Chapter 5

In vitro, Tissue-Based Models as a Replacement for Animal Models in Testing of Drugs at the Preclinical Stages

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1. Introduction

As early as 1950s, researchers began to apply *in vitro* culture technology in testing cytotoxicity of multiple drugs on different cells, which could basically determine the cytoxic dose range of these drugs. Regarding to the continuous development of novel chemotherapy drugs and large growth of chemical compounds closely related to human practice and life (including pharmaceutical, cosmetics, food additives, pesticide, industrial chemical, etc), there is great need to explore a convenient and effective way for selection, pre-clinical evaluation or pre-production safety assessment.

In vitro, tissue-based models are common and widely used for screening and ranking chemicals, especially in testing of drugs at the preclinical stages. The toxic effects include general cytotocixity, genotoxicity, mutagenesis and carcinogenesis. Cell-based assays are currently considered central to toxicity testing, biomaterial testing, and environmental material exposure testing. Nearly all of the assays could be adapted to other application for bioactivity test. For instance, the established cell lines have been successfully employed in a number of fields of medical research. Especially, many aspects of modern virology have been developed using animal cells in culture.

There are several strategies for using in vitro, tissue-based models in testing of drugs at the preclinical stages. One such strategy is to refine the choice of cells and end points of one method. For instance, human corneal cells are now used to screen for local eye toxicity of chemicals, with a method employing sophisticated end points. Another strategy is to use batteries of tests with different cell types, to cover most aspects of basic cell functions. A third strategy is to do more basic research into fundamental mechanisms of toxicity or bioactivity. When such mechanisms have been clarified, rational in vitro models could be set

up. By contrast, a fourth approach ignores whether the toxic mechanisms screened for is known or not. As long as the end point of the test correlates well with in vivo toxicity, the test may be used.

2. Cultures

The possibility of using cell or tissue cultures as suitable material for testing agents in pharmacology has often been suggested. The culture methods include organ culture, sphere culture, suspension culture, clone culture on soft agar and monolayer cell culture, etc.

Organ culture provides drug evaluation a model more close to in vivo situation, but this model are not suitable for efficacy quantitative experiment because of the size differentiation of organ implants and heterogeneity of the cells within the explants between repeated experiments. Sphere culture of the tumor cells is similar to tumor nodules in vivo and can be used to study the influence of three-dimensional relationship on drug sensitivity. Suspension culture is beneficial to furthest prevent the growth of "cell pollution source"-connective tissue cells (i.e. fibroblast), which is widely applied to chemosensitivity study and convenient to perform radionuclide analysis. Clone culture on soft agar is fit for cell with high capacity of self-renewal (i.e. tumor cell), other than cells with limited proliferation ability. Monolayer cell culture is best for cytotoxicity test of cell lines and chemosensitivity of various tumor biopsy materials. This test can implement automatic operation due to small cell amount requirement and its convenience and flexibility in drug treatment, reply and pharmacodynamic quantitative study.

Generally, a primary cell line possesses many characteristics of the original cells, such as similar chromosomal numbers and the specialized biochemical properties of the parent tissue, e.g. in the case of liver cells the ability to secrete albumin. On the contrary established cell lines invariably have different chromosome numbers and lose a number of specialized biochemical properties of the parent tissues. This latter fact imposes some restrictions on the utilization of established cultures in the design of tests of toxicity. However, established cell lines have been successfully employed in a number of fields of medical research. Especially, many aspects of modern virology have been developed using animal cells in culture.

3. Method used

3.1. Cytotoxicity

Cytotoxicity is considered primarily as the potential of a compound to induce cell death. Most in vitro cytotoxicity tests measure necrosis. However, an equally important mechanism of cell death is apoptosis, which requires different methods for its evaluation. Moreover, detailed studies on dose and time dependence of toxic effects to cells, together with the observation of effects on the cell cycle and their reversibility, can provide valuable information about mechanisms and type of toxicity, including necrosis, apoptosis or other events.

