The Use of Cell Culture Systems for the Assessment of General Cellular Toxicity and to Detect the Nature and Location of Free Radical Damage

K. HORÁKOVÁ

Department of Biochemistry and Microbiology, Faculty of Chemical Technology, Slovak University of Technology, Bratislava, Slovakia

Abstract. In the process of developing compounds to counteract the damaging effects of free radicals in biological systems it is important to determine the type and the intracellular location of specific toxic events. For that purpose cultured cells are used, because a properly designed cell culture system allows to asses specific damage at the cellular and subcellular level and can contribute to an evaluation of the biochemistry of free radical damage. A wide range of changes in cellular activities resulting from oxidative injury *in vitro* have been demonstrated. Data from many laboratories indicate that cell culture system coupled with appropriate analytical techniques can be used to explore cellular and biochemical details of damage induced by free radicals. The type of reactive oxygen species used to generate the radicals, the rate of radical production, and the location of action of the toxic species must be taken into account to understand the biochemistry of the system. An effort is made to analyse the considerable progress made in the development of appropriate *in vitro* models and end-points for use in testing and characterising the nature and location of free radical cytotoxicity.

Key words: Reactive oxygen species — General cellular toxicity — Free radical cytotoxicity — Cell culture systems — Analytical techniques

Introduction

During the last three decades it has been shown that cell culture systems offer an interesting empirical approach for toxicology especially in three research areas: for studying the metabolism of new drugs, for predicting their toxicity and for the explanation of their mode of action (Paganuzzi – Stammati et al. 1981). Toxicity test with mammalian cell cultures can be used to screen for toxicity both by estimation of the basal functions of the cell, or by tests on specialized cell functions. The cytotoxic concentrations of chemicals determined *in vitro* have been shown

Correspondence to: Prof. Dr. K. Horáková, Department of Biochemistry and Microbiology, Faculty of Chemical Technology, Slovak University of Technology, Radlinského 9, 812 37 Bratislava, Slovakia. E-mail: horakova@chtf.stuba.sk

to correlate well with lethal doses in laboratory animals and man for a range of selected drugs and chemicals (Ekwall 1983). Cytotoxicity tests using specialized cells have proved most useful when the *in vivo* toxicity of a chemical is already well established, and where *in vitro* investigations using specialized cell cultures have been used to clarify the mechanism of toxic action on the target tissue (Ekwall et al. 1990)

The aim of this paper was to illustrate the considerable progress made in the development of cell systems and end-points for use in characterising the mode of action induced by reactive oxygen species (ROS) generators.

In vitro methods for the assessment of general cellular toxicity

For general toxicity studies the more commonly used cell lines include the well-characterized diploid human fibroblast lines, WI-38 and tumour cell lines, HeLa. The first and most readily observed effect following exposure of cells to toxicants is morphological alteration in the cell layer and/or cell shape in monolayer culture. Gross modifications such as blebbing (suggesting injury of the cell membrane) or vacuolization (Table 1) can be observed using light microscopy (Horáková and Kalafut 1970) whereas fine ultrastructural modifications require analysis by transmission or scanning electron microscopy (Horáková et al. 1988). Another crude index of toxicity is cell viability measured using vital dyes. Counts of dead and vital cells in comparison with the control provide an index of lethality of the test compound.

The effects of drugs on the capability of cells to replicate are of fundamental importance for evaluating drug action. Numerous end-points have been used by different investigators to measure toxicity (Table 1). Most of these end-points are quantitative and can be used to plot dose-response curves. Our previous studies showed that cytotoxicity measured by indirect methods often differs from results obtained by direct cell counting, with lower cytotoxicity suggested by the former methods. The colorimetric proliferation assays measure the number of viable cells, however this approach is not always the most reliable for determining the presence

Table 1. End-points for the assessment of general cellular toxicity*

Cell morphology
Blebbing, vacuolisation, fine ultrastructural modification
Cell viability
Trypan blue (enters dead cells), neutral red (actively taken up by living cells), ^{51}Cr release
Cell growth
Cell count, plating efficiency, DNA or protein content, incorporation of radiolabelled precursors, glucose consumption, lactate production, NR-test, KB-test, MTT-test
Metabolic parameters
O_2 consumption or ATP level, pool of DNA and RNA precursors, NADH-NAD conversion

