Journal of Bioscience and Bioengineering
VOL. 116 No. 6, 647e652, 2013

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REVIEW

Cultured stem cells as tools for toxicological assays

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Received 17 August 2012; accepted 20 May 2013
Available online 1 July 2013

In the last 2 decades, cell culture techniques for both mammalian embryonic stem cells and adult stem cells have developed and improved, and are now widely available. These stem cells are either pluri- or multi-potent, which makes them favorable for use in vitro developmental toxicity assays. Recent studies have reported several applications for embryonic and adult stem cells in cytotoxicity and developmental toxicity testing. These applications have the potential to provide alternative assessment techniques for evaluating toxic substances, and possibly reveal novel toxic and developmental effects that are difficult to investigate in humans because of ethical considerations. In this review, we describe some of the new approaches that use mammalian embryonic and adult stem cells in toxicological safety testing.

[Key words: Developmental toxicity; Embryonic stem cell; Neural stem cell; Heavy metal; Safety assessment]

Recently, techniques for the proliferation of immature stem cells and the induction of these cells into terminally differentiated cells have been the subject of intense worldwide research, particularly after the successful isolation of human neural stem cells (1,2) and human embryonic stem (ES) cells (3), and the discovery in 2006e2007 of induced pluripotent stem (iPS) cells (4,5). The availability of human ES cells has improved substantially since the development of cell culture techniques using Rho kinase inhibitor (6). These stem cells contribute to tissue formation and organogenesis in vivo, and it is possible to induce them to generate several types of somatic cells in vitro. Therefore, they are potentially useful in regenerative medicine with the prospect of cell transplantation therapy or in in vitro drug-screening assays as target cells to evaluate drug candidates or investigate cell toxicity. The use of ES cells in toxicological assays has been reported for assessing the environmental impact of substances, and in addition, these cells are thought to be promising for use in developmental toxicity assays (7e10).

Stem cell-based toxicological assays are classified into the following 3 categories: (i) those that evaluate acute toxicity by determining viable cell count and hence cell survival rate (cytotoxicity assay); (ii) those that evaluate the inhibitory effect on the differentiation of stem cells into other cell types (developmental toxicity assay); and (iii) assays that determine the inhibitory or stimulatory effects on cell function of mature cells differentiated from stem cells (cell functional assay). For medical and/or ethical reasons, it is usually not possible to harvest somatic cells (such as neurons and cardiomyocytes) directly from humans for use in experimental assays. However, cells can be obtained through the differentiation of cultured stem cells. Table 1 shows various stem cell-based toxicological assays.

Stem cells can be categorized as pluripotent stem cells, which include ES cells and iPS cells, or somatic stem cells (sometimes referred to as adult stem cells). Pluripotent stem cells in an immature state have the ability to proliferate and differentiate into all tissue cell types (3e5). Recent studies have also reported that ES cells have the potential to differentiate into extra-embryonic tissue cells formed during fetal development (i.e., cells from the placenta and umbilical cord) (11,12). Somatic stem cells can proliferate in an immature state, and have the potential to differentiate into a limited number of cell types. As shown in Fig. 1, toxicological assay methods differ depending on the types of stem cells used. Methods for differentiating iPS cells into specific types of mature cells (e.g., cells of the nervous, digestive, circulatory, and reproductive systems) have been developed (13e17). Therefore, pluripotent stem cells have the potential for use in various types of assays.

APPLICATIONS FOR CYTOTOXICITY ASSAYS

Stem cell-based toxicological assays are classified into 3 categories, as previously described. Cytotoxicity assays are widely used to evaluate the cell-damaging effects of test substances. In most cases, cells are incubated in multi-well plates and treated with the serial dilutions of a test substance. Then cell survival rates are derived as a measure of cytotoxicity. Originally, tissue-specific cell lines with native cell functionality were used in such cytotoxicity assays, which enables the design of simple in vitro toxicity assays as alternatives for in vivo toxicity assays, and therefore allows for a reduction in the number of experimental animals (18e21). For example, epithelial cells in the digestive tract, and particularly in the intestine, are continuously renewed in a 3-stage process: (i)

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1389-1723/$ e see front matter
http://dx.doi.org/10.1016/j.jbiosc.2013.05.028

TABLE 1. Stem cell-based toxicological assays.

