Motoneuronal death during spinal cord development is mediated by oxidative stress

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Abstract

The involvement of reactive oxygen species (ROS) in neuronal death has been determined in culture, and in association with several neurodegenerative disorders. We examined whether ROS participate in the cell death observed during spinal cord development. We found that the general pattern of high ROS levels, gene expression for some antioxidant enzymes, and motoneuron death correlated positively along spinal cord development. ROS were reduced in spinal cords cultured in the presence of a synthetic superoxide dismutase and catalase mimetic, with a concomitant reduction in cell death and an increase in the number of motoneurons. The number of motoneurons was higher in spinal cords treated with the antioxidant than in those treated with caspase inhibitors. In general, the increase in motoneuron survival did not correlate with the reduction in cells undergoing DNA degradation in the motoneuronal region. These results suggest that ROS are signaling molecules controlling caspase-dependent and caspase-independent programmed motoneuron death, and support the hypothesis that this mechanism is abnormally turned on in some neurodegenerative disorders and aging.

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Abbreviations: ROS, reactive oxygen species; Gdnf, glial-derived neurotrophic factor; PI3K, phosphatidylinositol 3-kinase; AO, acridine orange; DHR-123, dihydrorhodamine 123; HET, hydroethidine

Introduction

During central nervous system development, about 50% of the neurons produced subsequently die by programmed cell death. Classically, it is thought that supernumerary neurons die because they fail to reach their target cell.¹ In more general terms, this hypothesis states that neuron survival during development depends on the availability of appropriate trophic support provided by the target cell. Many growth factors have shown survival activity on neurons; however, in most cases their role in development has not been determined. This 'trophic hypothesis' predicts that death is activated by 'death factors' that are either extrinsic or intrinsic to the neuron programmed to die. Presently, it is unclear whether this is a regulated (i.e., activation of a signaling pathway is required to turn the death process on) or a default process (i.e., a death pathway is always on) disclosed in the absence of survival factors. At least a portion of neurons die via apoptosis involving caspases and members of the Bcl2 family.²

Motoneuron survival is tightly linked to the presence of the target tissue. For example, removal of a limb causes the loss of almost all motoneurons,³ whereas addition of extra target tissue increases motoneuron survival.⁴ Growth factors such as glial cell line-derived neurotrophic factor (Gdnf) and brain-derived neurotrophic factor (Bdnf) promote motoneuron survival *in vitro*⁵ and prevent motoneuron death in the absence of target tissue³ or after axotomy.⁶ Confirming the *in vivo* role of Gdnf in motoneuron survival, loss of function mutations in *Gdnf*⁷ or in the gene coding one of its coreceptors (Gdnf-family receptor- α ; *Gfr α 1*) increase motoneuron death.⁸ Factors with the ability to promote motoneuron death have also recently been identified, including: Ngf (through its low affinity p75 receptor⁹), FasL (through Fas, a death domain containing receptor¹⁰), and Tgf β .¹¹

Neuronal survival has shown to be sensitive to oxidative stress. Studies *in vitro* have suggested that loss of trophic support induces a net increase in intracellular reactive oxygen species (ROS), which trigger death of sympathetic neurons.¹² In agreement with a role of ROS in cell death activation, superoxide dismutase shows a protective effect on cultured sympathetic neurons deprived of Ngf.¹² Likewise, the use of a variety of antioxidants promotes the survival of trophic factor-deprived neurons *in vitro*.^{13,14} Recently, it has been proposed that growth factors such as Bdnf¹⁵ and Gdnf¹⁶ promote neuron survival by increasing the levels of antioxidants.

Neuronal degeneration associated with several diseases appears to be mediated by an increase in ROS. There is evidence that ROS participate in the pathogenesis of sporadic Parkinson's disease and Alzheimer's disease.¹⁷ In addition, motoneuronal death associated with familial amyotrophic lateral sclerosis is linked to mutations in the gene coding for the superoxide dismutase 1 (Sod1), which appear to cause an increase in free radicals such as peroxynitrite.¹⁰ In the present work, we studied the role of ROS in the motoneuron death that

occurs during spinal cord development. We found a positive correlation between motoneuron death and indicators of oxidative stress *in vivo*. Furthermore, motoneuron death during development, reproduced in an explant culture system, was prevented by a catalase–superoxide dismutase mimetic. Interestingly, this mimetic showed a more widespread protection against motoneuron death than caspase inhibitors. Caspase-8- and caspase-9-specific inhibitors reduced motoneuron death, without affecting significantly DNA degradation. Hence, we propose that both caspase-dependent and caspase-independent programmed motoneuron death involve oxidative stress, and that regulation of ROS levels is a major event controlled by motoneuron death/survival-promoting factors.

Results

Regions of the developing spinal cord undergoing cell death show high ROS levels

Motoneuron death is highly regulated during vertebrate spinal cord development; yet, the signals involved in triggering this cell death remain to be identified. In order to elucidate the role of ROS in motoneuronal death *in vivo*, we first determined the pattern of ROS levels at the time motoneuron death is occurring. Motoneuron death during development follows an archetypal pattern in time and space, which is initiated just

after innervation of the target tissue.¹ Spinal cord motoneurons reach their targets from E11 to E15, this being the period of massive motoneuron death.¹⁸ We used whole mount staining with acridine orange (AO) or dihydrorhodamine 123 (DHR-123) to determine cell death or ROS levels, respectively. We stained E11.5–E14.5 fresh spinal cords and analyzed them as ‘open book’ preparations. The cell death pattern determined here with AO is in agreement with that reported using the TUNEL technique,¹⁸ exhibiting cell death first at cervical levels and last at sacral levels (Figure 1). At all stages, DHR-123 signal was detected in the ventral part of spinal cords corresponding to the motoneuronal population (Figure 1). At E11.5, cell death and high ROS levels were only observed in the cervical region, and later (E12.5) both extended to the posterior cervical region, and brachial, thoracic, and lumbar regions. By E13.5, cell death and high ROS levels were detected up to the anterior sacral region. At this stage, it is easy to identify brachial and lumbar regions by their characteristic lateral motor columns. By E14.5, both cell death and high ROS levels were reduced in the cervical and brachial regions, but remained high in the posterior thoracic region and the lumbar and sacral regions. Positive spatial and temporal correlation between cell death and high ROS levels suggest that ROS might be involved in spinal motoneuron death.

