Cell Death and Differentiation (2006) 13, 2150–2153

www.nature.com/cdd

Letter to the Editor

Bcl2a1 serves as a switch in death of motor neurons in amyotrophic lateral sclerosis

Cell Death and Differentiation (2006) **13,** 2150–2153. doi:10.1038/sj.cdd.4401943; published online 19 May 2006

Dear Editor,

Amyotrophic lateral sclerosis (ALS) is an adult-onset neurodegenerative disease, characterised by the progressive degeneration of motor neurons.[1] Although most of the ALS cases are sporadic, about 10% of the cases are familial (fALS). Among fALS cases, one-fifth are linked to mutations in the gene encoding Cu, Zn superoxide dismutase (SOD1). The mechanisms through which mutations in SOD1 lead to late-onset motor neuron degeneration are not fully understood, even though it is established that mutant SOD1s acquire novel or enhanced neurotoxic properties.[2] It is now well accepted that, even when due to a single gene defect, ALS is a multisystemic disease resulting from a complex neurotoxic cascade that involves a crosstalk between motor neurons and glia and between motor neurons and muscle.[1,3–5] Previous studies indicate that reactive oxygen species, nitric oxide, cytokines and growth factors are among the players involved in a cascade of events leading to apoptotic death of motor neurons.[1,6]

Alteration in the expression of pro- and antiapoptotic genes, activation of caspases and release of cytochrome *c* have been found in the spinal cord of transgenic mice expressing human SOD1 with the G93A mutation and in human ALS patients without SOD1 mutations.[7] In addition, survival of the G93A-SOD1 ALS mice is prolonged by treatment with the pan-caspase inhibitor ZVAD-fmk and by overexpression of Bcl2, and we have demonstrated that overexpression of G93A-SOD1 requires the expression of Apaf1 to induce cell death.[8,9] Intriguingly, evidence of a direct link between SOD1 and an apoptotic pathway has been provided by the demonstration that both wild-type (WT) and mutant SOD1 can bind Bcl2.[10] In this study, we have analysed the expression of genes in the Bcl2-family in transgenic mice expressing G93A-SOD1 and focused on Bcl2a1, the mouse homologue of human Bfl-1 gene. Bcl2a1 is a multifunctional regulator preferentially expressed in haematopoietic and endothelial cells and induced in appropriately stimulated mast, smooth muscle, T and myeloma cells. Bcl2a1 has protective effects in some experimental paradigms but promotes programmed cell death in others.[11] Furthermore, it responds to oxidative stress, is a direct transcriptional target of nuclear factor (NF)-κB[12,13] and is induced by the inflammatory cytokines tumour necrosis factor (TNF-α) and interleukin (IL)-1β[14,15] These unique characteristics of Bcl2a1 prompted us to study the role of this protein in ALS, as reactive oxygen species, NF-κB, TNF-α and IL-1β have been proposed by us and by others to contribute in the molecular crosstalk occurring among different cell types in the pathogenesis of ALS[1,6]

We have investigated expression of the Bcl2 family members in spinal cords of transgenic G93A-SOD1, WT-SOD1 and non-transgenic mice. As shown in Figure 1, *Bcl2a1* is the only member of this family to be upregulated in both asymptomatic and symptomatic G93A mice (Figure 1a), with a tendency to decrease in the final stages of disease. Interestingly, this upregulation is tissue specific, as *Bcl2a1* mRNA is increased neither in the brain nor in the muscle of G93A transgenic mice.

In Northern blot experiments, *Bcl2a1* is represented by a single mRNA band in two strains of G93A-SOD1 transgenic mice with different genetic backgrounds (Supplementary Figure S1A). Moreover, we have observed that whereas *Bcl2a1* mRNA is upregulated in the spinal cord of ALS mice, the mRNAs coding for *BclX* and for a member of the antioxidant defence (SOD2), which are both controlled by NF-κB,[16,17] are not, suggesting that Bcl2a1 increase is not due to a general activation of transcription by NF-κB (Supplementary Figure S1B).

To investigate whether increased *Bcl2a1* mRNA expression in G93A-SOD1 mice is functionally significant, we have performed Western blot analysis on total protein extracts from spinal cord of mice with different genotypes, using an antibody that can detect the mouse protein but not its human homologue (Supplementary Figure S1C). As reported in Figure 1b, Bcl2a1 was identified as a single immunoreactive band in transgenic mice overexpressing G93A-SOD1 but not in non-transgenic littermates or in transgenic mice overexpressing WT SOD1.