In vitro cytotoxicity tests are useful and necessary to define basal cytotoxicity, for example the intrinsic ability of a compound to cause cell death as a consequence of damage to basic cellular functions. Cytotoxicity tests are also necessary to define the concentration range for further and more detailed in vitro testing to provide meaningful information on parameters such as genotoxicity, induction of mutations or programmed cell death. By establishing the dose at which 50% of the cells are affected (i.e. TC_{50}), it is possible to compare quantitatitively responses of single compound in different systems or of several compound in individual systems.

The endpoint/parameters used in cellular toxicity testing including:

- Membrane permeability changes-dye exclusion(trypan blue);the release of intracellular enzymens like lactate dehydrogenase; preloaded ^{51}Cr; nucleoside release; uridine uptake;vital dye uptake, etc.
- Reduced mitochondrial function
- Changes in cell morphology
- Changes in cell replication
- Apoptosis evaluation-changes in morphology; membrane rearrangements; DNA fragmentation; caspase activation; cytochrome c release from mitochondria, etc

3.2. Cellular response and functional response regarding to general toxicity(protein/gene expression)

The basic methodology of general toxicity has changed little during past decades. Toxicity in laboratory animals has been evaluated by mainly using clinical chemistry, hematological and histological parameters as indicators of organ damage. The effect of a toxic chemical on a biological system in most cases is fundamentally reflected, at the cellular level, by its influence on gene expression. Consequently, measurement of the transcription (mRNA) and translation (protein) products of gene expression can explore valuable information about the potential toxicity of chemicals before the development of a toxic/pathological response.

The rapid progress in genomic (DNA sequence), transcriptomic (gene expression) and proteomic (the study of proteins expressed by a genome, tissue or cell) technologies, in combination with the ever-increasing power of bioinformatics, creates a unique opportunity to form the basis of improved hazard identification for more predictive safety evaluation.

For example, currently available methods for the study of gene expression at the transcript level include:

- Hybrdizization-based techniques: Northern blotting;S1-mapping/RNase protection; Differential plaque hybridization;
- PCR-based techniques: Subtraction cloning; DNA microarrays; Differential display; RDA(representational difference analysis); Quantitative (real time) PCR;
- Sequence-based techniques:ESTs(expressed sequence tags); SAGE(serial analysis of gene expression); MPSS(massively parallel signature sequencing); DNA-sequencing chip; Mass-spectrometry sequencing

Two-dimensional gel electrophoresis is a highly sensitive means of screening for toxicity and probing toxic mechanisms, which combines separation of proteins by isoelectric focusing(IEF) in the first dimension followed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis(SDS-PAGE) based on molecular weight in the second dimension.

By comparing gene/proteins expressed following exposure of a biological test system to a chemical with those present under untreated conditions, it is possible to identify changes in biochemical pathways via observed alteration in sets of gene/proteins that may be related to the tocicity, which provide the means to profile expression of thousands of messenger RNAs or proteins.

Over the last few decades, a large amount of research has resulted in an explosion of information regarding mechanisms of toxicity and new tools to study the biological responses to toxic stress. Some of the key cellular responses to toxicant exposure, which could potentially be used as early markers of toxicity, include the following:

- Responses following exposure to toxicants that form reactive electrophiles (e.g. oxidative stress) such as loss of glutathione (GSH), increased production and sensitivity to reactive oxygen species (ROS), increase in cellular calcium, lipid peroxidation, loss of ATP and mitochondrial/endoplasmic reticulum (ER) specific events.
- The cellular response to stress, including an increase in synthesis of the heat shock (Hsp) family of proteins, induction of the stress-activated protein kinases (SAPKs) and glucoseregulated proteins (Grps).
- Changes in the levels of key enzymes, such as the phase I and phase II metabolising enzymes involved in the detoxification of toxic chemicals.
- Induction of the metal-binding proteins, metallothioneins (MTs).
- Perturbations to cellular membranes, gap junctions and intercellular communication inhibition (involving the connexins Cx43, Cx32 and Cx26).
- Induction of cell proliferation (for which suitable markers could include TNF-α, TNF-β, plasminogen activator inhibitor-2 (PAI-2), the tumour proliferative marker Ki-67 antigen and proliferating cell nuclear antigen (PCNA).

