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Motor neuron disease in vitro: the use of cultured motor neurons to study amyotrophic lateral sclerosis

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Abstract

Amyotrophic lateral sclerosis (ALS) is a lethal neurodegenerative disease in which motor neurons in the nervous system die. The cause is unknown, and no effective treatment exists. Mutations in the gene for superoxide dismutase found in a subpopulation have led to an animal model, but research with these mice has not produced complete insight into the disease mechanism. Studies with isolated motor neurons may produce important information. This review discusses approaches to culture motor neurons — single cells, cocultured with other cells, and in intact preparations, such as the spinal or cortical slice. Motor neurons in monoculture are suitable for acute but not for chronic studies, whereas cocultures and slices survive up to months and are used for chronic studies. Results with toxic substances believed to play a role in the disease, such as oxidants and glutamate, and of studies where the energy status of the cells is manipulated, are presented.

Keywords: Amyotrophic lateral sclerosis (ALS); Motor neuron; Apoptosis; Cell culture; Tissue culture; Neurodegenerative disease

1. Introduction

The study of neurodegenerative diseases has been hampered by the fact that, for a long time, no animal model was available for such diseases as Alzheimer's, Parkinson's and Huntington's disease, and amyotrophic lateral sclerosis (ALS), the main motor neuron disease in adulthood. At present, there is still no model for Alzheimer's disease, while for Huntington's and Parkinson's disease, drug-induced syndromes in animals exist that are similar to the human disease (Brouillet and Hantraye, 1995). The discovery of gene mutations in a small proportion of all ALS patients (about 2%, namely, 20% of the 10% familial ALS patients (Rosen et al., 1993)) has led to an animal model in which the human mutation is brought to over-expression (Gurney et al., 1994). Still, these diseases are so complex that parallel in vitro models, in which specific cell types or specific cellular events can be tested, are important to find clues or to test hypotheses about the aetiology of the disease. Cell and tissue cultures allow researchers to study a specific cell or tissue type under controlled conditions, and to obtain tissue- or cell-specific effects under well-defined conditions. It can only be the combination of such studies with animal models, molecular biology and results from human studies, which will eventually allow us to understand the underlying principles of these devastating diseases, and to produce intervention strategies.

1.1. Nerve cell culture

The fact that most neurons lose the ability to divide early in development has complicated the culture of neuronal cells. Whereas many cells, under the right conditions, can divide in culture and produce ample number of cells suited for all kinds of experimental approaches, neurons, on the other hand, do not divide and are notoriously difficult to culture in pure form. The intimate relationship with their environment, specifically with the glial cells, requires special measures to compensate for the loss in trophic support from these neighbouring cells; for example, by adding purified trophic factors or extracts from target tissues, such as muscles. Several approaches, ranging from using purified cells to organotypic cultures, have

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been developed and extensively used in our laboratory. Although easier to obtain, we do not use established cell lines for our research on ALS. These cells are often derived from transformed cells, either from a tumour or from cells that are dedifferentiated and transformed in vitro. In general, there seems to be a trade-off between their degree of differentiation — important for close likeliness to the in vivo situation — and their propensity to proliferate — important for large- or even medium-scale studies. Therefore, although transformed cells may be suited to study certain well-defined steps in metabolism, they are less appropriate to mimic the behaviour of, in our case, motor neurons in situ. An example of a cultured cell type that resembles developing motor neurons is a fusion product of mouse neuroblastoma with primary mouse, embryonic spinal cord motor neurons, and neuroblastoma × spinal cord (NSC) cell line (Cashman et al., 1992).

We have performed all our experiments with primary cultures, i.e. cells or tissue from young (embryonic or early postnatal) animals. The early stage of development of the cells thus obtained allows survival in vitro, but it also has the disadvantage that certain features of the adult phenotype may not (yet) be present. This potential disadvantage can be compensated for by leaving the cells to develop in culture. Indeed, it has been shown that with time in vitro, characteristics of adult cells are expressed by cells. Also, profuse neurite outgrowth and, under the proper conditions, even synapse formation form an indication that cells can mature while in culture. Whether a cell obtained at embryonic day 14 and cultured for 2 weeks has the same properties of a comparable neuron in a 1-week old rat pup has not been looked at systematically. The culture conditions, e.g. with or without growth factors or serum, or highly purified neurons versus neurons that are cocultured with glial cells will determine, for a large part, what properties are going to be expressed. For example, it is known that embryonic motor neurons in single-cell culture do not express choline acetyltransferase (ChAT), a key enzyme in mature motor neurons. In slice cultures or in cocultures, this enzyme is expressed after some time in culture (Kaal et al., 1997). In this article, we will illustrate the possibilities and limitations of several spinal motor neuron culture types, namely, single-cell culture, coculture with other cell types, and organotypic slice culture, based on our studies to elucidate the underlying mechanisms of ALS. Finally, we will discuss some preliminary results obtained with cultured cortical slices, which contain the so-called upper motor neurons that are also affected in ALS.

