxicological approaches. Also, *in vivo* supravital micronucleus assay with peripheral reticulocytes by using acridine orange fluorescent staining[11,18] was introduced instead of mouse bone marrow micronucleus assay.

Moreover, the rapid progress in cellular and mole-

cular biology, like many other branches of biomedical research, toxicology is now experiencing a renaissance fueled by the application of "omic" technologies to gain a better understanding of the biological basis of toxicology of drugs and other environmental factors[37,38].

In this review on the rapid progress of cellular and molecular toxicological tools, several new cellular and molecular genotoxicity assay systems will be described briefly as follows.

Single Cell Gel Electrophoresis (Comet) Assay

Since Ostling and Johanson[39] introduced microelectrophoretic technique, Singh *et al.*[24] have modified and improved the microgel electrophoresis technique to evaluate DNA damage in single cells under alkaline conditions. The comet assay is rapid, sensitive, visual and simple technique to quantify DNA strand breaks in individual cells at least 3 major basic protocols[24-26,39-42]. If the agent can cause the strand break, we can see the extent of tail from the head (nucleus) like comet with staining of fluorescent dyes such as ethidium bromide, acridine orange and propidium iodide, etc.

Singh *et al.*[24] introduced a microgel technique involving electrophoresis under alkaline (pH 13) conditions for detecting DNA damage in single cells. At this pH, increased DNA migration is associated with increased levels of frank single strand break (SSB), SSB associated with incomplete excision repair sites, and alkali-labile sites (ALS). Because almost all genotoxic agents induce orders of magnitude more SSB and/or ALS than double strand break (DSB), this version of the assay offered greatly increased sensitivity for identifying genotoxic agents. Olive *et al.*[42] introduced another alkaline version of this assay in which DNA is electrophoresed at a pH of 12.3. Since the introduction of the alkaline (pH 13) Comet assay in 1988, the breadth of applications and the number of investigators using this technique have increased almost exponentially. Compared with other genotoxicity assays, the advantages of the technique include: (1) its demonstrated sensitivity for detecting low levels of DNA damage; (2) the requirement for small numbers of cells per sample; (3) flexibility; (4) low costs; (5) ease of application; (6) the ability to conduct studies using relatively small amounts of a test substance; and (7) the relatively short time period (a few days) needed to complete an experiment. During the last decade, this assay has developed into a basic tool for use by investigators interested in research areas ranging from human and environmental biomonitoring to DNA repair processes to genetic toxicology. Attractive uses of this assay in genetic toxicology include: (1) as a potentially high-throughput screening assay; (2) in mechanistic studies to distinguish between genotoxicity versus cytotoxicity induced chromosomal damage; (3) in mechanistic *in vivo* studies to distinguish between genotoxic versus non-genotoxic carcinogens; and (4) potentially, as part of a battery of *in vitro*/*in vivo* assays used for regulatory submissions.

To harmonize comet assay, International Workshop on Genotoxicity Test Procedure (IWGTP) was held at Washington D.C. on March, 1999 by Environmental Mutagen Society supported with OECD. Our laboratory[15,43,44] also involved in this harmonization and published as OECD guideline with Tice *et al.*[27]. General reviews on this technique have been published by Tice *et al.*[27] and Fairbairn *et al.*[45,46].

Mouse Lymphoma Thymidine Kinase (*tk*)$^{+/-}$ Gene Assay (MLA)

The mouse lymphoma assay (MLA) using the thymidine kinase (*tk*) locus has been widely used to detect the ability of chemicals to induce genetic damage in cultured mammalian cells. The assay was originally developed by Clive *et al.*[47,48] using soft agar medium to clone and enumerate mutants. Subsequently, Cole *et al.*[49] established the assay using a microwell protocol for cloning and mutant detection. Cole *et al.*[49] chose to use the microwell method because they had used this method successfully for a number of years with other cell mutation assays, including assays with L5178Y cells. While the soft agar and microwell versions of the assay are basically the same, there are some differences in the cytotoxicity measures, the calculation of the mutant frequency, and the counting and sizing of mutant colonies. A large body of information demonstrates the capability of the MLA *tk* assay to detect a broad spectrum of mutational events[50]. Therefore, the International Committee for Harmonization (ICH), in selecting a recommended test battery, discussed the MLA and the *in vitro* mammalian cytogenetic assays as possible alternatives. The cells used for the assay are mouse lymphoma cells (L5178Y $tk^{+/-}$ 3.7.2C), heterozygous at the thymidine kinase locus (*Tk1*) on chromosome 11. Inactivating the tk^{+} allele (this functional allele is also referred to as $Tk1^{b}$, on chromosome 11b) induces trifluorothymidine (TFT) resistance, and $tk^{-/-}$ mutants can be selected for in a background of $tk^{+/-}$ non-mutant cells. Mutant colonies have a bimodal size distribution, with so-called large colonies growing at the rate of $tk^{+/-}$ cells and small colonies growing at a slower rate[51]. Early cytogenetic studies demonstrated that small colony mutants are often associated with

chromosome aberrations involving chromosome 11 whereas large colony mutants are often cytogenetically normal[51-53]. Both large and small colony mutants are represented in spontaneous and induced mutants, and the proportion of small colony mutants is mutagen dependent. Extensive molecular and cytogenetic analysis has shown that mouse lymphoma cells can detect a variety of mutations, including point mutations and small mutations within *Tk1*, losses of $Tk1^b$ (the functional allele), larger deletions including $Tk1^b$ and cytogenetically detectable chromosome aberrations such as translocations[54].