*For references, see Ekwall et al (1990)

of proliferating cells. Such assay would fail when, for example protein, DNA, RNA, lysosomal or mitochondrial enzymes synthesis is induced in an arrested cell population without any change in cell numbers (Horáková et al. 1978). On the other hand, simultaneous use of the above mentioned methods combined with the morphological evaluation enables to estimate the mechanism of action if the metabolic activity affected by the cytotoxic compound is the principle of the indirect method (Horáková et al. 1993). Typically, when a reduced cytotoxic effect was determined by neutral red (NR-test), which shows the activity of lysosomal enzymes, microscopically multiplication and/or enlargement of lysosomes was observed. Similarly, the variation of the toxic effect measured by tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylformazan bromide (MTT-test), which shows the activity of mitochondrial reductases, was accompanied by the formation of formazan in the mitochondria of non-divided cells (Horáková et al. 1996).

MTT is known to interact with components of the mitochondrial respiratory chain (Slater et al. 1963) and its reduction has often been used as a means of assessing cell viability (Carmichael et al. 1987). It is able to enter cells, and by virtue of intracellular reduction to formazan offers in principle at least a means of also assessing any intracellular generation of superoxide (Nohl 1991; Rice-Evans et al. 1991). Burdon et al. (1993) proved that a significant portion of MTT formazan within cultured human tumour cells (HeLa) does result from intracellularly generated superoxide.

Specialized mammalian cell cultures for ROS toxicity testing

Free radical mediated damage is one of the mechanisms involved in the cytotoxic effect of many drugs (Burdon 1992). Such damage can be reduced by various cellular enzymatic and non-enzymatic systems (for a review see Johnson et al. 1994; Jantová et al. 1998).

Because cells consist of multiple compartments, it is necessary to understand the relationship between the source of free radicals, especially those generated extracellularly, and the location of damage within the cells. Different laboratories use

Table 2. Specialized cell culture systems used to demonstrate ROS generators and radical scavengers cytotoxicity

Mouse-rat:	neuronal hybridoma N18-RE-105	*Vroegop et al. (1995)*
Rat:	hepatoma cell lines,	*MacDonald et al. (1996)*
	blood-brain-barrier cells RBE-4	*Mertsch et al. (1996)*
Human:	different normal epithelial cells and fibroblasts,	*Grafström (1996)*
	experimentally transformed and tumorous variants (ME18),	*Anuszewska and Gruber (1996)*
	hepatoma HepG2, HeLa,	*Horáková et al. (1996)*
	skin fibroblasts (normal and Zellweger cells lacking peroxisomes)	*Hofer and Kremser (1996)*

different cell lines to investigate the nature and the location of free radical damage at the cellular level (Table 2). Cultures of a neuron-derived hybridoma, designated N18-RE-105 (N18) used by Vroegop et al.(1995), were derived from a mouse neuroblastoma and Fisher rat embryonic neural retina. Immortalized rat hepatocyte cell lines were isolated by introducing SV40 sequences into primary cells. They are investigated for the retention of phase I and phase II enzymes. The application of stable gene transfer techniques has served Grafström (1996) to establish metabolically competent, immortalized cells from various human epithelia. They express cell-type specific characteristics and reflect a multistep progression from normality to cancer.

Due to its naturally low SOD activity, HeLa cell line provides an excellent model to study cellular functions under conditions of reduced or enhanced oxidative stress, and the ability of antioxidants to protect the cells (Horáková et al. 1996). Three of the possible systems to generate ROS *in vivo*, namely Fenton reaction, photoreduction of riboflavin, and substances releasing NO were successfully adapted to fibroblast cell lines which lack functional peroxisomes (Köck and Kremser 1996). The fact that peroxisomal disorders are well defined biochemically and that peroxisomes consume a significant amount of the total cellular oxygen uptake, makes cells from patients suffering from these disorders (Zellweger Syndrome) suitable cell models for studying the peroxisomal participation in detoxification mechanisms (Hofer and Kremser 1996).