We also determined the expression pattern of genes encoding the antioxidant enzymes superoxide dismutase 1

Figure 1 Cell death and ROS detection in embryonic spinal cord. Spinal cords from E11.5–E14.5 embryos were stained with AO to detect cell death or with DHR-123 to detect ROS. Samples in ‘open book’ preparation (v, ventral; d, dorsal) were observed from the outside by confocal microscopy. The sum of 10–12 optical planes within 200 μ m is shown. Bright dots represent the specific signal produced by the dye (indicated by arrows in some areas), which can be distinguished from the diffuse artifactual brightness (examples indicated by arrowheads) caused by reflections on the thick sample. A positive correlation is observed when the two staining patterns are compared. Staining with both dyes began to be detected at the cervical level in E11 embryos; spread along the brachial, thoracic, and lumbar regions during E12 and E13; and finally reached the posterior sacral region at E14. Scale bar 250 μ m

Figure 2 Expression pattern of antioxidant enzymes in developing spinal cord. Whole-mount *in situ* hybridization was performed on E13.5 spinal cords using probes against *Sod1*, *Sod2*, *Cas1*, and *Gpx4*. Images are outside view of spinal cords after hybridization in an 'open book' preparation (v, ventral; d, dorsal). Note that, in general, higher expression signal (displayed in pictures as dark areas; arrowheads indicate some of these areas) coincides with areas of motoneuronal death; however, every gene has a characteristic expression pattern. Expression in motor columns of brachial and lumbar regions was evident for almost each gene, but expression in the thoracic region was better detected for *Sod2* than for the other genes. In fact, *Sod2* is the only gene whose expression coincided with all areas of motoneuronal death. Scale bar 100 μ m

(*Sod1*), superoxide dismutase 2 (*Sod2*), catalase (*Cas1*), and glutathione peroxidase 4 (*Gpx4*) (Figure 2). Areas of motoneuron death coincided with areas expressing one or more genes encoding antioxidant enzymes. Particularly, *Sod2* was expressed in the presumed brachial, thoracic, and lumbar motoneuron columns. These data suggest that some motoneurons are responding against signals that promote oxidative stress (see Discussion).

Antioxidant EUK-134 rescues motoneurons from death in thoracic spinal cord cultures

In order to test whether the pattern of motoneuronal death described above can be reproduced *in vitro*, E13.0 spinal cords were cultured in serum-free medium for 12 h, and distribution of cell death was evaluated at the end of culture. The general cell death pattern determined by AO staining was similar to that observed *in vivo* but, as expected due to the removal of all targets from the spinal cord in the explant culture, there was a significant increase in cell death (data not shown). In agreement with this observation, there was a reduction of 82% in the number of cells that were positive for the motoneuronal marker Islet 1/2 in the cultured thoracic region (Figure 3). This result contrasts with the natural

Figure 3 Motoneuron development in spinal cord in culture. Immunohistochemistry against Islet 1/2 (a motoneuronal marker) was performed on spinal cords of E13 and E13.5 embryos, or on spinal cord explants from E13 embryos cultured for 12 h ($t = 12$ h) in control media or in the presence of 400 ng/ml Gdnf (uncultured E13 spinal cords were used as reference; $t = 0$ h). Pictures show the typical patterns observed for motoneurons in each condition (a). The number of Islet 1/2-positive neurons in the motoneuron regions (Mn) of spinal cords treated as described above was determined. The average percentage of motoneurons per hemisection as compared with the number of motoneurons in a hemisection of slices from uncultured E13 spinal cords (100%) is shown (b). The number of experiments performed is indicated (numbers within bars). Observe that the natural decrease (33%) in motoneurons occurring during spinal cord development from E13 to E13.5 was lower than the decrease (82%) in motoneurons occurring during the 12-h culture of E13 spinal cords. When Gdnf was used, there was a recovery of motoneurons (43% of those in E13 spinal cords), which means an increase (138%) respect the number of motoneurons after culture without Gdnf. *** $P < 0.001$. Scale bar 50 μ m

decrease by 33% in the number of motoneurons in the thoracic region during the E13–E13.5 period (Figure 3).

To overcome the lack of trophic support provided by the target cell in the explants, Gdnf, a potent motoneuron survival factor,¹⁹ was provided. When the culture was supplemented with Gdnf, there was a reduction of 57% of the initial motoneurons (Figure 3), meaning that around 30% of motoneurons dying in the absence of growth factors were rescued by Gdnf. These data suggest that motoneuron survival in the spinal cord culture used in the present study depends on the same factors as *in vivo*, validating the cultured spinal cord as a suitable model to study natural motoneuron death.

