Limitations of cellular models in Parkinson's disease research

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Summary. Cell cultures for Parkinson's disease research have the advantage of virtually unlimited access, they allow rapid screening for disease pathogenesis and drug candidates, and they restrict the necessary number of animal experiments. Limitations of cell cultures, include that the survival of neurons is dependent upon the culture conditions; that the cells do not develop their natural neuronal networks. In most cases, neurons are deprived from the physiological afferent and efferent connections. In Parkinson's disease research, mesencephalic slice cultures, primary immature dopaminergic neurons and immortalized cell lines – either in a proliferating state or in a differentiated state – are used. Neuronal cultures may be plated in the presence or absence of glial cells and serum. These different culture conditions as well as the selection of outcome parameters (morphological evaluation, viability assays, biochemical assays, metabolic assays) have a strong influence on the results of the experiments and the conclusions drawn from them. A primary example is the question of whether L-Dopa is toxic to dopaminergic neurons or whether it provides neurotrophic effects: In pure, neuronal-like cultures, L-Dopa provides toxicity, whereas in the presence of glial cells, it provides trophic effects when applied. The multitude of factors that influence the data generated from cell culture experiments indicates that in order to obtain clear-cut and unambiguous results, investigators need to choose their model carefully and are encouraged to verify their main results with different models.

Introduction

Most new experimental therapeutic strategies for Parkinson's disease (PD) are first evaluated in cellular models. The most prominent advantages are (i) easy access of cells in culture to genetic and pharmacological interventions, (ii) easy and repeated access to cells for imaging and various biochemical assays to determine viability, metabolic state, protein aggregates and other parameters, (iii) the possibility to implement high-throughput screening for drug candidates and (iv) they restrict the number of animal experiments that need to be performed. Of course, these advantages come at the cost of several limitations. In this review, we will briefly discuss some cellular models of Parkinson's disease and methods used with them.

Cultures

Immortalized cell lines

Immortalized cell lines display the advantages of cell culture models to the greatest extent. They represent homogenous populations of continuously proliferating cells. This

allows large-scale experiments with reproducible results in a variety of tests. Genetic material can be brought into the cell effectively by chemical and physical means and cells can be frozen at $-150°$C. This allows to generate banks of cell lines, stably expressing various proteins of interest. Overexpression of a newly identified disease-causing gene or if a new mutation within a known gene is commonly the first step in its characterization since various biochemical and molecular studies can be readily performed. Freezing aliquots of low passage cell stocks is crucial to reproduce results since even cell lines are known change their appearance and behaviour with increasing passages as they accumulate genetic and epigenetic alterations. This effect, known as "replicative senescence", can

Table 1. Culture systems of dopaminergic neurons

Name	Origin	Neuronal differentiation	Dopaminergic phenotype[1]	α-Synuclein pathology
SH-SY5Y	human neuroblastoma	retinoic acid ±BDNF	DAT, D2R, DA, TH, vesicles, DA release[2]	eosinophilic cytoplasmic inclusions upon co-overexpression of α-synuclein and synphilin-1[3]
PC12	rat neuroblastoma	NGF	DAT, D2R, DA, TH, vesicles, DA release[4]	synuclein is upregulated following NGF[5]; cytoplasmic inclusions upon proteasome inhibition[6] and methamphetamine[7]
MN9D	mouse hybrid mesencephalic cells	GDNF, retinoic acid, overexpression of Nurr1[8]	DA, TH, D2R. no DAT[9] but sensitivity to low doses of MPP^{+}[10]	no record found
CSM14.1[11]	rat embryonal mesencephalon	39° (temperature-sensitive immortalization)	TH	no record found
MESC2.10[12]	8 week old human mesencephalon	tetracycline, GDNF, dibutyryl cyclic AMP	TH, DAT, DA release, electrically active	express endogenous human WT α-synuclein, inclusions following overexpression of synuclein and administration of amphetamine
primary midbrain cultures	E13 rodent embryo midbrain	without special treatment	5–10% dopaminergic neurons	endogenous synuclein-1[13]; inclusions following proteasome inhibition[14]
organotypic slice cultures	postnatal rat substantia nigra pars compacta	already differentiated	postnatal dopaminergic neurons in their natural environment	no record found