End-points for measuring cellular damage by ROS

To determine the site where a toxic agent produces damage, assays that relate damage to cell structure and functions are required. The fatty acids of membrane phospholipids are one cellular target of oxygen radicals, which as a consequence can undergo peroxidation and be broken down into several toxic carbonyl compounds (Canuto et al. 1995). The level of lipid peroxidation can be assessed in terms of malone-dialdehydes or 4-hydroxynonenal (Table 3). To determine the location and the degree of damage induced by ROS generators Vroegop et al. (1995) used two readily distinguishable cell surface transporters, namely, the active transport of alpha-aminoisobutyric acid and the facilitated diffusion of glucose as markers of membrane structural and functional integrity.

To define the relationship between the defence line against the toxic oxygen intermediates and the induction of cellular detoxification enzymes, glutathione peroxidase, catalase and superoxide dismutase are used. In a number of cases, differences in the activities of antioxidant enzymes have been found between normal and cancerous cells. Generally a deficiency of activity has been shown in cancerous cells. In this connection, recent studies have indicated that free radicals generated from anticancer drugs may play a critical role in their toxicity to human tumour cells (Anuszewska and Gruber 1996).

Previous studies have demonstrated a wide range of changes in cellular activities resulting from oxidative injury, including changes in membrane organisation, ATP levels, phospholipid metabolism, DNA damage (Carson et al. 1986),

Table 3. End-points used as markers of toxic effects induced by ROS in cultured cells

Synthesis or release of specific molecules
Malone-dialdehydes, 4-hydroxynonenal
Cell surface activities
Active transport of alpha-aminoisobutiric acid, facilitated diffusion of glucose, calcium influx
Synthesis, activity or release of specific enzymes
Phase I and II, glutathione peroxidase, catalase, superoxid dismutase, acyl-CoA oxidase, lactate dehydrogenase, cathepsin D, N-acetyl-beta-D-glucosaminidase, acid phosphatase
Alterations of metabolic pathways
Lipid peroxidation, ATP levels, phospholipid and LDL metabolism, DNA breakage, protein thiol cross-linking
Intracellular markers
Mitochondria, lysosomes, peroxisomes

and gene expression (Shibanuma et al. 1990). Changes in ion fluxes, particularly calcium homeostasis, also accompany oxidative injury and have been extensively documented in a number of cell types (Hyslop et al. 1986; Munns and Leach 1995). Data from several laboratories indicate that disruption in the homeostasis of Ca^{2+} and/or cell thiols plays a major role in the pathogenesis of cell injury associated with oxidative stress caused by several toxic agents or pathologic states such as ischemia/reperfusion or xenobiotic poisoning (Livrea et al. 1995). Other data indicate that mitochondrial damage is a crucial step in the sequence of events that leads to cell death under these conditions. Mitochondrial membrane permeabilization induced by Ca^{2+} is mediated by protein modification forming the so-called permeability transition pore sensitive to cyclosporin A rather than lipid peroxidation (Castilho et al. 1995).

The phospholipid-rich lysosomal membrane is a potential site of free radical attack. Leakage of lysosomal enzymes into cells and the surrounding extracellular space has been implicated in the pathogenesis of cell injury. To study the contribution of oxidative stress in cell cultures to lysosomal damage the release and activity of the lysosomal enzymes (e.g. acid phosphatase, N-acetyl-beta-D-glucosaminidase) were investigated (Navarová et al. 1998). A moderate degree of lysosomal rupture seems to result in cell damage of the apoptopic type, with pyknotic nuclei and shrunken cytoplasm. However, more extensive lysosomal damage is leading to autolytic degradation not only of cellular constituents, including nuclear DNA, but also to the rupture of cell plasma membrane, cellular swelling and necrosis (Zdolsek et al. 1993, Brunk et al. 1995).

Conclusion

Cell culture systems coupled with appropriate analytical techniques allow us to disclose differences in the mode of ROS action at different sites in the cells. The

results from different laboratories lead to following conclusions (a) There is a specificity to the location of damage produced in cells by particular reactive oxygen-generating species (b) The toxic activity involves multiple sites of action, including inactivation of metabolic activity of the cell as well as action at the cell membrane, however metabolic changes occur before the loss of plasma membrane integrity. (c) Radicals generated in the membrane can affect, though not necessarily in the same manner, a variety of membrane proteins.

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