Simulation of Stroke-related Damage in Cultured Human Nerve Cells

Gerd Bicker[1], Andrea Gierse[1], Saime Tan[1] and François Paquet-Durand[1,2]
[1]University of Veterinary Medicine, Div. Cell Biology, Hannover, Germany;
[2]University of Lund, Dept. Ophthalmology, Lund, Sweden

Summary
We describe a novel cell culture protocol for the generation of neurons from a human teratocarcinoma cell line. These neurons were used to investigate hypoxic-ischaemic cell damage and for developing neuroprotective strategies. Cultures of human model neurons should eventually serve to reduce the number of experimental animals in cerebral stroke research.

Keywords: Excitotoxicity, glutamate, ischaemia, neural differentiation, neuroprotection, human, brain-cell cultures, organ-specific, reduction, replacement, pharmacological testing

Background Information

Why animal models for ischaemic brain damage?

Two rodent models are widely employed: the transient global forebrain ischaemia model, in which the entire blood supply to the brain is transiently interrupted (imitating cardiac arrest), and the focal cerebral ischaemia model, in which the proximal middle cerebral artery is occluded. In addition to the initial damage in the immediate vicinity of occluded blood vessels, cerebral ischaemia also results in a wave of delayed cell death that spreads to surrounding tissue, the so-called penumbra. Whereas neuronal death occurs rapidly in the ischaemic core, neurons remain viable for many hours in the surrounding penumbra, providing a period of possible therapeutic intervention ("time to treatment" window). After inducing transient ischaemia, the animal is allowed to recover for some time, then is sacrificed and the brain damage studied in detail. In such studies, the bioavailability of a potential drug, its pharmacokinetics, effects on blood pressure, body temperature and motor activity are also determined.

Excitotoxic cascade during stroke

Stroke is the third leading cause of death and an important cause of adult disability in industrialised countries. Most strokes are caused by an acute interruption of the brain's blood supply, which leads to tissue ischaemia in the particularly vulnerable central nervous system. To investigate the cellular mechanisms occurring in ischaemic brain damage, a variety of rodent models have been developed that mimic the pathogenic environment of nerve cells during stroke. Cell culture models are also finding increasing use (Honegger and Pardo, 2007).

Ischaemic neurons deprived of oxygen and glucose rapidly lose their energy currency ATP, their intracellular pH then drops, and they depolarize. Among other consequences, the neurotransmitter substance glutamate is released. The accumulation of the excitatory transmitter glutamate excessively stimulates glutamate receptors of neighbouring cells, inducing increases in intracellular calcium levels and the production of free radicals, which in concert orchestrate cell injury. This cellular disorder is called excitotoxic cascade. One approach to reducing brain damage following a stroke might involve the application of neuroprotective substances that inhibit various stages of this cascade.

Generation of human nerve cells in the petri dish

We started with a well-characterised teratocarcinoma cell line (Ntera-2, NT-2) derived from a human testicular cancer. Upon treatment with the morphogen retinoic acid, the NT-2 line can be induced to differentiate into postmitotic neurons (Pleasure et al., 1992). This protocol involves a 5-7 weeks exposure with the morphogen, followed by two replates, a 7-10 days lasting treatment with mitotic inhibtors, and selective trypsinisation steps for neuronal purification. Taken together, the differentiation of NT-2 precursor cells into neurons requires between 44 and 56 days of cell culturing work and this is also the main disadvantage of this differentiation method.

www.forschung3r.ch/en/projects/pr_69_99.html

Time for differentiation is reduced by half

The conventional differentiation protocol uses adhesive substrates on which the cells are grown as monolayers. We seeded NT-2 precursor cells onto bacteriological grade petri dishes upon which these cells do not adhere. Under these conditions the cells proliferated as clusters in the shape of free floating spheres (Fig. 1a). At later stages of the neuronal differentiation process, the cells were again cultured as a monolayer. Allowing the cells to proliferate as free-floating cell spheres cuts the total time needed to obtain high yields of purified NT-2 neurons to about 24 - 28 days (Paquet-Durand et al., 2003). The cells obtained show neuronal morphology, migrate to form ganglion-like cell conglomerates, and are immunoreactive to neuronal cytoskeletal markers (Fig. 1b). Some epitheloid cells were also present in the petri dishes, but staining for neuronal markers indicated that after final plating the cell cultures were composed of approximately 90-95% human nerve cells (Fig. 1b). Calcium imaging techniques showed that NT-2 neurons express glutamate receptors including the N-methyl-D-aspartate type (Paquet-Durand and Bicker, 2004).