To determine whether increase in ROS levels contributes to motoneuron death, we evaluated the effect of the antioxidant EUK-134, a synthetic superoxide dismutase and catalase mimetic, on the survival of motoneurons. This antioxidant has been successfully used to prevent cell death in several neuropathological models.^{20–22} We carried out 12 h cultures of complete E13 spinal cords in the presence of increasing concentrations of EUK-134 and compared its effects with the effect of 150 μ M Z-VAD-FMK, a broad-spectrum caspase inhibitor, known to inhibit neuronal death. In agreement with our observations in fresh developing spinal cords, we found high ROS levels in the motoneuronal region of spinal cord sections (Figure 4, left panel), as detected by the ROS

Figure 4 ROS in spinal cord cultures. E13 spinal cords were cultured in control media for 12 h in the presence of 2 mM EUK-134 or 0.15 mM Z-VAD-FMK. In order to detect ROS, spinal cords were stained with HET, fixed and sectioned. There was a marked reduction in ROS levels in the presence of EUK-134 that was not seen in the presence of Z-VAD-FMK in the motoneuron region (Mn). Scale bar 50 μ m

sensitive dye hydroethidine (HET). HET was used instead of DHR-123 because it is able to withstand the fixing process. Antioxidant activity of EUK-134 was evident, since ROS levels in spinal cord were reduced when this compound was added to the culture medium in comparison with cultures treated with Z-VAD-FMK in which ROS levels remained high (Figure 4, middle and right panels). The different appearance of HET staining signal in Z-VAD-FMK-treated samples could be due to an artifact related to a more condensed state of the chromatin (oxidized HET binds to DNA), and/or to an actual increase in ROS due, for instance, to a mitochondrial failure. In agreement with a role of ROS in cell death activation in the spinal cord, EUK-134 decreased cell death in a dose-dependent manner as detected by both AO staining (Figure 5a) and TUNEL technique (Figure 5b). Using this latter technique, we estimate about 52% cell death reduction when 2 mM EUK-134 was used (Figure 5c). As expected, spinal cords cultured with Z-VAD-FMK showed 95% reduction in cell death detected by TUNEL (Figure 5b,c). Surprisingly, not such a dramatic reduction was observed when cell death was determined by AO staining (Figure 5a). These latter data suggest that a caspase-independent cell death that can be detected by AO staining is occurring during spinal cord development. In addition, the decrease in ROS levels caused by EUK-134 was not a consequence of reduction in cell death *per se*, since Z-VAD-FMK treatment did not reduce ROS levels (Figure 4).

As a consequence of a reduction in cell death, more motoneurons were predicted to survive. Using immunohistochemistry against Islet 1/2, there was a dose-dependent increment in motoneurons in cultures treated with EUK-134 or Z-VAD-FMK (Figure 6a, b). Interestingly, despite the stronger effect of Z-VAD-FMK on cell death than EUK-134, as detected by the TUNEL technique (Figure 5c), EUK-134 caused a larger increase in the number of motoneurons than Z-VAD-FMK. These data support the previous proposal indicating that a subpopulation of motoneurons die by a caspase-independent mechanism. In addition to the motoneuron population, the dorsal D2-interneuron population (also detected with the Islet 1/2 marker;²³ increased in number in the presence of EUK-134, but not when Z-VAD-FMK was used (Figure 6a). Since we were unable to detect positive cells in this area with the TUNEL technique (see Figure 5b), it is possible that this population dies exclusively by a ROS-

mediated, caspase-independent pathway. The nature of this latter death process is still under investigation.

Autophagy and a caspase-dependent mechanism control motoneuron death not detected with the TUNEL technique

A possible death pathway independent of caspases and not associated with TUNEL-positive cells is autophagy.²⁴ Inhibition of phosphatidylinositol 3-kinase III (PI3K-III) with reagents such as 3-MA and LY294002 has been used to determine the participation of autophagy in cell death.^{24,25} As shown in Figure 7, the number of motoneurons in spinal cords treated with LY294002 increased by 66% when compared with the number of motoneurons in spinal cords of control cultures. No significant change was found in the number of TUNEL-positive cells (Figure 7). Therefore, at least a fraction of motoneuron death (up to 50%) could occur by autophagy (see Discussion).

To get insights into the caspase-regulated pathway leading to motoneuron death, we treated spinal cords with inhibitors of the initiator caspases, caspase-8 and caspase-9. Treatments with either Ac-IETD-CHO (a caspase-8-specific inhibitor) or Ac-LEHD-CHO (a caspase-9 specific inhibitor) caused an increase in the number of motoneurons (68 and 77%, respectively; Figure 8), similar to the one obtained with the pan-caspase inhibitor Z-VAD-FMK (68%; Figure 8). Unexpectedly, neither caspase-8 nor caspase-9 inhibitor produced a significant decrease in the number of TUNEL-positive cells (Figure 8), which contrasts with the strong reduction observed for Z-VAD-FMK (more than 90%; Figures 5 and 8). These results are consistent with a motoneuron death mechanism involving caspase-8 and caspase-9, probably within a single pathway.

De novo generation of motoneurons does not occur in E13 spinal cord cultures

Although the generation of motoneurons *in vivo* starts around E9.5 and ends by E11.5,²⁶ at least a day before samples used for explant cultures, we wanted to discard the possibility that *de novo* generation of motoneurons in culture was being induced by EUK-134. Two types of experiments were performed. In one, spinal cords were cultured in the presence of BrdU in order to estimate the number of proliferating cells and their fate in cultures with or without EUK-134. As shown in Figure 9, no significant change in BrdU-positive cells was found when control spinal cord cultures were compared with those cultured with EUK-134 (Figure 9a) or Z-VAD-FMK (data not shown). Furthermore, no labeled cells were detected in the motoneuronal regions, indicating that proliferating cells in culture do not generate motoneurons during the 12 h culture period (Figure 9a). Since *de novo* generation of motoneurons in culture could initiate from nonproliferating progenitors, in the second experiment, proliferating cells were BrdU-labeled *in vivo* and their fate determined *in vitro*. BrdU was injected intraperitoneally to pregnant females 12 or 24 h before embryonic spinal cord dissection. Some BrdU-positive cells in the region of motoneurons were detected (Figure 9b), the

Figure 5 Cell death in spinal cord cultures in the presence of the antioxidant EUK-134. E13 spinal cords were cultured for 12 h in control medium or in the presence of 0.5 mM EUK-134, 1 mM EUK-134, 2 mM EUK-134 or 0.15 mM Z-VAD-FMK. To detect cell death, spinal cords were stained with AO and observed as 'open book' preparations (**a**; scale bar 250 μ m), or were fixed, sectioned and labeled using the TUNEL technique (**b**). Representative pictures are shown. Amplification of the boxed area containing motoneurons (Mn) in (**b**) is shown in right panels (scale bar 25 μ m). The TUNEL positive cells per hemisection in the motoneuron region were quantified, and the average percentage from the number obtained in untreated cultures determined (**c**). Note that the decrease in AO staining was greater than the reduction in TUNEL-positive cells when EUK-134 was used. Conversely, there was a dramatic reduction (95%) in TUNEL-positive cells but not in AO staining when Z-VAD-FMK was used. *** $P < 0.001$

number of which decreases as injection was administered at more advanced developmental stage (24 *versus* 12 h). However, no significant changes were found when cultures in the presence or absence of EUK-134 were compared. Thus, very few (if any) motoneurons were likely generated in culture, and EUK-134 did not appear to induce the differentiation and migration of progenitor cells present at the beginning of culture.