1.2. ALS

This disease is characterised by the death of motor neurons in the cortex, the brain stem, and the spinal cord. ALS is familial in about 10% of the cases and is sporadic in the rest. The course of the disease is undistinguishable between familial and sporadic ALS. The disease manifests itself late in life (average age of 56 years), and once diagnosed, leads to complete paralysis and death within 2–5 years (Robberecht and Van den Bosch, 1997). In about 20% of the familial patients, mutations have been found in a gene coding for Cu^{2+}/Zn^{2+} superoxide dismutase 1 (SOD1), a cytosolic enzyme involved in detoxification of free radicals (Rosen et al., 1993). Even if this discovery was seemingly relevant for only 2% of all ALS patients, it has led to an increased attention for the disease as a whole, and especially for the role of free radicals in its aetiology, not to mention the development of the first animal model for ALS. Other earlier findings (Plaitakis and Caroscio, 1987) had already pointed at the involvement of the neurotransmitter glutamate in causing motor neuron death. The only drug registered for ALS since 1998 is riluzole, which marginally prolongs the life of ALS patients and acts through blockade of the Na channel, resulting in the inhibition of glutamate release (Doble, 1996). Most attention in fundamental ALS research is focused on the role of glutamate and glutamate-induced toxicity or excitotoxicity (Shaw, 1994), glutamate transporters (Rothstein et al., 1992), and free radicals (Cookson and Shaw, 1999). Until quite recently, no suitable animal model existed for ALS (reviewed in Kaal et al., 1999a), and a lot of energy has been invested in producing motor neurons in culture in order to study their behaviour under, for example, high glutamate conditions or oxidative stress.

2. Motor neuron death

We have chosen to study motor neuron cell death when the cultures are exposed to conditions believed to occur in ALS, mostly, conditions related to oxidative stress. Using three different culture systems, we studied acute as well as chronic treatment of the cells. The variable that was usually measured to quantify the effect of treatment was cell death. Although it is not precisely known how motor neurons die in patients with ALS, it is generally accepted that this occurs via apoptosis (Fig. 1). Necrosis would cause an inflammatory response (Bär, 1996), and even though the number of cells dying over time is so low that it would probably cause a moderate chronic inflammation rather than a highly localised reaction, no signs of inflammation have ever been observed in postmortem studies. Support for apoptotic cell death in ALS comes from the recent finding that blockers of caspases, the enzymes that are the actual executioners of cell death, are able to prolong the life of transgenic ALS mice (Li et al., 2000). Interestingly, in humans, apoptosis has never been convincingly shown, but there may be several good reasons for this: first of all, the number of cells dying at any given time point is not very high, so the chance of observing apoptotic cells is low, especially as the time span during

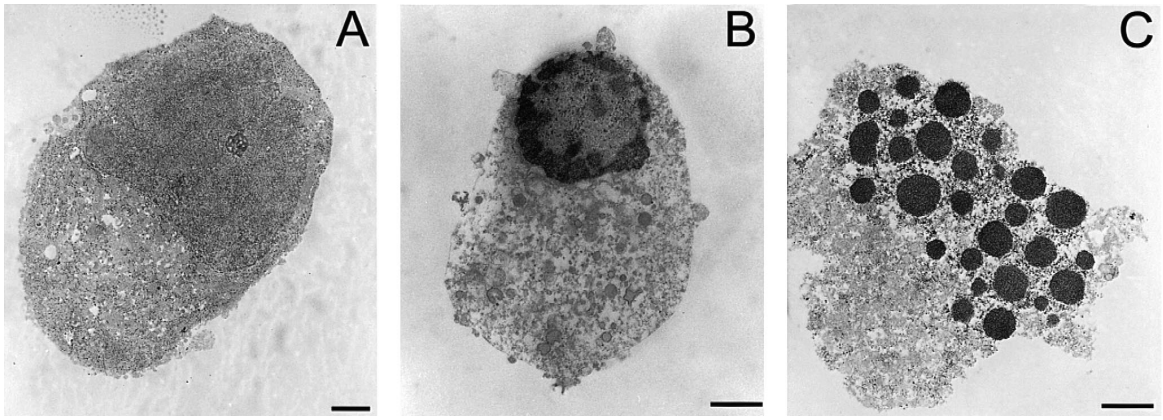

Fig. 1. Characteristic phases of apoptosis in a motor neuron visualised with electron microscopy. (A) Normal cell, with a kidney-shaped nucleus. (B) The cell is somewhat contracted, the nucleus is clearly contracted, and the chromatin is condensed along the wall of the nucleus. Some blebs — cell membrane protrusions — can be seen. (C) The nuclear material is broken down and packaged in so-called apoptotic bodies that can be phagocytosed by neighbouring cells. The cell membrane is still intact, and no leakage of cytosolic components takes place. Bars represent 2 μm.

which apoptosis can be recognised by immunohistochemical techniques, is probably less than an hour (Ellis et al., 1991). Furthermore, such studies are necessarily performed with autopsy material after a patient has died of his or her disease. In the final stages of ALS, most motor neurons have disappeared, making the chance of positive identification of apoptotic cells even more difficult. Also, in the time between death and autopsy, changes that occur in the tissue may obscure the morphological and cytochemical characteristics of apoptosis. This, in fact, is a general problem of neurodegenerative diseases: although apoptotic death is the most logical exit for neurons, it is difficult to obtain positive proof for its occurrence (reviewed in Savitz and Rosenbaum, 1998).

There are several ways to visualise apoptotic cell death, and the choice is dependent on the model system used. When single cells were cultured, we were able to follow the fate of individual cells by a mapping technique that identified each motor neuron in a given culture (Kaal et al., 1998a). In cocultures, this mapping technique can be used as well, but in organotypic cultures, because of the thickness of the slice and the resulting overlap of motor neurons with the other cells below or above it, we had to use other techniques. This problem can be circumvented by treating cells with drugs that specifically inhibit a key step in apoptosis, as shown below. It is, of course, still possible to recognise and count motor neurons in slice cultures with other staining techniques, for example, for ChAT or the cytoskeletal protein neurofilament, which is highly enriched in motor neurons.