Assay	Cell type	Reference
Cytotoxicity assay	hMSC or mMSC	24,29,30,31
	hNSC, hNPC, or mNPC	32,35
	hESC or mESC	9,25,36,37
Developmental toxicity assay	hESC or mESC	9,25,36e38,43,44
	hNPC or mNPC	32,35
Cell functional assay	Cardiomyocytes derived from hESC, hiPSC, or mESC	13,47,48
	Neural cells derived from hESC	49,50
	Hepatocytes derived from hESC or hiPSC	45,46

h/mMSC: human/mouse mesenchymal stem cells; hNSC: human neural stem cells, h/mNPC: human/mouse neural progenitor cells; h/mESC: human/mouse embryonic stem cells; hiPSC: human induced pluripotent stem cells.

differentiation from somatic stem cells in the crypt; (ii) migration into the tips of the villi; and (iii) detachment from the villi into the lumen of the digestive tract. However, it is difficult to establish a primary culture of cells from intestinal epithelial cells. Therefore, the Caco-2 cell line, which is derived from human epithelial colorectal adenocarcinoma, has been widely used as an alternative in cytotoxicity assays (22).

When metal ions or xenobiotics of low molecular weight are introduced into the human body through oral or percutaneous administration, they are metabolized and excreted into urine through the complex multi-step processes that make up the drug and xenobiotics metabolic pathways. These processes consist of digestion, absorption into the blood, distribution to the body tissues through the blood stream, enzymatic conversion and metabolism in the liver (some compounds are excreted into the bile that then re-enter the digestive tract), and excretion by the kidneys; substances in the blood can also diffuse into extracellular tissues and interact with cell membrane or intracellular receptors.

The uptake of xenobiotics into the central nervous system (CNS) is more tightly controlled than that into other organs, because of the presence of the blood–brain barrier (BBB). The delivery of xenobiotics into target organs/tissues depends on multiple factors, including the initial steps of absorption and distribution across different tissues of the body as described above. Sometimes, it is necessary to carefully evaluate the concentration of each substance to assess its toxicity. Generally, immature stem cells will proliferate under suitable cell culture conditions, and strongly express drug transporter molecules that pump xenobiotics out from the cells (23). In cytotoxicity tests, these cells have been reported to show different responses in comparison to fully differentiated cells (24,25).

More than 10 years ago, the European Center for the Validation of Alternative Methods (ECVAM) published a protocol describing the embryonic stem cell test (EST), a test that investigates embryonic toxicity using mouse ES cells (The use of scientifically-validated in vitro tests for embryotoxicity, http://ihcp.jrc.ec.europa.eu/our_labs/eurl-ecvam/publication/Embryotoxicity_statements.pdf), and that has been validated by several studies (9,10,26,27). Cytotoxicity assays and assays that assess the extent of differentiation are used to evaluate the toxic risk of compounds, by using a formula to calculate risk. Many other reports on toxicity assays using ES cells are principally based on this method.

Somatic stem cells are also used to test for embryonic toxicity. The toxicity of metals used as biomaterials in medical devices has been investigated using mesenchymal and neural stem cells (24,28e34). Neural stem cells proliferate as neurospheres (clusters of cultured cells in suspension). Therefore, in addition to the cell survival rates, the number and sizes of neurospheres can also used as criteria for evaluation (32,35). In many studies using pluripotent and somatic stem cells, both the cytotoxicity assay and the test for differentiation potential have been combined to evaluate the risk of developmental toxicity (9,25,27,31,35e37). Cytotoxicity assays are widely used to evaluate cell-damaging effects. However, the half-survival concentration (IC_{50}) value of a substance has been found to vary if nonconsistent cell culture media are used. Therefore, it is important to take this into consideration when comparing the data from a stem cell assay with results obtained using established cell lines as a control.

APPLICATIONS OF DEVELOPMENTAL TOXICITY ASSAYS

During fetal development, abnormal differentiation of immature stem cells can lead to developmental abnormalities and carcinogenesis. Developmental toxicity assays are used to assess the risk of abnormal stem cell differentiation by exposure to a test material. In developmental toxicity assay testing, the ratio of specific cell types after differentiation is determined as the assay result.