[1]Abbreviations: *DAT* Dopamine transporter, *D2R* D2 dopamine receptor, *DA* dopamine, *TH* tyrosine hydroxylase; [2]Presgraves et al. (2004a, b); [3]Marx et al. (2003); Smith et al. (2005); [4]Pothos et al. (2000); Beaujean et al. (2003); [5]Stefanis et al. (2001); [6]Rideout et al. (2001); [7]Fornai et al. (2004); [8]Transcription factor that is active in dopaminergic neurons; [9]Hermanson et al. (2003); [10]Kim et al. (2001); [11]Haas and Wree (2002); [12]Lotharius et al. (2002); [13]Rat homologue of human alpha-synuclein; [14]McNaught et al. (2002)

make it hard to reproduce results generated with one special batch of cells. Another drawback of proliferating cells is the difficulty to differentiate between growth arrest and real cell death as both ultimately result in a difference in cell number between "treated" and "untreated" cells. In our hands, SH-SY5Y exposed to $250\,\mu M$ 1-methyl-4-phenylpyridium (MPP$^+$) displayed only growth arrest after 48 and actual cell death not before 72 h (Soldner et al., 1999). We therefore encourage to establish the occurrence of cell death in each paradigm using a morphological marker (see below) before drawing conclusions from biochemical data or overall cell numbers.

As researchers in Parkinson's disease pathogenesis we are interested in the survival of mature dopaminergic neurons of the human substantia nigra pars compacta (SNc). Major characteristics of some widely used or upcoming "dopaminergic" cell lines are summarized in Table 1, assuming that neuronal differentiation, dopaminergic phenotype and the ability to form aggregates of alpha-synuclein – the histological hallmark of Parkinson's disease – are relevant traits to model PD. It is important to keep in mind, however, that even midbrain dopaminergic neurons are not a homogeneous population. Neurons in the SNc are much more strongly affected in PD patients and more vulnerable to MPP$^+$ toxicity than neurons in the ventral tegmental area (VTA). Gene expression profiles of these two populations have been performed recently (Chung et al., 2005) and may indicate some further susceptibility traits specific for PD.

All of the immortalized cell lines listed in Table 1 can be differentiated to a more neuron-like phenotype including cellular processes. Neuroblastoma cells can be differentiated by retinoic acid and/or growth factors. CSM14.1 cells have been immortalized by a temperature-sensitive SV40 large T antigen. These cells differentiate at the imperimissive temperature of 39°C. Similarly, MESC2.10 cells have been immortalized

with a LINX v-myc retroviral vector under control of a tet-system, allowing differentiation by tetracycline. Neuronal differentiation and in particular the growth of neuronal processes is an important feature to make a better model for neurodegenerative diseases. We and others have shown that neurotoxins such as MPP$^+$ damage neurites long before they kill neuronal somata and therapeutic strategies may vary greatly in their effect on neuritic integrity and functional parameters on one hand and survival of the cell body on the other hand (Herkenham et al., 1991; von Coelln et al., 2001; Berliocchi et al., 2005). After differentiation, however, cells are generally more delicate and gene transfer is made more difficult, often leaving viral vectors as the only possibility. They are also no longer amenable to FACS sorting (see below) and some other techniques requiring cell suspensions.

Primary immature dopaminergic neurons

Primary mesencephalic cultures are typically prepared from E13 rouse or rat embryos. They contain midbrain dopamine neurons cultured in the context of their physiological neighbours. As other primary neuronal cultures, neurons readily differentiate and form neurites and synapses. Even though these cultures are often referred to as "primary dopaminergic neurons", TH positive neurons actually make up only 5 to 10 percent of the total population. This constitutes an often invincible obstacle for viability assays and even more for many biochemical assays because significant changes that may occur in dopaminergic neurons are diluted or cancelled by changes in non-dopaminergic neurons. Moreover, there is more variability between cultures prepared on different days and by different investigators than in cell lines due to variations in preparation and processing of the tissue.

Gene transfer in primary neuronal cultures is more difficult than in cell lines. Calcium

phosphate transfection works in some cases; in most cases viral gene transfer has to be used. In our hands, lentiviruses have been successfully used whereas adenoviruses and adeno-associated viruses infect GABAergic but not dopaminergic neurons in primary midbrain cultures.

Help for the use of primary mesencephalic cultures may come from the growing field of functional and automated fluorescence microscopy (for a recent review see Bunt and Wouters, 2004). These techniques will eventually allow to specifically follow individual cells in culture, determine on a single cell level activation of second messenger cascades or protein aggregation – features that thus far relied on western blots and other "bulk" techniques. Once this has been accomplished, the low percentage of dopaminergic neurons in primary midbrain cultures and the often low transfection efficacy in these cultures will not be a problem for productive research on these neurons anymore.