3. Causes of motor neuron death

As mentioned above, it is widely believed that free radicals or oxidants play a role in the final stage of cell death in ALS. Although a disturbance in the free radical defence system is only positively shown in the familial ALS patients with a mutation in the gene coding for superoxide dismutase, the clinical symptoms of this small group (about 2% of all ALS patients) are so similar to those of other patients that it is reasonable to assume some final common pathway in ALS exists that leads to motor neuron death. The primary cause may be different for the different forms, and remains, so far, unknown. However, as stated above, there is reason to believe that the neurotransmitter glutamate is involved (Plaitakis and Caroscio, 1987; Shaw, 1994). Glutamate can cause cell death by overstimulating its receptors, a process called excitotoxicity, which probably plays a role in many neurological conditions, such as stroke, trauma, and neurodegenerative diseases (reviewed in Holt, 1997; Doble, 1999; Lee et al., 1999; Obrenovitch and Urenjak, 1997; Obrenovitch, 1999). The main consequence of glutamate receptor overstimulation is Ca^{2+} overload. The main port of entry is the NMDA receptor-linked Ca^{2+}/Na^{+} channel, which, once unblocked by membrane depolarisation, allows the entry of large amount of Ca^{2+} into cells. Motor neurons are extra sensitive to glutamate because the (adult) α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor, which, in most cells, only depolarises the postsynaptic cell, lacks the so-called GluR2 subunit, which makes the normally Ca^{2+}-impermeable AMPA receptor permeable to Ca^{2+} (Greig et al., 2000). Ca^{2+} overload is often the central issue in cell death (Siesjö, 1988): Ca^{2+} stimulates many enzymes and, while some enzymes (phospholipase A2, calpain) degrade structural elements of the cell (Bazan and Allan, 1997; Bartus, 1997), others (xanthine oxidase, phospholipase A2, cyclooxygenase) produce radicals as by-products (Bazan and Allan, 1997). Under these conditions, when the normal Ca^{2+}-pumping and removal systems fail, mitochondria will sequester Ca^{2+}, and thereby

lose their capacity to produce energy. This biochemical cascade occurs in many diseases (trauma, stroke), and leads to cell breakdown and death.

4. Role of the mitochondrion

Recent findings show that the mitochondrion has a remarkable double role in cell death. Classically, complete blockade of mitochondrial activity, i.e. in energy production, results in acute, necrotic cell death, as seen, for example, in the focus of an infarct. However, it appears that the mitochondrion also plays a central role in apoptotic cell death by releasing apoptosis-inducing factors under certain conditions (Susin et al., 1998). A high Ca^{2+} concentration and oxidative stress are both stimuli for the so-called mitochondrial megapore to open and release these factors (Hirsch et al., 1998). Surprisingly, one of these pro-apoptotic factors is cytochrome *c*, which normally floats around in the mitochondrial inner membrane space and shuttles electrons from complex II to complex IV. However, when released into the cytosol, cytochrome *c* is capable of activating the caspase cascade that leads to the enzymatic breakdown of key proteins in the cell (Cai et al., 1998). Furthermore, the energy status of the cell may also determine the final mode of its death: a good energy status allows the cell to finish the apoptotic process, whereas a low energy status may lead to direct necrosis. Intermediate stages have also been observed in vivo (Nicotera and Leist, 1997; Nicotera et al., 1999a). This new look at cellular energy and the mitochondrion in relation to cell death is discussed in some excellent recent reviews (Kroemer et al., 1998; Nicotera et al., 1999b). The main message is that the mitochondrion may be a central station where several signals converge, and where the fate of cells under difficult conditions is decided.

5. Oxidative stress in vitro

We have chosen to study the effects of oxidants and substances which affect the cellular energy supply of motor neurons, as model for the final steps in ALS. With this approach we hoped to gain insight into the processes that are occurring at the level of the motor neuron, and which may provide clues for pharmacological interventions. Free radicals, or rather, reactive oxygen species (because not all oxidants are radicals), have to be generated in situ. We have used several ways to do this. A simple (and dirty!) way is to mix iron(III) ions and ascorbate. Ascorbate, or vitamin C, is a well-known anti-oxidant, but can act — as can all anti-oxidants — as pro-oxidant as well (Bast and Bär, 1997). This mixture produces an uncontrolled burst of superoxides ions followed by secondary radical production. Even simpler is to add hydrogen peroxide, which is not a free radical, but which can generate one of the fastest-reacting physiological substances, the hydroxyl radical. Both methods are crude, and probably generate a mixture of radicals outside the cells, whereas it would be better to produce oxidants more slowly, and preferably inside the cells. Thus, as a first step, we used these two radical-generating systems to monitor the response of motor neurons to acute oxidative stress. We compared the effect of these oxidants on single-cell motor neurons and cocultures, and found some interesting differences. Later, more sophisticated ways of introducing oxidative stress were used; for example, by using a slow NO-donor, or by manipulating the energy levels of the neurons in culture.