Discussion

The present study is the first report demonstrating the involvement of ROS in the control of motoneuron death during spinal cord development. We have shown that there is a spatial and temporal correlation between cell death and high ROS levels during spinal cord development. In spinal cord cultures, the antioxidant EUK-134 promoted motoneuron

survival in greater extent than the pan-caspase inhibitor Z-VAD-FMK, implying that more than one form of cell death occurs among the motoneuron population. Our data show that DNA degradation is not a good indicator of motoneuron death. We propose that ROS participate in the activation of different forms of cell death in the developing spinal cord.

ROS as inducers of motoneuron death

During spinal cord development, motoneurons send axons to their postsynaptic targets and form synapses. Cells that do not reach their targets die. In the explants used here, all connections with their targets are absent, therefore, it is expected that more motoneurons die in these cultures than during *in vivo* development after a similar period of time. This situation resembles what happens *in vivo* when the targets (e.g., the limbs) are removed.³ Gdnf is a growth factor that

Figure 6 Survival of motoneurons when cultured in the presence of the antioxidant EUK-134. E13 embryonic spinal cords were cultured for 12 h in different concentrations of EUK-134 or Z-VAD-FMK. Immunohistochemistry against Islet 1/2 was performed after culture; representative pictures are shown (a). Amplification of the boxed area containing motoneurons (Mn) is shown in right panels (scale bar 25 μ m); D2-interneuron area is indicated (In). The Islet 1/2-positive cells per hemisphere in the motoneuron region were quantified and the average percentage from the number obtained in untreated cultures determined (b). The number of experiments performed in each condition is indicated (numbers within bars). Note the increase in motoneurons after treatment with either EUK-134 or Z-VAD-FMK. ** $P < 0.01$; *** $P < 0.001$

rescues motoneurons from dying when deprived of their targets.^{3,6} Gdnf added to the cultures used here rescued about 30% of motoneurons. As motoneurons depend on several neurotrophic factors, 100% rescue is not expected, even in cultures of purified motoneurons.⁵ Therefore, the spinal cord cultures used here are responding as expected, which permits the study of programmed motoneuron death.

Several evidences suggest that the cellular redox state has a role in different forms of cell death: (i) there is an increase in ROS before the appearance of typical characteristics of cell death;¹² (ii) the use of antioxidants prevents cell death,^{13,14} and; (iii) overexpression of antioxidant enzymes prevents cells from dying in models of neurodegenerative diseases or ischemia.¹⁷ Most of the previous studies have used adult cell cultures or animals under pathological conditions, and only few reports show the involvement of ROS during embryonic programmed cell death. Salas-Vidal *et al.* reported a positive correlation between high ROS levels and massive cell death during development of the limb, the palate, the sternum, the otic vesicle, and the eye. They also showed that death in the interdigits is prevented by antioxidants.²⁷ In the present work, we investigated whether ROS participate in the death of motoneurons observed during spinal cord development. Using DHR-123, a redox sensitive dye, we found a positive spatiotemporal correlation between the regions of cell death and the areas of elevated ROS in the spinal cord. We also found a positive correlation between the areas of cell death and those expressing *Sod2*, *Cas1* and *Gpx4*. Antioxidant enzymes are known to upregulate upon oxidative stress.²⁸ Particularly, *Sod2* and *Cas1* are regulated by *Foxo* genes, which could be direct targets of stress sensors including those detecting oxidative stress.²⁹ On the other hand, some growth factors may promote survival by activating the expression of antioxidant enzymes. For instance, Gdnf and Bdnf, growth factors with survival activity on motoneurons, upregulate activity of catalase, glutathione peroxidase, and CuZn-superoxide dismutase.^{15,16} In either case, whether antioxidant enzyme expression is stimulated by the oxidative stress that promotes cell death or by the growth factors that promote survival, high expression levels of genes encoding antioxidant enzymes are indicative of an oxidative stress condition. However, the correlations described above do not distinguish between the participation of ROS as a cause or as a consequence of cell death. Therefore, we tested the effect of an antioxidant on cell death.

EUK-134 is a synthetic compound with a combined activity of superoxide dismutase and catalase, being able to remove both superoxide anion and hydrogen peroxide.²¹ It protects neurons from dying in models of stroke and amyotrophic lateral sclerosis,²² and increases the life expectancy of *Sod2* mutant mice.²⁰ When EUK-134 was added to the cultures, we observed a decrease in the number of cells under oxidative stress and of those dying (detected by TUNEL technique or AO staining). An increase in ROS levels was not a consequence of cell death since Z-VAD-FMK reduced cell death without a decrease in oxidative stress. In agreement with the reduction in cell death, the number of motoneurons increased when EUK-134 or Z-VAD-FMK was present in the culture medium. Since the generation of motoneurons *in vivo* ends between E11 and E12,²⁶ it would be unexpected that