Cultured nerve cells are sensitive to ischaemic injury

In cell cultures the cell densities are lower than in intact tissue. Conversely, the volume of the surrounding medium is considerably larger than the extracellular tissue volume. Thus, only traces of oxygen are sufficient for cell survival. To mimic anoxia in culture, the NT-2 neurons were kept under an atmosphere of 95% argon and 5% CO_2; to ensure complete anoxia, remaining traces of oxygen in the medium were removed with non-toxic concentrations of dithionite. Glutamate served as excitotoxin. Cell viability was assessed using the Alamar Blue assay, which measures metabolic activity. The reduction in the cells' viability over time increased with the duration of anoxia (Fig. 2) and increasing glutamate concentrations. Anoxia times of four hours resulted in the destruction of more than 80% of the cells. Control experiments with undifferentiated NT-2 teratocarcinoma cells showed no vulnerability to anoxia (Paquet-Durant and Bicker, 2004).

An important parameter that influences the vulnerability of the mature postmitotic NT-2 neurons is the time in culture (Fig. 3) following the differentiation process. Older NT-2 neuronal cultures are more vulnerable to ischaemic insult, reflecting ongoing maturation of neurochemical properties *in vitro*. The cell culture model can be used to assay for potential neuroprotective compounds. We have shown that low doses of diltiazem, a licensed drug that is commonly used to treat cardiovascular disorders, protects the human model neurons against ischaemic damage (Paquet-Durand et al., 2006).

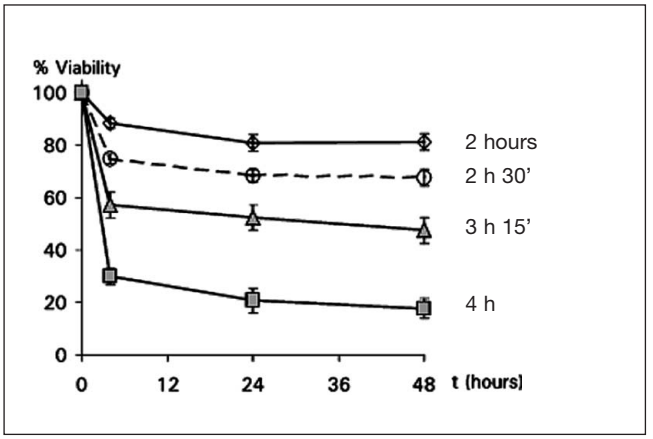

Fig. 2: Dependence of viability on duration of anoxia.
Cultured NT-2 neurons were subjected to anoxia in the presence of dithionite and 1mM glutamate. Duration of anoxia ranged from 2h to 4h. Viabilities were followed for 48 hours post anoxia treatment and plotted as percentage of internal control (100 %).

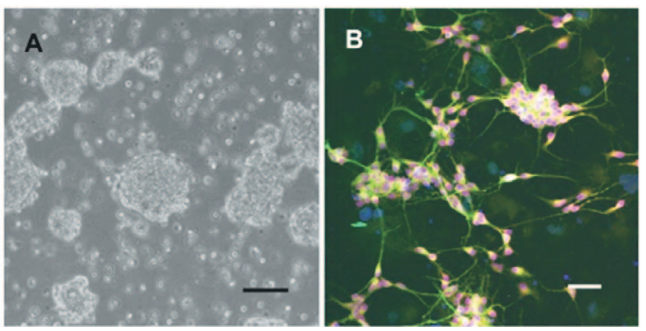

Fig. 1: Differentiation of NT-2 cells into neurons.
(A) Expansion of NT-2 precursors as free floating cell spheres. This technique resembles the sphere culture method used in embryonic stem cell differentiation. Scale 100 µm.
(B) Differentiated NT-2 neurons stained by immunofluorescence. Spindle shaped cell bodies send out neural processes.
Scale 50 µm