6. Experiments with cultured motor neurons

6.1. Motor neurons in single-cell culture

The first description of a motor neuron culture was published in 1981 (Schnaar and Schaffner, 1981). The embryonic spinal cord was used as source, and a differential centrifugation technique was developed to remove the other cells, mainly astrocytes and small (inter)neurons. Motor neurons, being large cells, are less dense than all other cells. Thus, when using the right density of a neutral substance, in this case metrizamide, it was possible to obtain relatively pure motor neurons floating on top of the gradient after gentle centrifugation, while most other cells were collected at the bottom of the tube. This method has been adapted and modified by several authors, but the principle is always as described above. Further purification is possible, e.g. by "pulling out" the motor neurons by putting them in a plate that is covered with antibodies to the low-affinity nerve growth factor (NGF) receptor. This receptor is present in motor neurons, and can also be used to detect them or to establish their purity in culture (Camu and Henderson, 1992). Motor neurons remain on the plate and can be collected, whereas other cells that do not attach to the antibodies are removed by washing. Once isolated, motor neurons are cultured on a suitable substratum, usually containing laminin, and a polymer of a basic amino acid, such as ornithine. Many motor neurons, up to 90%, die in the first few hours after being dissociated, and the surviving ones remain alive for a week, sometimes even longer. Depending on the methodology and rat strains used, their survival can be as long as several weeks, but this is the exception rather than the rule. Long-term survival, a necessity when one studies a chronic disease and wants to expose the cells chronically to toxic substances, is not possible with single-cell motor neuron cultures, or requires the presence of a cocktail of several neurotrophic factors, which may affect the defence status of the cells, and therefore affect the outcome of such experiments. Taken together, this system seems hardly suited to study long-term effects, and, with some exaggeration, it seems fair to say that the motor neurons that survive the procedure are dying from the very first day in culture and are

only kept alive by external support, such as trophic factors. Although the system allows many studies, especially acute ones (see below), this should be kept in mind. Thus, when one is interested to know the effect of long-term treatment of motor neurons with toxic substances, or when one wants to mimic a condition that may exist for years, single motor neuron culture is not the best choice of model.

Our results with monocultures, in line with the results of others (Henderson et al., 1993), have shown that isolated motor neurons in vitro are very sensitive to the presence of trophic factors: without them, motor neurons die apoptotically (Kaal et al., 1997). We found that a homogenate of embryonic muscle was most effective in stimulating motor neuron survival in a dose-dependent fashion. Apparently, the target tissue contains very effective factors for survival, as predicted by the early work on NGF and studies on the chicken embryo (Levi Montalcini, 1987). In fact, purified trophic factors, such as brain-derived neurotrophic factor (BDNF) and neurotrophin-4 (NT-4), which are supposed to be more or less specific for motor neurons, supported survival only when small amounts of the embryonic muscle extract were also present. On their own, NT-4 and BDNF did not keep isolated motor neurons alive. Studies in which these factors were found to be sufficient for survival used a different protocol, with a different reference point for counting survival, as discussed by Kaal et al. (1997). In the absence of muscle extract, our cells died apoptotically, as visualised by a technique that stains the nucleus of dying cells. The shape of the nucleus, which is normally round or kidney-shaped, changes from contracted/round to a stage in which both the fragmented DNA and cytosolic components are packaged into small so-called apoptotic bodies (Fig. 1). This process can be observed quite easily in isolated cells, thereby making other techniques, such as the terminal transferase-mediated deoxyuridine triphosphate-biotin nick-end labeling (TUNEL) assay, unnecessary. This is clearly an advantage of the single-cell culture: after one simple, in vivo staining procedure, not only can the fate of cells be observed and easily quantified, but all motor neurons can be mapped as well, so that each individual cell can be followed over time, even after days. In this way, each cell is its own control, and reliable and reproducible results can be obtained with a relatively small number of cells.

Treatment of such cultures with radical-generating systems (Fe(III)/ascorbate mixture — produces superoxide; hydrogen peroxide — produces the HO radical) showed that motor neurons under oxidative stress die apoptotically, if the dose of the oxidants is not too high. By quantifying the stages of apoptosis (normal nucleus, condensed nucleus, and fragmented nucleus), we could show that when high doses of oxidant are used, the cells may start the apoptotic cascade, but if during this process the cells run out of energy, they become leaky and disintegrate before apoptosis is complete (Fig. 2). This leads to cells which combine the characteristics of both apoptosis (shrunken nucleus) and necrosis (leaky cells). When cells are depleted of energy, they die necrotically, and when their energy supply is normal and mitochondrial function is undisturbed, they can finish the time- and energy-consuming process of apoptosis. It should be kept in mind that cells in culture which have finished the complete apoptotic pathway can still become leaky and have characteristics of necrosis. The reason for this is that, in culture, there are often no neighbouring cells to phagocytose the apoptotic bodies. This phenomenon, an artifact of cell culture, is called secondary necrosis. The shape of the nucleus helps to recognise secondary necrotic cells: a leaky cell with a contracted but not fragmented nucleus indicates that the cell was stopped in its apoptotic pathway, probably because of lack of energy (Kaal et al., 1998a).

Later, we used a more subtle way of exposing motor neurons to radicals by using a substance that slowly releases nitric oxide, or NO. NO, a physiologically occurring, gaseous radical, is a neurotransmitter and neuromodulator involved in many physiological processes (Dawson et al., 1992), and in neurodegeneration (Heales et al., 1999). Under oxidative stress, it can react with superoxide to form an aggressive oxidant, peroxinitrite (Beckman and Koppenol, 1996). NO, possibly through peroxinitrite, is thought to play a role in ALS pathology (Beckman et al., 1993). Treatment with deta-nonoate (NOC-18), which releases NO over a long time, probably reflects a more physiological exposure to oxidants, as the two other systems mentioned above produce high concentrations of radicals over a short time, whereas NOC-18 has a $t_{1/2} > 500$ min. NO has two contrasting effects: at low concentrations, it is neurotrophic, but at higher concentrations, it becomes toxic. It also causes apoptotic death of motor neurons, probably by inhibiting the cellular energy supply. However, as no glutamate is present in the culture, and none is produced by neighbouring innervating cells, the motor neurons probably die of Ca^{2+} overload due to insufficient pumping. This may explain why, in this system, cobalt, which blocks Ca^{2+} entry, was the only effective neuroprotective substance (Kaal et al., 1999b).