Until now, expression of *Bcl2a1* has never been described in neuronal tissues. This observation raised the question of which cell type was upregulating *Bcl2a1* during ALS progression in G93A transgenic mice. By a combined *in situ*/immunohybridisation technique (Figure 1c), we have observed that motor neurons specifically express this mRNA, as indicated by labelling of many NeuN-positive cell bodies appearing typically large in the same cross-section where small neuronal bodies are not stained. This observation indicates that the slight decrease in *Bcl2a1* expression observed in spinal cord from late symptomatic G93A-SOD1 mice with respect to earlier stages may be owing to the decrease in the number of motor neurons rather than to diminished level of mRNA.

Bcl2a1 has a dual role as an anti- or proapoptotic factor depending on the experimental paradigm. We asked which is

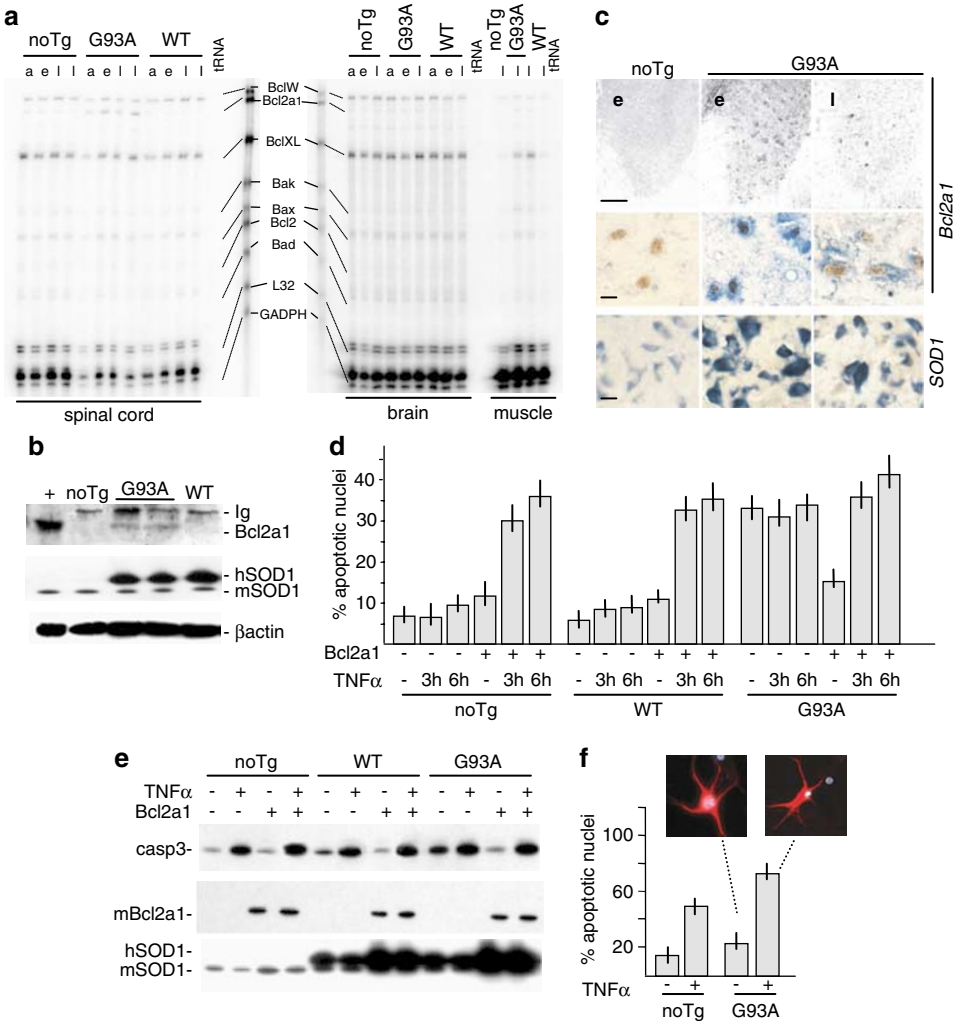

Figure 1 Bcl2a1 is overexpressed only in motor neurons of in G93A-SOD1 mice and it switches from anti- to proapoptotic in neuronal cells overexpressing G93A-SOD1, after treatment with TNF-α. (**a**) RNAse protection assay (RPA) was performed on 5 μg of total RNA extracted from spinal cord, brain and muscle of non-transgenic (noTg), G93A-SOD1 (G93A) and WT-SOD1 (WT) transgenic mice (Jackson Laboratory), using multiprobe APO-1 (Ambion). Tissues were collected from age-matched mice according to G93A-SOD1 phenotype: asymptomatic (a), early symptomatic (e) and late symptomatic (l). The positions of the probes for the different mRNAs are identified with lines, whereas the dotted lines point at the protected fragments. In all samples, the presence of the transgene was verified using hSOD1 riboprobe (not shown). (**b**) Forty micrograms of total protein extracted from the spinal cord of early symptomatic G93A-SOD1 mice and age-matched non-transgenic or WT-SOD1 mice was separated in SDS/PAGE and revealed using anti-mouse Bcl2a1 (R&D System), -SOD1 (Stressgene) and -β-actin (Sigma) antibodies. Bands corresponding to Bcl2a1 and the light chain of immunoglubulins (Ig) are indicated. The symbol (+) indicates a protein extract obtained from NSC-34 cells transfected with a plasmid coding for mouse Bcl2a1, used as positive control. (**c**) Cryosections obtained from early symptomatic (e) or late symptomatic (l) G93A-SOD1 mice and age-matched non-transgenic mice were used for *in situ* immunohybridization, using *Bcl2a1* DIG-labelled riboprobe (blue staining) or human *SOD1* DIG-labelled