In all cellular models, neurons are evidently deprived of their natural environment, of afferent and efferent connections. As these connections are an integral part of a neuron's life in the brain, this is no trivial change. Even though cultured cell do engage in cellular contacts, these are two dimensional only. One side of the cell is usually occupied by the glass or plastic support and another side by the liquid cell culture medium. Much of the "natural" environment is substituted by this culture medium. Consequently, the exact culture conditions largely determine the survival of specific types of cells. For example, primary cultures are almost purely neuronal when plated without serum. With serum, there is glial growth as well, helping the survival of neurons but complicating biochemical and viability assays even further – unless cocultures of neurons and glia are intended to study their reciprocal interactions. Another critical factor in primary cultures is cell density with cells plated at a higher density surviving better (Falkenburger

and Schulz, unpublished observation) and being more resistant to serum withdrawal (Collier et al., 2003).

Organotypic cultures

Some of the drawbacks just mentioned may be avoided by culturing not dissociated neurons but brain slices (Sherer et al., 2003b; Jakobsen et al., 2005). Typically, brain slices from postnatal rat pups are used, cultured either on coverslips in "roller tubes" as originally described by Gähwiler (1988) or on membranes at the interface between medium and the air of the incubator (Stoppini et al., 1991). Organotypic cultures combine features of cell culture and intact animals: Neurons are more easily accessible for pharmacology, gene transfer and – most importantly – repeated imaging than inside the intact animal. Gene transfer has to rely on viral vectors, however, which are either injected to locally in the slice or added to the culture medium (for a recent review see Teschemacher et al., 2005). By choosing appropriate slicing planes, important neuronal projection (such as the nigrostratal tract) can be preserved in the culture, in addition to the local neuron-glia microenvironment. Moreover, postnatal neurons are more fully differentiated than embryonic tissue used for primary cell cultures. Similar as in primary dopaminergic cultures, "bulk" techniques to determine viability and biochemical alterations are generally difficult, measuring dopamine production can be useful in some paradigms. It may be expected that with the advancement of imaging techniques such as two-photon microscopy this culture will gain relevance for some applications.

Methods

Inducing oxidative stress

The use of 6-hydroxydopamine (6-OHDA) and MPP^+ to study cell death of dopaminergic neurons in culture has been reviewed recently by others (Collier et al., 2003; Dauer and Przedborski, 2003). Therefore, we want to focus on two critical aspects of these widely

used paradigms. Both neurotoxins are taken up into dopaminergic neurons through the dopamine transporter (DAT). Inside the cell, they lead to the production of reactive oxygen species, inhibition of oxidative phosphorylation, and cell death. The mode of cell death depends on the concentrations that are used and is controversially discussed. As they very selectively kill dopaminergic neurons, 6-OHDA and MPTP (which is metabolized into MPP^+) are commonly used to generate animal models of PD. Interestingly, the regional distribution of neuronal cell death following systemic MPTP treatment parallels that observed in PD patients which has been correlated to the regional distribution of the DAT (Uhl et al., 1994). Therefore, a large part of the selectivity of MPP^+ and 6-OHDA results from their uptake through the dopamine transporter. As a consequence, inhibitors and modulators of the DAT will give false positive results in a neuroprotection study using MPP^+ or 6-OHDA. In addition, it cannot be inferred from MPP^+ and 6-OHDA data alone whether dopaminergic neurons are just sensitive to these toxins because they are accumulated by the DAT or whether these neurons are also particularly vulnerable to this mechanism of toxicity – that is mitochondrial impairment and oxidative stress. Since oxidative damage and mitochondrial impairment have been observed in the substantia nigra of PD patients (Dauer and Przedborski, 2003), this is nonetheless most likely the case. In addition, it is convenient to selectively challenge dopaminergic neurons in a mixed primary or organotypic culture and appealing to move from the MPP^+ cell culture to the MPTP mouse model.

To overcome this dependence on the dopamine transporter, the non selective mitochondrial complex I inhibitor rotenone has been used by several groups. Rotenone induces oxidative stress in cell lines, slice cultures and animals (Sherer et al., 2003b). Dopaminergic neurons appear to be particularly vulnerable to rotenone toxicity again indicating that oxidative stress may represent an important pathogenic factor in PD.

Inducing protein aggregates

Histologically, PD is characterized by depletion of dopaminergic neurons in the substantia nigra and by cytoplasmic inclusions of aggregated protein, so called Lewy bodies. As mentioned above, selective degeneration of dopaminergic neurons can be mimicked by MPP^+. Additionally, chronic but not acute exposure to MPP^+ and rotenone have been shown to induce Lewy-body like inclusions in animals (Sherer et al., 2003a; Fornai et al., 2005). In cell culture, protein aggregation is commonly induced by proteasome inhibition or by overexpression of mutant genes hat have been linked to PD in patients (see Table 1). The intri-

guing result that aggregates may also be induced by cytoplasmic dopamine following methamphetamine treatment (Fornai et al., 2004) along with the role of dopamine adducts for protein aggregation indicate that dopaminergic neurons are particularly susceptible to aggregate formation.