The experiments with single motor neurons have shown that motor neurons in culture are very vulnerable, highly dependent on trophic factors and are easily damaged by physiological oxidants. When the oxidative stress is not too severe, motor neurons will die apoptotically. The disadvantage of motor neuron monocultures is that long treatment times cannot be evaluated, as the motor neurons are, in fact, doomed to die from the very first moment that they are in culture.

6.2. Motor neuron cocultures

To obtain more stable cultures, several authors have chosen to culture motor neurons on top of astrocytes.

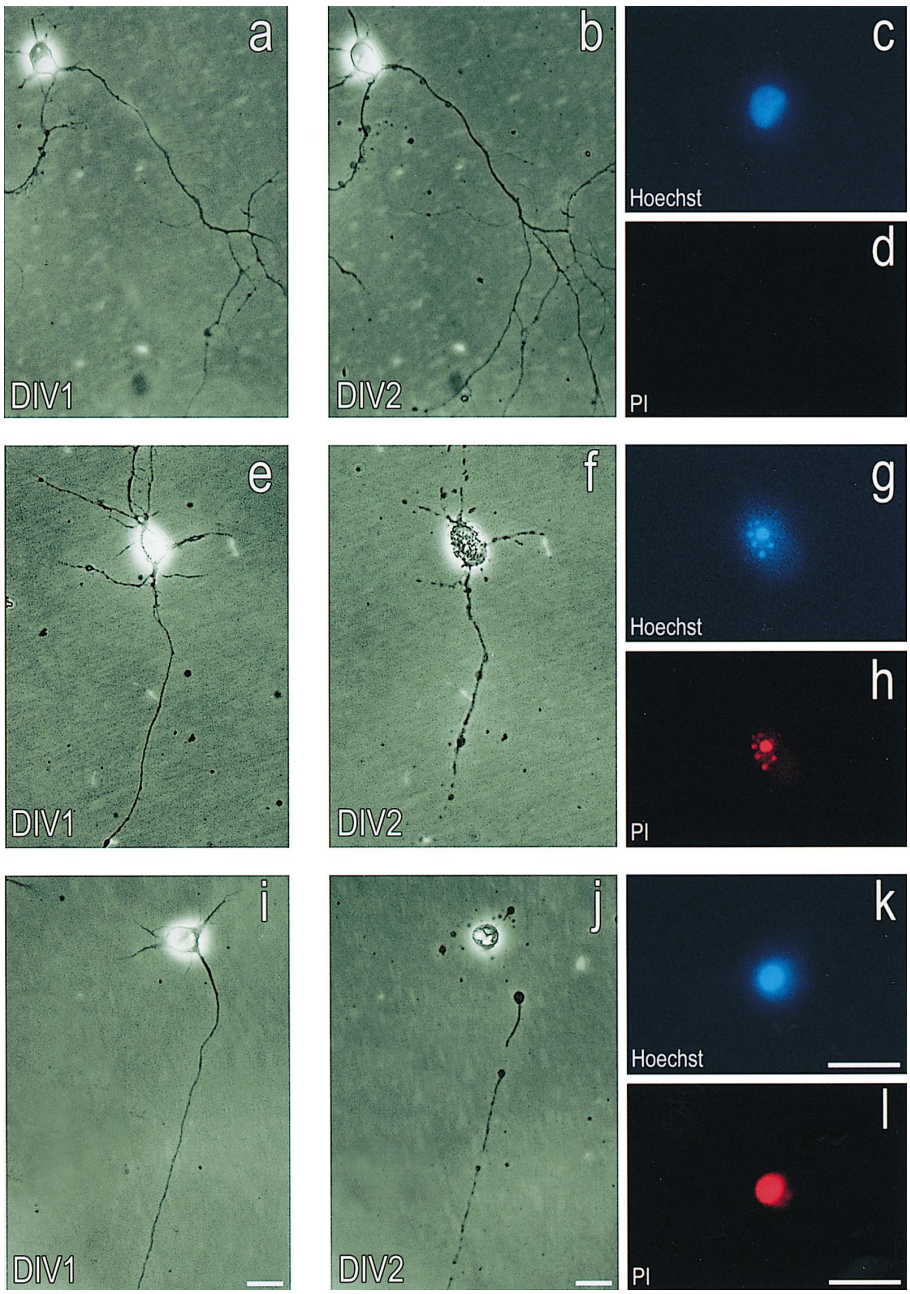

Fig. 2. Motor neurons in single-cell culture, treated with two concentrations of a combination of Fe(III) and ascorbate. Each of the three rows shows pictures of the same cell under phase contrast (large pictures), and after Hoechst nuclear staining (blue) and propidium iodide staining (red). (a–d) Motor neuron after 1 (a) and 2 days (b) in culture. The outgrowth has increased overnight, the soma is bright under phase contrast microscopy and has a halo, and the nucleus is kidney-shaped, as visualised by staining with a fluorescent DNA marker (c). The cell membrane is not leaky, as the propidium iodide nuclear stain is negative (d). (e–h) Treatment with low doses of oxidants: a normal cell before treatment (e), and a vacuolised cell after treatment (f). The cell has a reduced phase contrast halo, and the neurites are affected. Nuclear staining shows fragmentation of the nucleus, indicative of late-stage apoptosis (g). The same cell stained with propidium iodide indicates that the cell membrane is leaky (h). (i–l) Treatment with high doses of oxidants: the cell is clearly affected (j), and shows a contracted nucleus (k) and a leaky membrane (l). This combination indicates that the oxidative stress was so severe that the apoptotic cascade could not be finished and stopped before DNA fragmentation. This figure is printed with permission from Kaal et al. (1998a). Bars represent 20 μm.