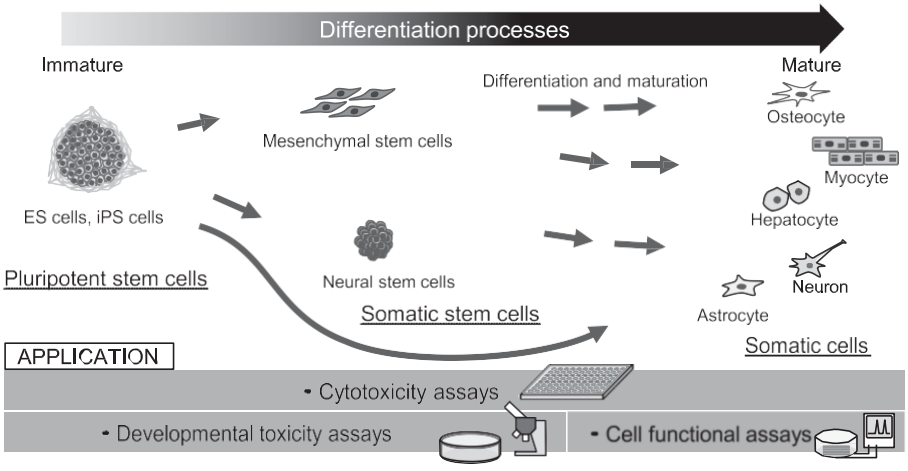

FIG. 1. Developmental stage of stem cells and their applications in toxicological assays. Stem cells are pluripotent or multipotent depending on their stage of development. Immature pluripotent stem cells and somatic stem cells can be used in cytotoxicity assays to evaluate the cell-damaging effect of test substances, and in developmental toxicity assays to evaluate effects on cell differentiation. In most cases, cells are incubated in multi-well plates and treated with serial dilutions of the test substance, after which cell survival rates are determined. Somatic cells can be used in cytotoxicity assays, but not in developmental toxicity assays. However, mature cells displaying readily detectable functional changes, such as action potential generation or albumin secretion, can be used in functional assays to evaluate changes in physiological status of the cells.

To obtain this ratio, techniques such as flow cytometry, colony analysis, gene expression, and fluorescent microscopic analysis are used (9,32,38). The ECVAM has recommended that the EST uses a combination of cytotoxicity and developmental toxicity assay testing with ES cells and 3T3 fibroblasts, as described previously (9,25,27,36,37). In this method, the IC_{50} value of a test compound is measured in both 3T3 fibroblasts and ES cells, to allow comparison. Additionally, the ID_{50} value (50% inhibition of ES cell differentiation) obtained by the measurement of the ratios of differentiation of ES cells into beating cardiomyocytes after the induction are then calculated. Both values are used to evaluate the risk of embryotoxicity. With regard to the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) legislation in Europe, and similar legislation in other parts of the world, the chemical industry faces the challenge of developing alternative methods to animal testing for risk assessing chemicals, and the EST protocol is part of this development. Specifically, the European cosmetics industry has to meet deadlines for the replacement of animal testing, and so must consider in vitro developmental toxicity assays, including the EST (39). However, the cell type is limited to myocardial cells only, and only the differentiation ratio is measured in the EST protocol. For the evaluation of neurodevelopmental toxicity, assays involving neural differentiation should also be carried out, as has been reported (25).

Neurodevelopmental toxicity assays are still under development. Many methods remain to be fully evaluated for use in environmental toxicity testing, and in screening for new drugs for CNS diseases (40e42). Novel methods using flow cytometry and transcriptome analysis have recently been reported (43,44). In addition to pluripotent stem cells, neural stem cells (a type of somatic stem cells) have also been used in testing for neurodevelopmental toxicity. In neurotoxicity studies, the three-dimensional (3D) structures of neurospheres were investigated and used to analyze cell differentiation, cell migration, and cell growth (32,35).

Embryo bodies (EBs) are usually prepared prior to somatic stem cells being generated from pluripotent stem cells. However, using this method, only a limited number of cells in EBs differentiate into somatic stem cells. Therefore, other cell types are always present. If the endpoint of the risk assessment or the drug screening protocol is related to specific cell types, e.g., neural or blood cells, the use of somatic stem cells that differentiate into these specific cell types are preferred; this avoids erroneous results from cellular heterogeneity.

APPLICATIONS FOR CELL FUNCTIONAL ASSAYS

The use of pluripotent and somatic stem cells that can differentiate into somatic cells avoids ethical issues associated with the harvesting of cells from human adult tissue. For the most part, cellular functions have been investigated to date using cell lines and primary cells harvested from animals. Recently, the electrophysiological and metabolic functions of somatic cells that had been differentiated from either ES cells or iPS cells were measured (13,45e51). Neurons and myocardial cells are 2 groups of cells that are suitable for cell function assays using electrophysiological measurements because the action potential is easily measured. In this assay, EBs were prepared via differentiation from ES cells. Myocardial cells were then generated from the EBs and cultured on the surface of a multi-electrode measurement system to monitor action potential propagation (13,47,48).