Protein aggregates can be assayed and defined in various ways: (i) high-molecular smear on gel electrophoresis from cell lysates, (ii) SDS insoluble pellet or proteinase K resistant fraction of cell lysates, (iii) ''bright fluorescent spots'' following overexpression of GFP fusion proteins, (iv) areas of FRET between two fluorescently labelled proteins, (v) thioflavine-S or thiazin red positive inclusions, (vi) aggregates of fibrils in electron microscopy. Which of these methods is used depends on the experimental design and the technical equipment available at the institution. In general, fluorescent imaging has the advantage that live cells can be repeatedly assayed whereas techniques such as the assay of oligomeres on gel electrophoresis or of fibrils in electron microscopy provide a better picture of what we are actually looking at. To what extent the different methods target overlapping species has unfortunately not been investigated in detail. Interestingly, protein aggregation seems to be a specific process occurring more readily in differentiated than in undifferentiated neurons (Hasegawa et al., 2004), suggesting that undifferentiated and non-neuronal cell lines most likely are less suited models to study protein aggregation.

Assays of cell viability

In most studies concerning the pathogenesis or experimental therapies of PD, cell viability or survival is the main target outcome. Therefore, the method used to assay cell viability must be carefully chosen. In undifferentiated cell lines, surviving cells can be counted using a Neubauer chamber or automated devices. This does, however, not differentiate between effects on cell proliferation and effects on cell viability (see above). Viable cells are characterized by their capacity to exclude and prevent staining by trypan blue (blue staining in light microscopy) and propidium iodine (red fluorescent nuclear staining) and their capacity to contain overexpressed proteins such as GFP and its fusion proteins. Other viability stainings use cellular enzymes to generate fluorescent, membrane-impermeable molecules from membrane-permeable precursors such as fluorescein diacetate or different calcein probes. Biochemical assays measure LDH which is contained in viable and released from dying cells or probe mitochondrial function (MTT and alamar blue assay for example). Generally, biochemical assays are better suited for large-scale and high-throughout analysis be-

cause they can be performed in a mircotiter plate format. Morphological methods have the advantage that they look more directly at what we are interested in: the survival of neurons. Also, they allow differentiation between effects on proliferation and viability as mentioned above (Soldner et al., 1999).

Fluorescence activated cell sorting (FACS) is increasingly used to conveniently measure a variety of parameters. It allows assays of (i) cell number: total cell number, number of fluorescently labelled cells, fraction of double-labeled cells, etc. (ii) cell cycle analysis by quantifying the amount of propidium-iodine-DNA staining per (permeabilized) cell (iii) detection of dying cells by propidium iodine staining of unpermeabilized cells, (iv) detection of apoptosis through the recognition of surface expression of annexin. It is therefore a very powerful technique to assay cell proliferation and cell death, which has been used in primary neuronal cultures (Garcia et al., 2005). However, we and others have not been able to use FACS in differentiated neuronal cultures since neurites tend to rupture and neurons die when they are manually detached from their support.

In differentiated cell lines, primary and organotpyic cultures, most of the morphological and biochemical analysis mentioned above habe been used, in addition to more more specific functional test such as dopamine uptake. In primary mesencephalic cultures, staining or co-staining for tyrosine hydroxylase is commonly performed to assay surviving dopaminergic neurons since assays of total viability are dominated by the 95% non-dopaminergic neurons in these cultures. However, numbers get very low (and variability is high) when one has to compare the viability of TH-positive and successfully transfected cells between two different conditions. Advances in automated microscopy and statistics coming from clinical trials might provide an escape to this. Recently developed systems allow us to automatically and repeatedly find a large number of specific areas within a multiwell plate, take a picture and analyze it offline. It is therefore conceivable that we will in the future not compare the number of surviving neurons in treated and untreated cultures but rather follow a large number of defined cells in culture and correlate their survival with the treatment they have obtained. However, the statistical analysis of these data will also be considerably more challenging (for a recent example, see Arrasate et al., 2004).

Conclusions

In this review, we briefly mentioned some cellular models used in PD research. We discussed some of the methodological limitations associated with them and in some cases indicated possible solutions. The selection of models and limitations was necessarily subjective and incomplete. However, similar arguments may apply to other models as well. We strongly believe that cellular models have an important role for investigating general principles of cell stress and cell death, but also for exploring new treatment strategies. Undifferentiated cell lines can be used for initial and screening experiments. Positive results should be validated in differentiated or primary cultures before moving to animal models. It should be kept in mind, however that some aspects of neuronal degeneration are specific to postmitotic neurons and therefore have to be investigated in primary or organotypic cultures.

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