These glial cells surround the motor neurons in the spinal cord and are thought to interact with and supply trophic support to motor neurons. When freshly isolated motor neurons, as described above, are plated on top of confluent asytrocyte cultures that have been prepared 1 or 2 weeks earlier, the motor neurons live longer (weeks) than single-cell motor neurons, and are clearly in a better state: they grow long neurites, have densely branched dendritic trees, and show characteristics of adult motor neurons (Fig. 3). Apparently, the physical contact between astrocytes and

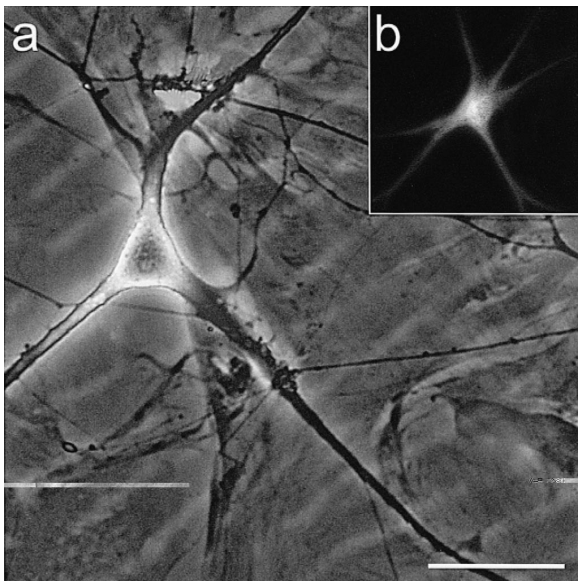

Fig. 3. A rat motor neuron in coculture with rat astrocytes. After a week in culture on top of a 3-week-old confluent layer of astrocytes, the neuron has grown broad neurites, of which one very long axon and a dense dendritic tree. The insert shows the expression of SMI-32, a marker for neurofilament. This figure is printed with permission from Herpers et al. (1999). Bar represents 50 μm.

neurons produces a more physiological environment than when neurons are cultured on a laminin–polyornithine-coated plastic substratum. Some groups use a total spinal cord culture, i.e. dissociated cells of the spinal cord without purification. Although all neighbouring cells of the motor neurons are present in such cultures and are potentially capable of supporting the survival of the motor neurons, the actual concentration of this cell within the culture is very low ($< 1\%$), and therefore, this approach is not ideal to study the responses of motor neurons. Purification of motor neurons, followed by coculturing on confluent astrocyte layers, yields better characterised systems that are enriched in the target cell, the motor neuron. We have used this coculture system as well as motor neurons cocultured with fibroblasts. On top of fibroblasts, motor neurons can be cultured longer than single-cell cultures, but not as long as those on top of astrocytes. Thus, it is not only the presence of another cell as substratum that is important, but also the type of cell. Still, even with the astrocyte/motor neuron system, culture times longer than 4 weeks are difficult to obtain. Interestingly, the glutamate analogue kainic acid (an AMPA/kainic acid receptor agonist) is far more toxic in motor neuron/astrocyte cocultures than in monocultures as a consequence of the disappearance of GluR2 from the AMPA receptor (Vandenberghe et al., 1998), a sign that the motor neurons mature in culture. Also interesting, and very recently shown, is that insulin, a common ingredient in culture media, may induce a decreased cell surface expression of the GluR2-containing AMPA receptor (Man et al., 2000). While this observation may provide a tool to manipulate in culture systems the occurrence of this important receptor, it is, at the same time, a caveat and a reason for researchers to carefully check insulin effects in their model systems.

We found some interesting differences when we compared the effect of hydrogen peroxide on single cells, motor neurons cultured on astrocytes, and motor neurons cultured on fibroblasts. Apart from the prolonged neuronal survival per se, as mentioned above, we found that the sensitivity towards hydrogen peroxide treatment became less when going from monoculture via culture with fibroblasts to coculture with astrocytes. Thus, the environment has a strong influence on how well motor neurons can cope with oxidative stress. On their own, they died quickly, but in the presence of astrocytes, their physiological supporters, they survived at much higher concentrations of the oxidant. The data obtained with fibroblast cocultures were in-between those obtained with single-cell culture and those with astrocyte coculture (Fig. 4). The explanation for these differences probably lies in the presence of a well-developed oxidant defence system in astrocytes. Superoxide dismutase and catalase are present in high amounts in these cells and, apparently, less so in fibroblasts. Also, it cannot be ruled out that neuron-specific trophic factors, which can be produced by astrocytes but not by fibro-

Fig. 4. The effect of several doses of hydrogen peroxide (μM, *x*-axis) on the survival of cultured motor neurons (%, *y*-axis). The motor neurons were cultured as single cells (circles), on top of fibroblasts (triangles), and on top of astrocytes (squares). Single motor neurons barely survive hydrogen peroxide treatment, whereas in coculture, they are protected by the presence of the other cell type: astrocytes are clearly more protective than fibroblasts (data from Herpers et al. (1999) and unpublished data).

blasts, play a role in the protective presence of the astrocytes. Using the same system, that of motor neurons on astrocytes, we were able to transfect motor neurons with a DNA construct for catalase, thus providing them with an effective radical scavenger. We found that the transfected cells were capable of dealing with hydrogen peroxide much better: whereas 80% of the nontransfected cells died after exposure to 600 μM hydrogen peroxide, only 25–30% of the transfected cells died (Herpers et al., 1999). As the extra catalase is produced within the cell, and as hydrogen peroxide is capable of passing through cell membranes, intracellular processes in this model were studied, whereas in many experiments, also in the ones described above, protective agent and oxidant are supplied from the outside.