Neurons can be differentiated from either EBs or neurospheres of neural stem cells. In other published studies, neurons were generated and cultured on the surface of a multi-electrode measurement system to monitor the action potential generation from a spontaneously formed network of neurons (49e51). The patterns of neuronal activity (e.g., action potential frequency and synchronization between different neurons) were modified by adding substances to the medium, which allowed the toxicity of test substances to be evaluated (49).

Hepatocytes are important cells in toxicological assays and drug screening protocols because of their drug metabolizing activities. Recent studies have reported the generation of metabolically functioning hepatocytes from human ES cells and iPS cells (45,46). In these studies, the ES cell- or iPS cell-derived hepatocytes were characterized for albumin secretion and glycogen storage, as well as drug metabolizing capacities. Functional assays such as electrophysiological measurements and metabolic functional assays are applied to the limited cell types whose functions are found to be very evident. Therefore, other criteria such as changes in cell shape can also be used to evaluate the physiological state of cells and their functions in the native state.

STEM CELL-BASED TOXICOLOGICAL ASSAYS FOR HAZARDOUS ASSESSMENT OF HEAVY METAL IONS

In humans, the toxicity of heavy metal ions, such as lead (Pb) and mercury (Hg), is well recognized following poisoning incidents from pollution related to mining and industrial activities at the time of the industrial revolution, and even back to Roman times (52). The diseases caused by heavy metal poisoning are a recurrent issue that occurred not only in the past, but also occur today in many countries, particularly in developing nations as they become industrialized (53). Indeed, incidents of pollution caused by mining and housing paints have been recently reported in Brazil and China (54,55).

Heavy metals in the environment are ingested not only through food, but also through accidental consumption of everyday objects (e.g., from eating utensils, children's toys, and dental prosthesis). Recently, it was reported that lead poisoning occurred in children who accidentally swallowed toys. Epidemiological studies have evaluated toxicological data for heavy metals, but mostly in adults. Included in the mechanisms of toxicity for heavy metals are the inhibition of essential trace elements into cells, and the elevation of concentrations of reactive oxygen species that leads to cell death. However, many mechanisms remain unknown.

The mechanisms underlying the neurodevelopmental toxicity of heavy metals on fetuses and newborn babies have not been fully elucidated. However, the number of associated reports is currently increasing alongside progress in stem cell research (25,32,43,44). The mechanisms underlying neurodevelopmental toxicity in neural stem cells are being intensively studied because this toxicity can results in developmental disorders within the CNS (52). Studies are investigating not only the well-known toxic heavy metals, such as cadmium (Cd) and Hg, but also others such as zinc (Zn), which is an essential micronutrient that can affect somatic stem cells. These studies will clarify the effect of heavy metal ions on the differentiation of somatic stem cells, and also their migration during developmental tissue formation.

IMPORTANCE OF CONDITIONING FOR STEM CELL CULTURE IN TOXICOLOGICAL ASSAYS

A difficult challenge in the use of cultured stem cells in toxicological assays is to culture the cells under ideal conditions. Human ES and iPS cells require feeder layers to maintain them in an immature state. In many experiments, mouse fibroblasts are used as feeder layers. If the addition of a substance decreases the number of viable cells, it is difficult to determine whether the substance affected the stem cells directly, or whether it affected the feeder layer so affecting the stem cells indirectly. Recently, feeder-free

culture systems using extracellular matrices (ECMs) and other effective compounds have been developed, thus circumventing this problem (56). However, whether in toxicological assays the stem cells in a novel feeder-free culture system yield the same or slightly different results compared to a conventional system with feeder layers remains to be seen. Of course, appropriate standardization will be required.

DOWNSIZING OF TOXICOLOGICAL ASSAY SYSTEMS COMBINED WITH THE USE OF CELL TIPS AND MICROREACTORS

Significant advances are currently being made in technologies for cell manipulation. Micro-electrical/mechanical systems (MEMS), microreactors, tip technologies, and bio-sensing are technologies that are finding combined usage for manipulating cells and evaluating their properties and functions in culture. Examples of microreactors and cell tips being used in this way were described by Kaur and Singhal (57). When clusters of cells such as neurospheres or EBs are formed on a cell tip, their cellular functions can be improved so making the identification of their 3D location easier (58,59). Cell tips with grooves of 200 mm width on which clusters of cells are attached have been designed and are useful for detecting the clustered response of cells to stimuli. A scaffold consisting of gels or sponges supporting mesenchymal stem cells has also been used to construct a 3D-model of mesenchymal tissue (60). ES cells have been cultured on a tip that itself was fabricated using MEMS technologies (61). Concentration gradients of the analyte can be included in a tip device (or in microchannels), and so used to estimate the concentration threshold for an effect on cells (62,63). This type of measuring device is interesting because a test substance may yield different effects on the cells depending on its concentration relative to the threshold, similar to morphogens in developmental biology.