3.3. Toxicokinetic study

Toxicokinetic modelling describes the absorption, distribution, metabolism and elimination of xenobiotics as a function of dose and time within an organism. Toxicokinetic models can be divided into two main classes: data-based compartmental models and physiologically-based compartmental models. In vitro approaches can be used to obtain useful information in this area. One representative model, the physiologically-based toxicokinetic (PB-TK) model, can be obtained by studies in vitro, including tissue–blood partition coefficients, the kinetics of any active transport processes, and the kinetics of metabolism by the liver and any other organ capable of biotransforming the compound (e.g. the lung). The output of toxicokinetic models is the prediction of concentration/time courses in different tissues. This information can be combined with the basal cytotoxicity data to make a prediction of the acute systemic toxicity of the chemical.

3.4. Specific toxicity

3.4.1. Genotoxicity

In vitro system can also be used to determine genotoxicity for identifying potential carcinogens, which includes three levels of mutation, namely *gene, chromosome and genomic* mutations. It is acknowledged that the generally accepted objectives of genotoxicity testing of chemicals are: (a) identification of germ cell mutagens, because of their possible involvement in the etiology of human heritable genetic defects; (b) identification of somatic cell mutagens, because of their involvement in neoplastic transformation.

As far as test methods are concerned, it is recommended that OECD protocols be used, which updated six previous guidelines and introduced a new one in 1997. These guidelines provide guidance for the conduct of in vitro screening tests (e.g. gene mutations in bacteria and in mammalian cells, chromosomal aberrations in vitro) as well as for the in vivo assays (e.g. micronuclei and chromosomal aberrations in rodent bone marrow, rat liver unscheduled DNA synthesis, chromosomal aberrations in spermatogonia).

A number of useful techniques have been developed with which it is also possible to determine the genetic toxicology. For example, a protocol for in vitro micronucleus test is currently being evaluated for inclusion, which might be considered in test batteries as an alternative to in vitro chromosomal aberration assay. By using centromeric specific probes based on fluorescence in situ hybridization (FISH), chromosome loss and non-disjunction specific probes allow rapid scoring of aneuploidy in a variety of cell types, including human cells. mammalian cell assays should be routinely performed according to the standard updated protocols for the detection of either gene mutation (at *tk*, HPRT, or other loci), or structuralchromosomal aberrations by metaphase analysis.

On the other hand, non-genotoxic carcinogenicity of the compound is able to be evaluated through the following common mechanism:

- Persistent cytotoxicity accompanied by proliferative regeneration.
- Chronic inflammation
- Hormones
- Ligands for xenobiotic receptors
- DNA methylation

Furthermore, there are also some potential short-term tests designed for non-genotoxic carcinogens. They are:

- Detection of mitogenesis
- The application of gene arrays and other approaches to transcription profiling
- In vitro cell transformation assays
- Transgenic cell systems
- Cytosine methylation
- Quantitative structure-activity relationships(QSAR) and other computational approaches

Overall, no single system will be will be adequate to detect all non-genotoxic carcinogens or even a large number of them. However, by focusing on those mechanisms that appear to be of relevance to humans, it may be possible to identify key toxicological responses, which provide a clear indication of carcinogenic potential.

3.4.2. Developmental toxicity

Reproductive toxicology embraces studies on male and female fertility and on developmental toxicity, with special emphasis on embryotoxicity and teratogenicity. Over the past 20 years, more than 30 different culture systems have been proposed as tests for developmental toxicity. The culture systems fall into the following categories:

- Tests on non-vertebrate species, including Hydra, slime moulds, brine shrimps and Drosophila.
- Tests on lower vertebrate embryos or embryonic cell aggregates, including fish, amphibians and birds.
- Tests on whole mammalian embryos.
- Tests on micromass cultures from mammalian embryos (limb buds, midbrain).
- Tests on embryonic stem cells or embryonic stem cell lines.
- Tests on other mammalian cell lines (e.g. human embryonic palate mesenchymal cells, mouse ovarian tumour cells, neuroblastoma cells, teratocarcinoma cells).