Figure 7 Effect of inhibition of autophagy on motoneuron survival in spinal cord cultures. E13 embryonic spinal cords were cultured for 12 h in different concentrations of LY294002 (15–60 μ M) an inhibitor of the positive regulator of autophagy PI3K-III; only the results obtained with 30 μ M are shown. In the same experiments, some spinal cords were cultured with EUK-134 or Z-VAD-FMK. Immunohistochemistry against Islet 1/2 and TUNEL procedure were performed after culture; the representative pictures show the area containing motoneurons (a; scale bar 25 μ m). The Islet 1/2-positive and TUNEL-positive cells per hemisection in the motoneuron region were quantified, and the average percentage from the numbers obtained in untreated cultures determined (panels b and c, respectively). The number of experiments performed in each condition is indicated (numbers within bars). Observe that LY294002 treatment increased the survival of motoneurons (a, b) without altering the number of TUNEL-positive cells (a, c); however, the antioxidant EUK-134 was more effective than LY294002 in protecting motoneurons from death, indicating that only a fraction of the population could degenerate by autophagy. ** $P < 0.01$

differentiation in culture accounts for the increments observed. Accordingly, no labeled cells were detected in the motoneuron region after *in vivo* and *in vitro* BrdU labeling. Furthermore, EUK-134 had not a significant effect on the number of proliferating cells. Therefore, the most plausible explanation is that EUK-134 diminishes ROS levels, and in this way prevents cell death. These data strongly support the idea that ROS participate in the activation of motoneuronal death during development.

Death of motoneuron by a ROS-regulated caspase-dependent and caspase-independent mechanism

On the basis of morphological characteristics, the cell death that occurs during development has been classified into three types:³⁰ (i) type 1 follows the typical characteristics of apoptosis (e.g., chromatin condensation, cell shrinkage) and is known to be regulated by caspases and Bcl2 family members among others;² however, it is important to note that caspase-independent apoptotic death has been reported.³¹ (ii) Type 2 resembles autophagy, which is characterized by the presence of many phagolysosomal vacuoles without apparent changes in the nucleus; a key autophagy positive regulator is a complex containing the PI3K-III kinase, while a negative regulator is the kinase mTOR.^{2,24} (iii) Type 3 is a nonlysosomal vesiculated degeneration, which is less well characterized. Our data are in agreement with the participation of at least two death mechanisms (type 1 and type 2) in the degeneration of motoneurons during spinal cord development.

It is generally assumed that the major natural death mechanism is caspase-dependent. Furthermore, it is commonly expected that an essential end of the cascade of activation of caspases is DNA degradation (Figure 10). This DNA degradation process (usually nucleosomal) is frequently detected by agarose gel electrophoresis or by the TUNEL technique, which detects it *in situ*. Using specific caspase inhibitors, we evidenced the participation of caspases in motoneuron death (manifested by the number of surviving motoneurons). DNA degradation in the motoneuron region also appears to involve caspases since the pan-caspase inhibitor Z-VAD-FMK produced a significant reduction in TUNEL-positive cells. However, neither caspase-8- nor caspase-9-specific inhibitors produced a significant reduction in the number of TUNEL-positive cells, despite the similar increase in the number of surviving motoneurons with the three caspase inhibitors. Therefore, caspase-8 and caspase-9 appear to act within the same death pathway (see below), but they do not target directly any molecule involved in DNA degradation (Figure 10). The greater reduction in TUNEL-positive cells than the increase in surviving motoneurons with the pan-caspase inhibitor (34.9 ± 2.2 cells TUNEL-negative/hemisection recovered ($n = 4$) versus 11.0 ± 1.3 motoneurons/hemisection recovered ($n = 5$); see also Figures 5–8) may indicate that DNA degradation can be prevented at a stage in which motoneurons are already dead (previous to spinal cord culture). Alternatively, blocking caspases could force motoneurons to switch to a death pathway that is caspase-independent.^{32,33}

Figure 8 Motoneuron survival and DNA fragmentation in spinal cord cultures treated with caspase-8 or caspase-9 inhibitors. E13 embryonic spinal cords were cultured for 12 h in the presence of caspase-8 (Ac-IETD-CHO), caspase-9 (Ac-LEHD-CHO) or pan-caspase (Z-VAD-FMK) inhibitors. Cultures in the presence of EUK-134 were also performed as reference. Immunohistochemistry against Islet 1/2 and TUNEL procedure were performed after culture; representative pictures of the motoneuron area are shown (a; scale bar 25 μm). The Islet 1/2-positive and TUNEL-positive cells per hemisection were quantified in the motoneuron region, and the average percentage from the numbers obtained in untreated cultures determined (panels b and c, respectively). The number of experiments performed in each condition is indicated (numbers within bars). Note the similar increase in motoneurons after treatment with Ac-IETD-CHO, Ac-LEHD-CHO or Z-VAD-FMK, which was about half of that obtained after treatment with the antioxidant, while the only caspase inhibitor able to produce a significant decrease in TUNEL-positive cells was Z-VAD-FMK. ** $P < 0.01$; *** $P < 0.001$

It is interesting to note that EUK-134 caused a larger increase in the number of motoneurons than caspase inhibitors (143 versus 72%). These data suggest that a caspase-independent form of cell death is occurring in a subpopulation of motoneurons, which is not detected by the TUNEL technique (up to 50%). In concordance, Oppenheim *et al.* has reported that motoneurons die in mice null for the genes coding caspase-3 and caspase-9³³ and in chick spinal cords treated with caspase inhibitors.³⁴ AO staining has been shown to detect apoptotic cell death, or at least to coincide with regions where abundant apoptotic cell death is occurring.^{27,35} In fact, the AO staining pattern of spinal cord determined here is very similar to the pattern determined by TUNEL on whole mount samples.¹⁸ However, it is interesting to note that EUK-134 almost completely abolished AO staining, whereas Z-VAD-FMK only produced a partial reduction. In contrast, the inverse effect was observed when cell death was detected by TUNEL. We suggest that AO is able to detect, perhaps in addition to apoptotic cells, cells dying by a nonapoptotic mechanism, or at least by a mechanism that does not correlate with DNA degradation. It has been reported that AO stains autophagic cells by

