

Letter to the Editor

The proapoptotic BCL-2 family member BIM mediates motoneuron loss in a model of amyotrophic lateral sclerosis

Cell Death and Differentiation (2007) 14, 1386–1389; doi:10.1038/sj.cdd.4402166; published online 18 May 2007

Dear Editor,

Amyotrophic lateral sclerosis (ALS) is a progressive adult-onset motoneuron disease characterized by muscle weakness, atrophy, paralysis and premature death. The pathological hallmark of ALS is the selective degeneration of motoneurons in the spinal ventral horn, most brainstem nuclei and cerebral cortex.¹ The majority of ALS patients lack a defined hereditary genetic component and are considered sporadic, whereas approximately 10% of cases are familial (FALS). Over 100 mutations in the gene encoding superoxide dismutase-1 (SOD1), which trigger the misfolding and abnormal aggregation of SOD1, have been genetically linked with FALS.¹ Overexpression of human FALS-linked SOD1 mutations in transgenic mice provokes age-dependent protein aggregation, paralysis and motoneuron degeneration suggesting a direct involvement of misfolded SOD1 in the disease process.¹ The primary mechanism by which mutations in SOD1 contribute to progressive motoneuron loss in FALS remains unknown. It has been proposed that motoneuron apoptosis is mediated by different mechanisms including mitochondrial dysfunction, altered axonal transport, endoplasmic reticulum stress and other non-neuronal components (reviewed by Pasinelli and Brown¹).

The BCL-2 family of proteins is a group of evolutionarily conserved regulators of cell death that operate at the mitochondrial membrane to control caspase activation. The BCL-2 family is comprised of pro- and antiapoptotic members, and is defined by the presence of up to four conserved domains within their primary structure.² Antiapoptotic BCL-2 family members display sequence homology in four α -helical domains called BCL-2 homology (BH)1 to BH4 (i.e. BCL-2 and BCL-X_L). Proapoptotic members can be further subdivided into more fully conserved, 'multidomain' members containing BH1-3 (i.e. BAX and BAK) or the 'BH3-only' members (i.e. BID, BIM, PUMA and BAD) that contain a single α -helical domain critical for activation of apoptosis. Multidomain BAX and BAK function in concert as an essential gateway to the intrinsic cell death pathways operating at the mitochondria, constituting the main core proapoptotic pathway.² The upstream BH3-only members respond to particular apoptotic signals and subsequently trigger the conformational activation of BAX and BAK, inducing their intramembranous homooligomerization and resultant permeabilization of the mitochondrial outer membrane.² Released mitochondrial proteins, such as cytochrome *c*, trigger the activation of caspases through the apoptosome complex.

Alterations in the expression of different BCL-2 family members have been described in the spinal cord of transgenic mice expressing mutant SOD1 and in humans affected with ALS.^{3,4} Moreover, survival of SOD1^{G93A} mice is prolonged by the administration of caspase inhibitors or by the overexpression of BCL-2.^{5–7} A direct interaction between BCL-2 and mutant SOD1 has been proposed to occur at mitochondria, which may contribute to FALS pathogenesis.⁸ A recent report showed that BAX deletion slightly increases the survival of SOD1^{G93A} mice, but it strongly protects motoneurons against apoptosis.⁹ This suggests that, in addition to cell death, loss of neuronal functionality is responsible for motoneuron dysfunction in FALS. Although it is known that BAX mediates motoneuron apoptosis in FALS, the upstream death signals that activate the core proapoptotic pathway remain unidentified.