3.4.3. Cell lines and embryonic stem cells

A number of established cell lines have been used for screening purposes in 1980s. These include: human embryonic palate mesenchymal cells, mouse ovarian tumour cells and neuroblastoma cells. The results show a high number of false positives.

However, the use of omnipotent embryonic stem cell lines shows more promising results. For instance, blastocyst totipotent embryonic stem cells (ES) can be cultured under conditions in which the cells form several types of differentiated cells, such as muscle cells or haematopoietic cells. These culture systems can be used to determine the two essential features of embryotoxicity: inhibition of differentiation and/or a higher sensitivity to cytotoxic effects in embryonic cells than adult tissues. Results of such a test were comparable to the outcome of an embryotoxicity test with rat whole embryo cultures. The use of ES cells in the production of transgenic cells with targeted mutations and reporter constructs should enable the development of tests with simplified endpoints, which can be used in robotised assay systems. What's more, new developments in which multipotent (or even totipotent) stem cells can be isolated from adult tissues are very promising. For example, nervous tissue stem cells can give raise to haematopoietic stem cells and vice versa.

Besides, testing systems using aggregate and micromass cultures, embryos of lower order species, avian and mammaliam whole embryo culture for detecting the developmental toxicity have been described and widely used. They allow the detection of dysmorphogenesis in many organs and the comparison of specific dysmorphogenic effects with general adverse effects on growth and differentiation. In addition, they enable the

potencies of structurally related compounds to be ranked. Limitations of these systems are related to the fact that they are relatively complex, cover only a part of organogenesis, require high technical skills and they also can be costly.

In conclusion, in vitro culture detection system is more suitable for anti-cancer drug and teratogenic, carcinogenic, and mutagenic chemicals. Main application area include that 1) Identify potential active compounds; 2) Study the mechanism of chemical toxicity; 3) Predict effective toxicity drug possibly used in treatment for cancer patients; 4) Screen effective component and its active range from a variety of compounds; 5) Determine the types of effector cells; 6) Confirm toxicity range; 7) Investigate relationship between the drug concentration and exposure time.

Although the drug efficacy, adverse reaction and safety evaluation of need certain animal experiment, the *in vitro* culture technology has already become common tool for testing cytotoxicity and efficacy globally. The advantages are that 1) established cell lines provide cell sources with uniform or similar genetic background for in vitro test, which make the drug selection more stable, convenient and economic.2) This technique can precisely control the object, time and dose of drug action as well as the cell growth conditions. For example, clinical medicine study can choose human cells. Tissue-specific drugs need to select targeted and relevant cells. Researchers employ tumor cell lines to evaluate anti-cancer drugs by observing the cell reaction to drug from cytological view. Therefore, this in vitro detection avoid the drawbacks of in vivo experiment about lack of specific effector cell, drug metabolism reaction, different drug resistance between species and individuals. 3) It's easier to distinguish the direct drug effect and indirect internal effect by adding drugs or other chemicals directly to cell culture system, or even injecting drug into the cell. 4) Animal protection moral appeal reduction of animal experiment internationally. Thus the application of in vitro technology in drug test becomes more and more widespread.

Definitely, determining the action of different drugs on the cell in vitro under various dosages is not completely suitable for in vivo status. The disadvantages of in vitro test are 1) in vivo growing environment is quite complicated compared to the simple in vitro culture condition. The in vivo experiments possess not only comprehensive functional regulating system, but also the metabolism, modification (such as increase or decrease of pharmaceutical biochemistry through the liver, kidneys and other organs), immunological effect. 2) Cytotoxicity and activity test using in vitro system is suitable for monomer drug other than compound medicines and Chinese patent medicine. 3) It is easier to test water-soluble drug compared to the water insoluble drug, which may need suitable solvent to dissolve and control group in experimental design to exclude the effect of the solvent itself to the cell. 4) In vitro system is mainly applicable to acute toxicity research, thus analysis of chronic effect requires improving the culture system.

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