riboprobe (blue staining) and NeuN monoclonal antibody (Zymed, brown staining). Scale bar = 300 μM (upper panel), 20 μM (middle and lower panels). The presence of the transgene was verified by using a human *SOD1* riboprobe (lower panel). (**d**) ETNA cells were transiently transfected with the indicated plasmid coding for human WT-SOD1 or G93A-SOD1 or murine Bcl2a1. At 48 h after transfection, cell were treated for the indicated time with 10 ng/ml mouse recombinant TNF-α (Sigma). Quantification of cell death was performed by counting apoptotic nuclei stained with Hoechst 33342. (**e**) ETNA cells were trasfected as in (**d**); 24 h after transfection, they were exposed to a differentiating medium for 48 h as described previously.[8] Protein extracts were analysed by Western blotting for the presence of cleaved caspase-3, Bcl2a1 and SOD1. (**f**) Primary cultures from the spinal cord[4] of non-transgenic (noTg) or G93A embryos (E13.5) were treated with TNF-α for 4 h. Quantitative analysis of apoptotic nuclei is shown as mean $\pm$ S.D. of $n = 3$ independent experiments. A representative double staining with a motor neuronal marker (SMI32 antibody, Sternberger) and with Hoechst 33342 is shown

its role in motor neurons expressing G93A-SOD1 and/or exposed to TNF-α, that is, in a situation mimicking the neuro-inflammatory condition associated with degeneration in ALS.

In ETNA neuronal precursors or NSC-34 motoneuronal cells expressing Bcl2a1 and either G93A-SOD1 or WT SOD1, Bcl2a1 upregulation is a protective, rather than detrimental consequence of the expression of toxic G93A-SOD1, as in the case of treatment with staurosporine (Supplementary Figure S1D–G). On the contrary, when neuronal cells expressing Bcl2a1 were exposed to TNF-α, the level of cell death increases (Supplementary Figure S1H–I) and cotransfection of ETNA cells with G93A-SOD1 and Bcl2a1 and simultaneous treatment with TNF-α clearly induces an increase in cell death (Figure 1d), indicating that in neuronal cells stimulated with TNF-α, Bcl2a1 loses its antiapoptotic properties against cell death induced by G93A-SOD1 and becomes proapoptotic.

TNF-α-induced degeneration in the presence of Bcl2a1 is not prevented by overexpression of WT SOD1 (Figure 1d). These results are consistently confirmed also in differentiated ETNA cells (Figure 1e) and in primary cultures from the spinal cord of G93A mice (Figure 1f), where only a modest increase in *Bcl2a1* mRNA is observed with respect to control (Supplementary Figure S1J).