We therefore chose to investigate the involvement of upstream BH3-only proteins in FALS, as these proteins are good candidates as Bax modulators. We first determined the expression levels of different BCL-2 family members in the spinal cord of hSOD1^{G93A} and mSOD1^{G86R} transgenic mice including several BH3-only proteins (BIM, BMF, BAD, PUMA, NOXA, BID and BIK), the proapoptotic BAX and BAK, and the antiapoptotic BCL-X_L and BCL-2 proteins (Supplementary Figure S1). Using quantitative RT-PCR, we observed an increase in the mRNA levels of the proapoptotic BH3-only member *bim* in the spinal cord of post-symptomatic SOD1^{G86R} transgenic mice, when paralysis of the hind legs was observed (Supplementary Figure S1), whereas only a slight increase in the mRNA levels of the BH3-only proteins *bid* and *noxa* were detected in the same samples. No significant changes were observed in the mRNA levels of *bad*, *bmf*, *bik*, *puma*, *bax* and *bak*. In contrast, an increase in the mRNA levels of *bcl-2* and *bcl-xL* mRNA was observed. Consistent with the mRNA induction of *bim*, Western blot analysis of spinal cord extracts revealed that the BIM extra long (BIM_{EL}) is induced at the protein level in the spinal cord of post-symptomatic SOD1^{G93A} and SOD1^{G86R} transgenic mice (Figure 1a). A slight increase in the expression levels of PUMA was observed, whereas the levels of other proapoptotic proteins, such as BAX, BID or BAK, were not significantly altered (Figure 1a and Supplementary 2S1). In addition, an upregulation of antiapoptotic BCL-X_L and BCL-2 was observed to different extents in the two mutant SOD1-transgenic mice analyzed (Figure 1a and Supplementary S2a). These