detecting acidic lysosomes,³⁶ therefore, autophagy could be one of the cell death mechanisms regulated by ROS occurring in the developing spinal cord that cannot be detected by the TUNEL technique. Supporting the contribution of autophagy to developmental motoneuron death, inhibition of PI3K-III increased the number of motoneurons without affecting significantly the number of TUNEL-positive cells. Type 2 or type 3 neuronal death has been observed in normal frog³⁰ and chick³⁷ spinal cord, as well as in other regions of the developing central nervous system.³⁰ Electron microscopy has been considered the definitive way to identify autophagic cells; however, a handicap of this technique is the difficulty to perform a reliable quantitative analysis. Recently, a molecule (LC3) has been described whose distribution changes markedly during autophagy, allowing the detection of this process *in vivo*.³⁸ LC3, therefore, will be a very useful marker to estimate the number of autophagic motoneurons during development.

Taken all data together, we conclude that ROS are involved in the control of different forms of motoneuron death (Figure 10). One of these forms, probably typical apoptosis, involves caspases and cells undergoing this type of death can

Figure 9 Cell proliferation and fate of proliferating cells in spinal cord cultures. BrdU was added to E13 spinal cords cultured for 12 h in 2 mM EUK-134 or control media (a), or injected intraperitoneally to pregnant mice 12 or 24 h before spinal cord dissection from E13 embryos (b). Representative pictures of spinal cord sections after BrdU-specific immunohistochemistry are shown; motoneuron (Mn) area is limited by white dots (scale bar 50 μ m). The distribution and average percentage of total BrdU-positive cells per hemisection (a), or the average percentage of BrdU-positive cells within the motoneuron area per hemisection (b) was determined; 100% is the number of BrdU-positive cells in a hemisection of slices from spinal cords cultured in medium without EUK-134. BrdU-labeled cells were not found within the motoneuron area when BrdU was given to the culture medium (a), but some BrdU-labeled cells were found in motoneuron area when BrdU was injected into pregnant mice (b). There was no significant difference in cell proliferation (estimated by the number of cells labeled with BrdU in culture) between the different culture conditions (a). Also, no significant difference was found in the number of BrdU-labeled cells within the motoneuron region when cultures with or without EUK-134 are compared (b).

be visualized by detecting DNA degradation. Another form also involves caspases, at least caspase-8 and caspase-9, but dying cells cannot be visualized by detecting DNA degradation. The mechanism that triggers these two latter forms of cell death may represent at least part of the extrinsic pathway, which is commonly initiated by dead domain-containing receptors. Recently, the participation of caspase-8 and Fas in motoneuron death was reported (see below³⁹). In addition, it has been shown that ROS can activate caspase-8^{40,41} and together with caspase-9 can trigger nonapoptotic cell death.⁴⁰ Further experiments are required to establish whether caspase-8 is regulated by ROS in motoneurons and the type of cell death associated with it. A third form, most likely autophagy, does not involve caspases and cells undergoing this type of cell death cannot be visualized by detecting DNA degradation.²⁴ In any cell degeneration process, the degradation of DNA is implicit; however, substantial differences in how and when this occurs could exist among them.

For instance, it is thought that apoptosis is a fast cell degeneration process in which DNA degradation could start soon after the initial death signals are received. In contrast, autophagy (or other nonapoptotic mechanisms) is a slow degeneration process in which DNA appears to degrade at the late stages. This conclusion implies that an important number of dying motoneurons at one given time during the degeneration process cannot be detected by sensing DNA degradation (e.g., by the TUNEL technique). It is not possible to estimate the contribution of each death mechanism referred above to the whole degeneration process of motoneurons, since the presence of caspase inhibitors could deviate the death pathway to caspase-independent mechanisms (e.g., autophagy^{32,33}). Nonetheless, we can estimate that the recovery with EUK-134 represents near 80% of the expected total recovery (44.6 ± 1.3 motoneurons/hemisection before culture ($n=4$) versus 34.0 ± 1.6 motoneurons/hemisection after culture with EUK-134 ($n=14$)); this means that about 20%

Figure 10 Possible ROS-regulated mechanisms of motoneuron death. ROS can be generated in motoneurons and/or neighboring cells in response to 'death factors' such as FasL or to the lack of survival factors. ROS can activate different death pathways through its oxidative activity on key triggering molecules. These death pathways can be grouped in two: caspase-dependent (left and center pathways) and caspase-independent (right pathway). The caspase-dependent pathways initially involve caspase-8 activation followed by activation of caspase-9, probably through the activity of caspase-8-processed t-bid on pro-apoptotic Bcl2 family members, finally triggering events in the mitochondria. Caspase-9 appears to activate two death pathways. One involves other caspases, particularly those that activate the enzymes involved in DNA degradation (e.g., ICAD). This pathway corresponds to the typical apoptosis, which includes DNA degradation as an early event during this fast death process (left pathway). The other does not involve additional caspases and activation of DNAses should be a late event since dead cells cannot be detected by the TUNEL technique. This pathway could share some characteristics with apoptosis except early DNA degradation. Finally, autophagy (right pathway) appears to be a major caspase-independent death process involved in motoneuron death, in which activation of DNA degradation occurs at a late stage of the degeneration process. The section of the proposed pathways within the shadow area includes those events during which the degeneration process may be irreversible. The reagents used in the present study as well as the expected major observation after the TUNEL technique is performed at one given time during development are shown (gray letters)

of motoneurons are not protected by the antioxidant at the developmental stage used in our experiments. Although the protective effect on motoneurons of EUK-134 could be due to a partial accessibility or efficiency of the compound, the possibility of a nonoverlapping motoneuron population between those dying by a caspase-dependent and those dying by a ROS-regulated mechanism should be considered.