The cytotoxicity of chemical was determined by relative survival (RS) after 3 hr treatment at concentrations up to 5,000 μg/ml with and without S-9 mixture. The highest concentration chosen was one with a 10-20% RS. Cultures were exposed to the test chemical for 3 hr, then cultured for 2 days before plating in 96-well microtiter plates at 2000 cells/well with trifluorothymidine for mutant selection and at 1.6 cells/well for cell viability. The number of wells containing colonies was counted on day 12 after plating, and large and small colonies were scored. Mutation frequencies were analyzed by the statistical package, Mutant V2.31 program (Hazleton, England) in accordance with the UKEMS guidelines

Supravital Staining *In Vivo* Micronucleus Assay with Peripheral Reticulocytes

Micronuclei were described in the cytoplasm of erythrocytes more than a century ago and called "fragment of nuclear material" by Howell or "corpuscules intraglobulaires" in the terminology of Jolly in the late 1800s and early 1900s. These structures are known to the hematologist as "Howell-Jolly bodies". Similar structures were described in other cell types (e.g. in mouse and rat embryos by Brenneke in 1937[55] or in Vicia faba by Thoday in 1951[56]) and called "fragment nuclei" or "micronuclei". These micronuclei were consistently found after radiation exposure of cells, and it was assumed that they originated from acentric fragments, which were excluded from the two daughter nuclei at the late stages of mitosis[57]. Evans *et al.* discovered in 1959[57] the usefulness of micronuclei as markers for cytogenetic damage, when they compared the efficiency of neutrons to that of gamma-rays in *Vicia faba* roots. The decisive breakthrough of micronuclei as assay system for the genotoxic potential of agents came with the work of Boller and Schmid[58] (suggestion of the term micronucleus (MN) test for the first time) and Heddle[59] by using bone marrow erythrocytes. Shortly thereafter, Countryman and Heddle[60] introduced lymphocytes as another useful cellular system for detection of chromosomal damage by determination of MN production, and recommended using micronuclei as a biomarker in testing schemes.

Micronuclei result from lesions/adducts at the level of DNA or chromosomes, or at the level of proteins directly or indirectly involved in chromosome segregation (e.g. tubulin). Formation of micronuclei originating from chromosome fragments or chromosome loss events requires a mitotic or meiotic division. The MN assay *in vivo* is a method devised primarily for screening chemicals for chromosome-breaking effects. In the monitoring of chromosome breakage, the test is at least as sensitive as the metaphase method; in addition it includes effects on the spindle apparatus. This MN assay using peripheral blood erythrocytes was introduced by MacGregor *et al.*[61] and developed by Hayashi *et al.*[11,12] having more simple and convenient compared to conventional bone marrow assay by the introduction of supravital stain-ing with acridine orange[18]. The conventional Giemsa staining method, however, has some disadvantages because not only MN but also some cell inclusions containing RNA and other acidic materials are stained dark blue by Giemsa; it is occasionally difficult to identify MN from these inclusions. Acridine orange metachromatic fluorochrome discriminates between DNA and RNA by green and red fluorescence, respectively.

In Vitro Cytokinesis-Block Micronucleus Assay

The micronucleus assays have emerged as one of the preferred methods for assessing chromosome damage because they enable both chromosome loss and chromosome breakage to be measured reliably[62]. Development of the cytokinesis-block methodology by Fenech and Morley[63] by addition of the actin inhibitor cytochalasin-B during the targeted *in vitro* mitosis allowed the identification of once-divided nuclei as binucleates and provided an efficient approach to study the mechanism leading to the induction of micronuclei. By restricting scoring of micronuclei in once-divided cells, the cytokinesis-block micronucleus assay solved the problem of variation in micronucleus frequency because the data obtained are not confounded by altered cell division kinetics caused by cytotoxicity of agents tested or sub-optimal cell culture conditions[64].

Transgenic Mutagenesis Assay System

The transgenic mutagenesis system is a useful and powerful tool to evaluate the genotoxicity, and it also provides a window of carcinogenesis and mutagenesis mechanisms of chemicals based on information

such as mutation pattern, frequency, and location in sequence context of the *lacI* target gene[65]. For example, the *lacI* transgenic Big Blue Rat2 fibroblast cell line carries over 40 copies of lambda shuttle vector[66] containing *lacI* gene as a target[67,68]. The *lacI* gene, as a mutational target, is very useful for the study of the mutational characteristics of a carcinogen for several reasons. First, the relatively small size (1,080 bp of coding region) *of lacI* gene facilitates sequence analysis. Second, the expression of repressor protein permits a rapid colorimetric assay to screen for mutations. The mutations induced in the *lacI* gene can easily be quantified by mutant frequency (MF), and the precise mutation type and distribution can quickly be identified by direct sequencing. Moreover, considering that mutations in *lacI* gene induced by chemicals reflect the effects of mutagens on other endogenous genes such as proto-oncogenes and tumor suppressor genes, and that mutations occurred in these genes are the most common events in many types of human cancer[30,69], this assay may provide a powerful tool to predict the mutation spectrum induced in cancer-related genes more accurately[31-34].

In conclusion, we anticipate that these new technologies will (1) lead to new families of biomarkers that permit characterization and efficient monitoring of cellular perturbations, (2) provide an increased understanding of the influence of genetic variation on toxicological outcomes, and (3) allow definition of environmental causes of genetic alterations and their relationship to human disease. The broad application of these new approaches will likely erase the current distinctions among the fields of toxicology, pathology, genetic toxicology, and molecular genetics. Instead, a new integrated approach will likely emerge that involves a comprehensive understanding of genetic control of cellular functions, and of cellular responses to alterations in normal molecular structure and function.

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