6.3. Spinal cord organotypic cultures

One (large) step closer to the in vivo architecture and surroundings of the motor neuron is to leave the motor neurons in situ, with all the other cells and connections, in place. Some tissues can be cultured in toto, such as the dorsal root ganglion (Tabor et al., 1997), while others have to be sliced. This technique has been extensively used in neuroscience by neurophysiologists (Richards and Sercombe, 1968; Tielen et al., 1982) and neurobiologists (Gahwiler et al., 1997). For example, the hippocampal slice, cut in such a way that all synaptic connections remain intact, can be kept alive for a day under special conditions (temperature, buffer, glucose, humidity) and used for electrophysiological experiments (Skrede and Westgaard, 1971). Using this model system, long-term potentiation has been, and still is, studied extensively (Tielen et al., 1982; Bär et al., 1980; Bliss and Collingridge, 1993).

By using the so-called interphase technique, it is possible to keep such slices in good condition for much longer than 1 day (Stoppini et al., 1991). The migration of glial cells, resulting in disruption of the architecture of the slice, is strongly reduced in the interphase system. Such slices can be kept for months with the cells remaining alive, as evidenced by staining for several enzymes and extensive axonal outgrowth from the motor neurons within the slice (Fig. 5). The organotypic slice has been used earlier in ALS research, namely, to test the effect of chronic glutamate toxicity on motor neurons (Rothstein et al., 1993). Chronic inhibition of glutamate transporters led to slow motor neuron degeneration that could be blocked by non-NMDA receptor antagonists. Apparently, motor neurons in the slice preparation also have the adult GluR2 subunit constellation of their AMPA receptors, as NMDA receptor antagonists were not effective in protecting against motor neuron death. Later, several other substances were shown to be neuroprotective as well (Rothstein and Kuncl, 1995). In these studies, neither the nature of motor neuron death was established nor was selective motor neuron death quantified.

Using the interphase slice culture, we tried to induce selective neuronal death and developed an indirect method to introduce oxidative stress: we down-regulated the cellular energy production, thus mimicking the situation that may arise in cells that are chronically exposed to slightly increased concentrations of glutamate, leading to chronic

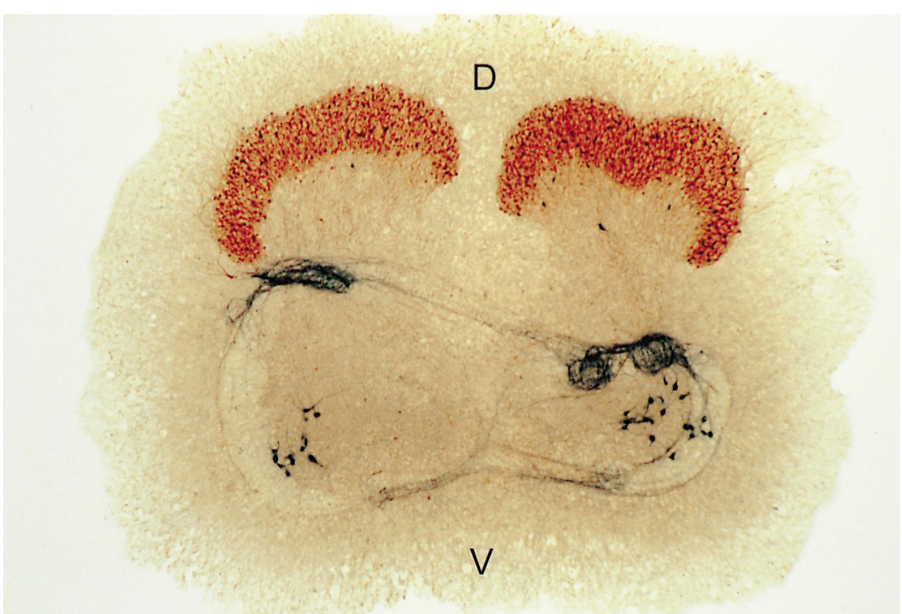

Fig. 5. A spinal cord slice, derived from an 8-day-old rat pup, after 3 weeks in culture. The slice is double-stained for ChAT (black staining) and calretinin (brownish red). The calretinin-positive cells in the dorsal (D) horn are interneurons, the black cells in the ventral (V) part are motor neurons. From this picture, it can be seen that there are few motor neurons per slice, and that they are quite active: not only is their marker enzyme ChAT present, they also grow long, circling neurites that stay within the slice. The in situ architecture of the slices is well-preserved. This picture is reprinted with permission from Kaal et al. (2000).