Caspase-dependent and caspase-independent cell death could be linked to the same death activation process. Apoptotic and nonapoptotic dying cells are commonly

observed in regions where abundant cell death occurs.³⁰ In addition, it has been shown that reduction in caspase activity can disclose a nonapoptotic type of cell death in several systems.^{32,33} A death signal could activate rapid caspase-dependent apoptotic cell death in some circumstances, but in others, several weak signals might be required for efficient cell degradation. Cells receiving sufficient signals could reach the caspase activation threshold necessary to initiate apoptosis, whereas those not reaching that threshold would die by alternative slow mechanisms. ROS appear to be an early signal regulating both caspase-dependent and caspase-independent types of motoneuron death. The inability of EUK-134 to reduce the number of TUNEL-positive cells at comparable levels as Z-VAD-FMK might indicate that the antioxidant is no longer able to protect at late stages of the apoptotic process, reinforcing the idea that ROS act upstream activation of the caspase(s) triggering DNA degradation.

How ROS regulate motoneuron death?

Cell death can be regulated by either survival- or death-promoting factors. Commonly, it is considered that survival signaling cascades originate from extrinsic sources to the cell programmed to die, whereas cell death activators could arise from intrinsic or extrinsic sources, and in some instances could be activated by default in the absence of survival factors. Neurotrophic factors support the survival of neurons both *in vivo* and *in vitro*. A number of growth factors protect neurons against oxidative insults.^{16,42,43} It is known that neurons in culture in the absence of Ngf induce their NADPH oxidase⁴⁴ and neuronal nitric oxide synthase,¹³ two sources of ROS. On the other hand, Ngf, and Gdnf rescue neurons from dying apparently due to an increase in glutathione, and/or by inducing the activity of antioxidant enzyme such as catalase, superoxide dismutase, and glutathione peroxidase.^{16,45} Moreover, it has been shown that apoptosis induced by Tgf β 1 is accompanied by ROS generation;⁴⁶ and that cell death induced by growth factor deprivation or by Tnf α is accompanied by an increase in the cellular levels of superoxide anion and inhibited by a number of antioxidants and ROS scavengers.⁴⁷

During the stages used in the present study, motoneurons are reaching their targets, requiring growth factors such as Bdnf, Gdnf, Nt3 and others to survive.^{5,8,9} Traditionally, death has been visualized as passive, happening only in the absence of a survival factor. However, recently the active role of 'death factors' such as Ngf/p75, Fas, and Tgf β have been considered relevant during motoneuron death.^{9,11,39,48,49} In particular, Fas has been involved in motoneuron death *in vitro* and *in vivo*.^{10,39} Fas appears to activate motoneuron death by the increase in nitric oxide synthase and the generation of peroxynitrite; MnTBAP, a ROS scavenger, can rescue motoneurons from Fas-triggered motoneuron death.¹⁰ Putative 'death factors' promoting the increase in ROS levels must be produced by the spinal cord, since ROS appeared elevated in our cultures, which lack target cells. All this evidence suggests that, during normal development, motoneurons are exposed to oxidative stress, through the action of specific 'death factors' produced in the spinal cord, and growth factors produced by the target cell

prevent motoneurons from dying by upregulating antioxidant mechanisms.

ROS can act cell autonomously to control motoneuron death such that either the absence of growth factors increase ROS levels (e.g., by increasing NADPH oxidase activity) or the presence of growth factors decrease ROS levels (e.g., by increasing antioxidant enzyme activity). Another alternative is that ROS arise from non-neuronal neighboring cells that directly or indirectly act on motoneurons. From this latter perspective, it is interesting to consider the role of microglial cells. These cells have been demonstrated to be a source of superoxide anion and participate in the natural death of Purkinje neurons during cerebellum development.⁵⁰ In the nervous system, microglia are probably the most important cell type used to clear dead cells; thus, as macrophages, microglial cells appear to undergo a respiratory burst that increases the production of ROS during phagocytosis. In *Caenorhabditis elegans*, engulfment contributes critically to cell death activation, which is evident under conditions of low caspase activation.⁵¹ Therefore, noncell autonomous death activation may be a general feature of developmental cell death. The role of non-neuronal cells as source of ROS is a tempting possibility in the case of motoneurons, since it has been recently shown that the mutant form of Sod1 associated to the amyotrophic lateral sclerosis disease acts noncell autonomously.⁵² Therefore, natural and pathological motoneuron death may be mediated by a similar mechanism.

Our data suggest that several forms of cell death are simultaneously present during spinal cord development, sharing ROS elevation as one early activation event. It is noteworthy that nonapoptotic caspase-independent death appears to be the major mechanism for the neuronal degeneration observed in many diseases that affect humans. In addition, oxidative stress is considered a relevant process associated to those diseases. Therefore, a common mechanism could be used in developmental and pathological neuronal death. Consequently, defining the mechanism of cell death regulated by ROS during normal development will be important to understand neurodegenerative disorders and find the best therapeutic procedures.

Materials and Methods

Animals

Mouse strain CD-1 was used in this study. Pregnant females were killed by cervical dislocation from 11.5 to 15.5 days *post coitum* (the morning on which the vaginal positive plug is observed was taken as embryonic (E) day 0.5). The embryos were removed and the spinal cords dissected in phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10.1 mM Na₂HPO₄ and 1.8 mM KH₂PO₄).

Detection of cell death and oxidative stress with fluorescent dyes

Regions of cell death in freshly dissected embryonic spinal cords were visualized by AO (Sigma-Aldrich) staining using the protocol described by Salas-Vidal *et al.*²⁷ Briefly, the tissues were rinsed in PBS and then stained with AO at 5 μ g/ml in the same buffer for 30 min at 37°C. They were observed and analyzed by confocal microscopy as described below.