The physiological state of cells can be evaluated using high-throughput analytical methods, such as cell morphology imaging, cytotoxicity assays, fluorescent microscopy combined with fluorescent probes, among other methods (62,64,65). The measuring system should be simple and easy to use by operators, and provide repeatable results for it to be suitable for use in environmental toxin evaluation and quality control. The miniaturization of such systems and the standardization of evaluation techniques will of course be necessary to make the systems fit for purpose.

Recently, an increasing number of studies have shown examples of different cell types being cultured together on a micro-scale. Functional and support cells co-exist in most types of living tissue, for example the vascular endothelial cells of microvessels (61,66–68). The generation of 3D tissue-like structures with microvascular network can prolong the viability of artificial tissues in culture, as demonstrated in vascular tissue (69). A functioning tissue-based assay, albeit artificially generated, will be helpful in the risk evaluation of toxic substances.

Takahashi et al. showed that human iPS cells can differentiate into several types of cells (4). Therefore, in theory, a patient's own cells could be used to establish a tailor-made assay system using stem cells (70). In the future, it will be possible to evaluate the specific potential risk of a toxic substance prospectively before the body is exposed to the substance. However, there are many technical problems and obstacles that need to be overcome first. Controlling the growth of pluripotent stem cells and their induction into specific cell types on a micro-scale depends on experimental skill, which can be likened to a craftwork; both single cells and cell clusters need to be handled in this process. Automating the entire process using a microchannel connected to a microreactor containing the culture medium is difficult. Therefore more collaborative studies are needed between stem cell researchers and bioengineering researchers to develop standardized methodologies for use in toxicology assessment systems.

IMPORTANCE OF THE STEM CELL-BASED ASSAY SYSTEM IN TOXICOLOGY

If terminally differentiated cells are used in toxicological assays, it is relatively easy to carry out the cytotoxicity assay by measuring cell proliferation. However, evaluating the differentiation of cells into other cell types is difficult. Results for in vitro assays using stem cells are likely to be related to teratogenesis and developmental defects observed in in vivo experiments. If a toxicological assay is carried out with experimental animals in vivo, it is possible to test for teratogenicity and other developmental toxicities. However, estimating the exact concentration of the test substance for toxicity is difficult because of the varying absorption and distribution parameters in live animals. Whole embryo culture is a possible method for evaluating the teratogenicity of toxic substances. However, sophisticated culture techniques are required, and these are not easy to carry out. Of course, whole embryo culture is not possible with human embryos, for ethical reasons. To narrow the gap between the cell-based toxicological assays and assays using experimental animals, tissue engineering technologies should be used to produce artificial 3D tissues for toxicological assay testing comprising cells derived from pluripotent stem cells. This will enable a determination of the effect of the analyte substance on cell differentiation and tissue plasticity (e.g., formation of new functional synapses between cultured neurons).

If a valid and reliable toxicological assay system using stem cells becomes established, it can be used in the risk assessment of pollutants and food additives, thereby contributing to public safety. Stem cells attract much research attention in regenerative medicine with the prospect of cell transplantation therapy being used to treat intractable diseases. Another possibility from stem cell research is their use in toxicological assays, particularly in testing for heavy metals. In Japan, many pollution-related incidents have occurred, including the outbreak of Minamata disease in the district of Kyushu. Novel technologies for assessing heavy metal toxicity will contribute to the current and future safety of the Japanese society, as well as that of other countries.

CONCLUSION

New discoveries have been made for the use of pluripotent and somatic stem cells (such as neural and mesenchymal stem cells), including the potential for use in toxicity testing. The ECVAM published a protocol for testing embryonic toxicity using ES cells. This protocol is likely to be widely used as a standard method for evaluating developmental toxicity, although the protocol will require modification depending on the tissues being tested. An assay system for evaluating developmental toxicity should be simple, easy to use, and reproducible if it is to be widely adopted as a standard method.

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