Ca^{2+} overload and — probably a common mechanism in many neurodegenerative diseases — to a chronic shortage of ATP. Taken together, this leads to cells that are chronically partially depolarised, and therefore more sensitive than normal polarised cells to glutamate, even in physiological concentrations (Albin and Greenamyre, 1992). The circle is thus closed: glutamate, the levels of which are increased either by inhibited uptake or by facilitated release, increases the intracellular Ca^{2+} concentration, which means that there is more energy required to pump Ca^{2+} out of the cytosol. This continuous drain of energy makes the cell more vulnerable to glutamate. The model we set up appeared to mimic one of the most characteristic traits of ALS: that motor neurons died before other neurons did. In other words, there was selective motor neuron vulnerability, a specific characteristic of ALS (Kaal et al., 1998b, 2000).

Two-week-old slices were incubated with several concentrations of malonate, which is a competitive blocker of site II of the electron transport chain. This blocker reduces the efficacy of the mitochondrion to produce ATP, and indeed, we found a dose-dependent decrease in the ATP concentration with 0–30 mM malonate. At 5–10 mM malonate, the ATP levels in the slice were about 15–30% diminished, a reduction that is reported to occur in the affected regions in Huntington's (striatum) and Parkinson's diseases (substantia nigra) (Schapira, 1998; Tatton and Olanow, 1999). Such reductions in ATP have not been shown in patients with ALS, but, by using magnetic resonance spectroscopy techniques, it should be possible to test the hypothesis that the energy supply in motor neurons is compromised in ALS. In the presence of malonate, motor neurons died, whereas interneurons, visualised by staining with calretinin, survived: at 7 mM, only 10% of the motor neurons survived, whereas about 60% of the interneurons were still alive. This selective motor neuron death could be blocked by anti-oxidants, such as α-phenyl-*N*-*tert*-butylnitrone (PBN), a glutamate-release inhibitor, 2-amino-6-trifluoro-methoxy-benzothiazide (riluzole), and partially by an AMPA inhibitor, 6-cyano-7-nitroquinooxaline-2,3-dione (CNQX) (Kaal et al., 2000). Thus, although no glutamate or radicals were directly introduced into the culture, anti-oxidants and glutamate receptor antagonists were effective neuroprotectants. This strongly suggests that a lowering of the energy supply indeed leads to the cascade of events as sketched above. As it is impossible to observe the type of cell death as we did in other culture systems, we tested two caspase inhibitors, *N*-benzyloxy-carbonyl-Val-Ala-Asp-fluoromethyl ketone (*z*VAD-fmk, a broad-spectrum caspase inhibitor) and *z*-Asp-Glu-Val-Asp-fluoromethyl ketone (*z*DEVD-fmk, inhibits the key enzyme, caspase 3). Both inhibitors strongly reduced cell death, and we can conclude that motor neurons die apoptotically in the circumstances described, and possibly, by extrapolation, also in the spinal cord of ALS patients. Our observations are corroborated by recent findings that *z*VAD-fmk, infused directly into the brain, prolongs the survival of transgenic ALS mice by 70%, compared to the 30% by riluzole, the only registered drug for ALS treatment (Li et al., 2000). This also strengthens our belief in the predictive value of results obtained with the spinal cord slice model (Kaal et al., 2000).

Most attention is focused on the motor neurons in the spinal cord, probably because they are the most accessible for tissue culture studies. However, the motor neurons in layer V of the motor cortex also die in ALS. We have developed a cortical slice culture to study the fate of these so-called upper motor neurons. Preliminary results show that these slices can indeed be cultured, but identification of the motor neurons is not as easy as in the spinal cord slices. The combination of staining with SMI-32, a neurofilament antibody, with the shape and localisation of the cells within the slice are conditions for positive identification (van Westerlaak et al., accepted, Exp. Neurology). Experiments have shown that malonate kills these motor neurons in a dose-dependent manner (van Westerlaak et al., 1998), and that several drugs are neuroprotective. However, in these cultures, the NMDA antagonist MK801 (dizocilpine) was protective, whereas it was not in spinal cord slices. The meaning of this phenomenon in relation to ALS needs to be looked into.

7. Conclusions

It seems valid to conclude that cell culture systems can provide essential information about disease processes in cells that can be studied either all by themselves, in combination with another cell type, or more or less in their 'normal' environment, in an organotypic culture. As the choice of culture system and conditions influence the cell's response, it will always be necessary to check findings obtained in one system with those obtained in another and, finally, in a living animal. Quite recently, the issue of the relevance of in vitro findings to the ALS mouse model (and thus to the human disease) was brought up in a discussion on the website of Science. In a series of experiments, it was shown that the toxicity of mutated superoxide dismutase is determined by the loss of zinc from the enzyme (Estevez et al., 1999). However, several authors doubted the relevance of these findings for the in vivo situation and tested the zinc hypothesis in an ALS transgenic mouse (Williamson et al., 2000). Without going into detail, this is a good example of the principle that any finding, be it in vitro, in a culture system or in an animal model, needs to be tested for its general validity in other models before it can lead to generalisation or to extrapolation to the human clinical situation. In their conclusion, the 'defending' authors wrote: "... Transgenic mouse models have provided vital clues, but the existence of redundant compensatory systems limits their usefulness as a primary means of testing specific biochemical hypotheses. Results

obtained in animals should be interpreted cautiously to avoid premature closure of promising research avenues. The issues raised by Williamson et al. underscore the need for a combined approach, including human tissues, transgenic animals, neuronal culture models, and in vitro biochemistry . . . " (Beckman et al., 2000). In other words, all models have their limitations, and only the combination of validated models can lead to reliable answers.

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