Changes in the balance of pro- and antiapoptotic molecules belonging to the Bcl2 family, together with the modulation of their mitochondrial/cytosolic distribution reported in animal models, may contribute to degeneration of motor neurons in ALS. For instance, it has been observed that the translocation of Bax and Bak from the cytosol to the mitochondria correlates with a diseased progression in mutant SOD1 mice, and increased Bid cleavage is detected in mutant SOD1 mice as compared to WT controls.[7]

Our data extend previous findings in three important directions.

First, we have carried out a systematic survey to determine the role of the Bcl2-family members in the pathogenesis of ALS and found that Bcl2a1 is the only member to be markedly upregulated selectively in the spinal cord of G93A-SOD1 mice. This upregulation leads to accumulation of Bcl2a1 protein in motor neurons, that is, selectively in those cells that are lost during the course of the disease.

Second, induction of Bcl2a1 in G93A-SOD1 transgenic mice is in line with the idea that one of the toxic functions acquired by mutant SOD1 is to exert a pro-oxidant chemistry. In fact, it has been reported that Bcl2a1 is induced by hyperoxia in the lung of mice, where exposure to 100% O_2 causes alveolar cell death.[18] Because oxidative stress is known to trigger activation of NF-κB in several experimental paradigms,[19] we had hypothesized that the observed induction of the gene coding for Bcl2a1 might occur via a general upregulation of NF-κB-dependent pathway. However, this seems not to be the case, and clearly further studies are needed to elucidate the exact pathway of activation of Bcl2a1 in neurons.

Third, upregulation of Bcl2a1 may represent an early neuronal defense reaction to the toxic action of mutant SOD1, as demonstrated by a decrease in caspase-3 activity and decrease of picnotic nuclei upon transfection of G93A neuronal cells with the plasmid coding for Bcl2a1 and in line with the observation that upregulation of Bcl2a1 occurs already at the asymptomatic stage in G93A-SOD1 mice. However, at variance with cultured cells, motor neurons in the spinal cord are subjected and respond to a variety of signals from neighbouring cells. Among those signals, upregulation of TNF-α in reactive microglia and astroglia cells seems to play a crucial role in the 'non-cell autonomous' mechanism of motor neuron death in ALS.

It has been shown that Bcl2a1 has various effects in response to TNF-α in different cell cultures: it acts as an antiapoptotic in immune-derived cell lines,[13] in microvascular endothelial cells[20] and in HT1080 fibrosarcoma cells,[14] whereas it is clearly proapoptotic in B cells.[11] In this work, we demonstrate in different cell cultures (NSC-34, undifferentiated and differentiated ETNA cells and primary cultures from spinal cords) that the overexpression of Bcl2a1 and G93A-SOD1 increases the level of cell death after exposure to TNF-α, a situation mimicking the condition of neuroinflammation occurring in ALS.

These observations may open a new scenario to explain the selective vulnerability of motor neurons towards mutant SOD1 expression. The selective induction of Bcl2a1 in motor neurons may represent an early event aimed to protect cells from oxidative stress induced by mutant SOD1. At later stages, when neuroinflammation occurring in ALS is maximal and TNF-α is released from activated microglia, Bcl2a1 expressed early in G93A-SOD1 motor neurons may switch from being antiapoptotic to proapoptotic. Thus, the increase in Bcl2a1 may constitute a reinforcing event in the progressive loss of motor neurons occurring in ALS.

Further studies are needed to explore the mechanism underlying the proapoptotic behaviour of Bcl2a1 in neurons, for example, whether re-localization of this factor occurs or this factor is subject to proteolitic cleavage in SOD1-linked ALS, as suggested by studies in other models.[11]

Acknowledgements

We thank A Ferri and M Cozzolino for critical reading of the manuscript, M Nencini for help with cell cultures and M Rodriguez for help with cloning. NSC-34 cells were kindly provided by Neil R Cashman. This work was supported by Min. Salute (PF 'Studio delle alterazioni del processamento degli RNA messaggeri in patologie degenerative del motoneurone' to MTC), by Fondazione Cariplo (Project 'Role of Alterations In RNA Metabolism In Motor Neurone Diseases' to MTC) and by Min. Salute (PF 'Malattie neurodegenerative e processi di riparazione neuronale' to CC).

Note added in proof

During revision of this paper, expression of Bcl2a1 in the spinal cord from G93A mice has been reported by others (Pun, S *et al.* Nat Neurosci. 2006 Feb 12; [E-pub ahead of print]).

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Supplementary Information accompanies the paper on Cell Death and Differentiation website (http://www.nature.com/cdd)