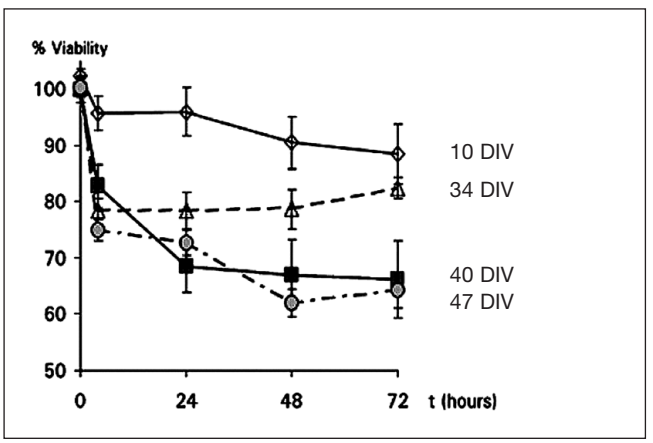

Fig. 3: Vulnerability of mature, post-mitotic NT-2 neurons depends on days *in vitro* (DIV) prior to anoxia treatment.
Times in culture ranged from 10 to 47 days. After 2h of anoxia, percentage of viabilities was followed for 72 h.

Reduction and replacement of laboratory animals

Stroke-related research often depends on animal models, because the interest is mainly focused on general brain damage after ischaemic lesions rather than on the effects on individual brain cells. The NT-2 cell culture system, in contrast, focuses more on the neuronal cell biology which has a number of advantages (Paquet-Durand and Bicker, 2007):

1) NT-2 neurons are derived from a human cell line, thus they are especially suitable for screening for neuroprotective drugs effective in the human brain.

2) The fairly homogeneous cellular composition of clonally derived neurons allows for large-scale cell-based assays.

3) Culturing NT-2 neurons in a monolayer readily allows electrophysiological recordings and optical imaging studies to investigate the excitotoxic cascade at a cellular level. Accordingly this cell culture system might

i) replace the use of primary cell cultures prepared from rodent embryos,

ii) reduce the number of laboratory animals needed in initial stages of drug screening and

iii) has the potential to replace laboratory animals in basic stroke research.

References

Honegger, P. and Pardo, B. (2007). Aggregating brain cell cultures: Investigation of stroke related brain damage. *ALTEX 24,* Spec. Issue, 32-34.

Paquet-Durand, F., Tan, S. and Bicker, G. (2003). Turning teratocarcinoma cells into neurons: Rapid differentiation of NT-2 cells in floating spheres. *Brain Res. Dev. Brain Res. 142*, 161-167.

Paquet-Durand, F. and Bicker, G. (2004). Hypoxic/ischaemic cell damage in cultured human NT-2 neurons. *Brain Res. 1011*, 33-47.

Paquet-Durand, F., Gierse, A. and Bicker, G. (2006). Diltiazem protects human NT-2 neurons against excitotoxic damage in a model of simulated ischemia. *Brain Res. 1124*, 45-54.

Paquet-Durand, F. and Bicker, G. (2007). Human model neurons in studies of brain cell damage and neural repair. *Current Molecular Medicine* (in press).

Pleasure, S. J., Page, C. and Lee, V. M. Y. (1992). Pure, postmitotic, polarized human neurons derived from NTera 2 cells provide a system for expressing exogenous proteins in terminally differentiated neurons. *J. Neurosci. 12*, 1802-1815.

Correspondence to

Prof. Dr. Gerd Bicker
University of Veterinary Medicine
Div. Cell Biology
Bischofsholerdamm 15/102
30173 Hannover
Germany
e-mail: gerd.bicker@tiho-hannover.de