For ROS detection, spinal cords were dissected free from meninges in cold PBS at different stages of development and incubated with DHR-123 (Molecular Probes), a ROS-sensitive dye,⁵³ at 5 μ g/ml in PBS for 30 min at 37°C. The spinal cords were flattened as 'open book' by opening them by the dorsal side. They were analyzed by confocal microscopy with the outer side facing the microscope. Alternatively, we used HET (hydroethidine; also called dihydroethidium; Molecular Probes) to detect ROS in slices of spinal cords, since staining is kept after fixing (see below).

Spinal cords were imaged using a Bio-Rad MRC-600 confocal laser scanning system equipped with a krypton/argon laser coupled to an Axioscope microscope (Zeiss) that has a PlanNeofluar 5 $\times$ (aperture 0.15) objective. AO or DHR-123, which were used to stain the tissues, were excited with blue light (488 nm); AO fluorescence was detected with the red high-sensitivity (RHS) filter, and DHR-123 fluorescence was detected with the blue high-sensitivity (BHS) filter. The pinhole aperture was maintained at 5. Serial optical sections were produced at 30–54 μ m through the z-step motor.

Whole-mount *in situ* hybridization

Plasmid cDNA clones were linearized and transcribed with T7, T3 or with SP6 polymerase using digoxigenin (DIG) labeling reagents following the manufacturer's instructions (Roche Diagnostics). Probes were used at a concentration of 0.5–1 μ g/ml. The antisense riboprobes were: *Cas1*, 580 nucleotides (nt) transcript corresponding to position 618–1198; *Sod1*, 338 nt transcript corresponding to position 152–490; *Sod2*, 344 nt transcript corresponding to position 235–574; and *Gpx4*, 382 nt transcript corresponding to position 273–655. Mouse spinal cords were dissected in PBS and fixed overnight at 4°C in 4% paraformaldehyde in PBT-Tween (0.1% Tween-20 in PBS). They were progressively dehydrated in increasing concentrations of ethanol/PBT-Tween, and stored at –20°C until they were used. Whole-mount *in situ* hybridization was performed as described by Wilkinson and Nieto.⁵⁴

Explant cultures

The thoracic part of spinal cords from E12.5 mouse embryos was dissected in PBS, opened dorsally, released from meninges and flattened or only removed from the embryo with meninges. They were embedded in collagen (Cohesion) in DMEM 1 $\times$ (Invitrogen) and then allowed to set 30–40 min at 37°C. The spinal cords were cultured on DMEM 1 $\times$, without serum, supplemented with 200 IU/ml penicillin G sodium, 200 mg/ml streptomycin sulfate and 2 μ M glutamine. Cultures were maintained 12 h in a humidified incubator at 37°C in 5% CO₂/95%-air. Explants were cultured for 12 h in the absence (control) or presence of the following molecules diluted in the culture medium: EUK-134 (0.5–2 mM; Eukarion, Inc.), z-Val-Ala-Asp(Ome)-fluoromethylketone (100–150 μ M; Z-VAD-FMK; Biomol), N-acetyl-Ile-Glu-Thr-Asp-aldehyde (100 μ M; Ac-IETD-CHO; Biomol), N-acetyl-Leu-Glu-His-Asp-aldehyde (100 μ M; Ac-LEHD-CHO; Biomol), and LY294002 (15–60 μ M; Calbiochem). In other experiments Gdnf (400 ng/ml, Preprotech) was supplemented to collagen before immersion of spinal cords.

Antibodies and immunofluorescence

The primary antibody used was the monoclonal anti-Islet 1/2 (containing 2D6 and 4D5 antibodies 1:1 supernatants; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, USA). The secondary antibody was Alexa-Fluor 594 nm goat anti-mouse (1/1000) (Molecular Probes). Explants were fixed by immersion in 4% paraformaldehyde/PBS

for 2 h, rinsed with PBS, transferred to 30% sucrose in PBS overnight at 4°C, and frozen in Cryo-M-Bed (Bright Instruments). The first transverse 10- μ m-thick sections, taken from every three sections, were mounted on Superfrost plus glass slides, dried for 30 min, and fixed with 4% paraformaldehyde in PBS for 2 min. Samples were rinsed with PBS, permeabilized with PBS-1% Triton X-100 (PBT-Triton), blocked with anti-mouse-IgG for 1 h and 10% sheep serum in PBS-0.2% Triton X-100 for 3 h, and finally incubated for 1 h at room temperature with the primary antibody. The slides were rinsed in PBT-Triton and incubated with the secondary antibody diluted in PBT-Triton for 30 min at room temperature. After washing the sections in PBT-Triton, they were mounted with 80% glycerol/PBS, and observed by standard epifluorescence microscopy.

TUNEL technique

For detection of DNA fragmentation, the explants were fixed as described above. After immunohistochemistry, we rinsed the sections with PBS three times. The sections were incubated in permeabilization solution (0.1% sodium citrate and 0.1% Triton X-100) for 5 min at room temperature and then in the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL⁵⁵) cocktail, containing FITC-dUTP, for 1 h at 37°C according to the manufacturer's instructions (Roche Diagnostics). Finally, the sections were washed in PBS and mounted for analysis.

BrdU labeling

Pregnant females at 12 or 12.5 days *post coitum* were injected intraperitoneally with bromodeoxyuridine (BrdU; 5 mg/ml in PBS injected at 50 μ g/g of body weight). Embryos were harvested and the spinal cords were removed and cultured for 12 h as described above. In other experiments, BrdU was present during culture. Then, the spinal cords were fixed and processed to detect BrdU as reported by Moran-Rivard *et al.*⁵⁶ with some modifications. Briefly, we used Alexa-Fluor 594 nm goat anti-mouse (1/1000) instead of a secondary antibody coupled to peroxidase.

Statistics

Statistical analysis was conducted with GraphPad Prism software (GraphPad Software, Inc.). An appropriate statistical procedure was used for each data set. When indicated, statistical significance was determined by a Student's *t*-test (one or two tail). Each value in all histograms represents the mean of means $\pm$ standard error of the median (S.E.M.).

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