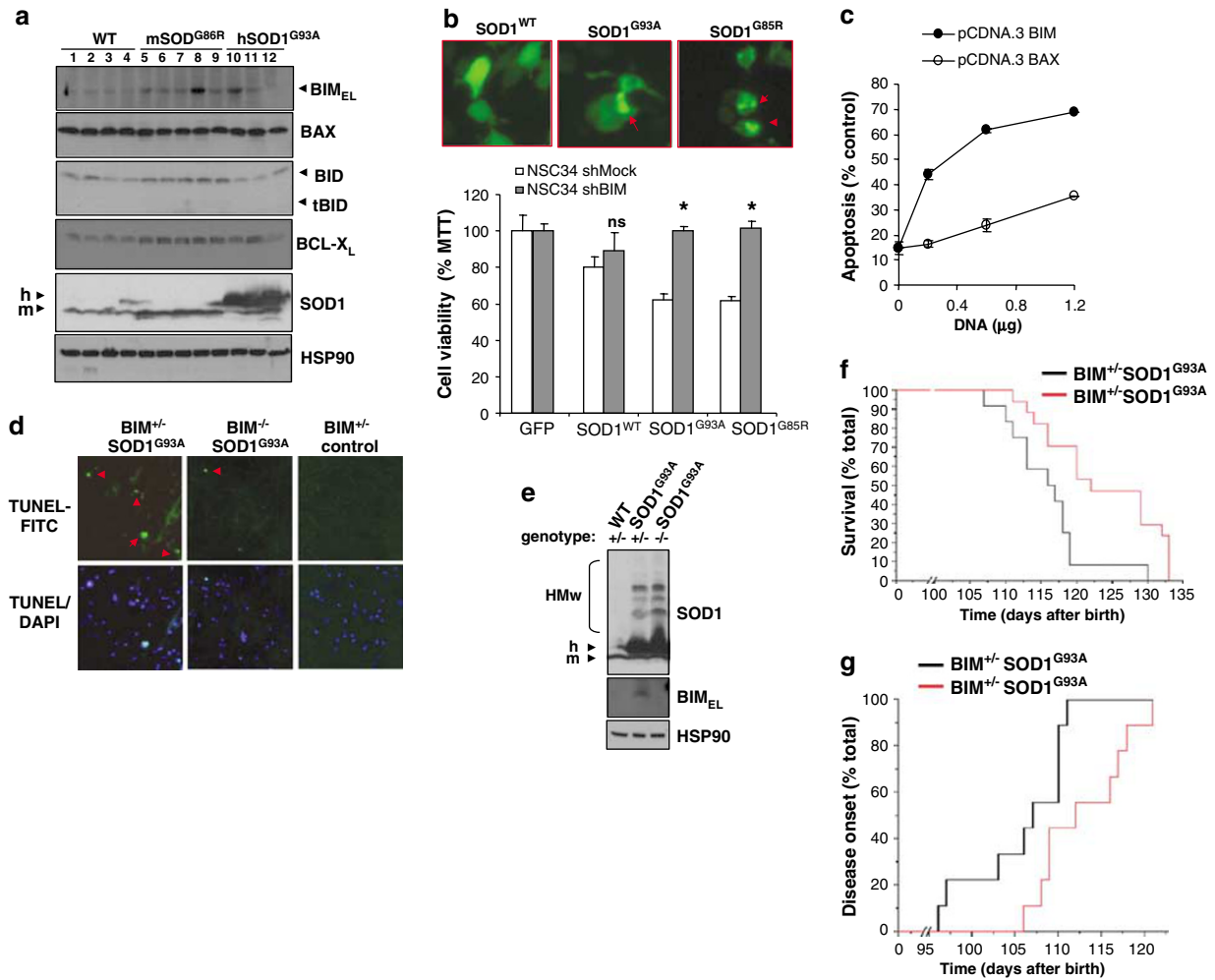


Figure 1 Upregulation of the pro-apoptotic protein BIM mediates motoneuron loss in cellular and mouse ALS models. (a) The expression levels of BIM, BAX, BID, BCL-X_L, and SOD1 were analyzed in spinal cord extracts from different symptomatic SOD1^{G93A}, SOD1^{G86R} or control mice by Western blot. Animals are between 115 to 125 days of age. Line 12 represents a *bim* deficient mouse. As loading controls, the expression levels of Hsp90 are shown. (b) NSC34 cells were stably transduced with lentiviral vectors expressing shRNA against the *bim* or *luciferase* mRNA (Mock), and cell viability was quantified 72 h after transfecting expression vectors for SOD1^{WT}, SOD1^{G93A} or SOD1^{G86R}-EGFP fusion proteins using the MTS viability assay. Experiments were performed in the presence of 0.5% serum. As control, cells were transfected with an EGFP expression vector and this is considered as 100% viability. Upper panel: The generation of SOD1-EGFP aggregates was visualized by fluorescent microscopy. Asterisks represent *P*-values < 0.001 obtained using Student's *t* test of comparing Mock versus shRNA for BIM (ns, not significantly different). (c) NSC34 cells were transiently transfected with indicated amounts of expression vectors for BIM or BAX, and the percentage of apoptosis was quantified after 24 h by FACS using Annexin-V-PE staining. (d) The number of TUNEL-FITC positive cells (apoptotic cells) was visualized in the spinal cord of BIM deficient mice or BIM heterozygous mice carrying a human SOD^{G93A} allele. Animals shown represent littermate controls of the same age (120 days). Results are representative of the analysis of three different mice per group. (e) The levels of hSOD1^{G93A} aggregation and BIM were determined by Western blot in BIM deficient and BIM heterozygous mice. As negative control non transgenic BIM heterozygous mice are shown. Of note, high molecular weight (HMw) SOD1 aggregates are observed in the analysis. As controls the expression levels of BIM and Hsp90 are shown. (f) Kaplan–Meier survival curves of BIM deficient (*N* = 15) or BIM heterozygous (*N* = 17) mice bearing a hSOD1^{G93A} allele. (g) The disease onset of BIM deficient (*N* = 15) or BIM heterozygous (*N* = 17) mice bearing a hSOD1^{G93A} allele was determined and plotted against time. Disease onset was defined as the appearance of abnormal limb-clasping, slight tremor felt in one of the hind-limbs, wobbly gait and the first signs of paralysis in one hind-limb

results contrast with previous findings in which BCL-2 and BCL-X_L levels were decreased in the model, possibly owing to analysis at different stages of the disease or variability in mouse genetic background.³

BIM is a potent activator of apoptosis and, in neurons, induces the direct activation of BAX.¹⁰ BIM upregulation is involved in neuronal loss induced by growth factor deprivation and brain seizures, and it is a critical mediator of ischemia-brain injury.^{10–12} The function of BIM in neuronal death may not represent a general pro-death activity, as BIM deficiency

does not protect mutant *Lurcher* mice from neurodegeneration.¹³ In order to establish the role of BIM in the toxicity of SOD1 mutants, we targeted *bim* mRNA with small hairpin RNA (shRNA) using lentiviral-based delivery in the motoneuron cell line NSC34 (Supplementary Figure S2b). Overexpression of SOD1^{G93A}- or SOD1^{G86R}-EGFP fusion proteins leads to the aggregation of SOD1 in NSC34 cells (Figure 1b and Supplementary Figure S2c) as described previously.¹⁴ Knockdown of BIM completely protected NSC34 cells from mutant SOD1 toxicity after transient transfection (Figure 1b),

suggesting that BIM is part of the proapoptotic pathway triggered by SOD1 aggregates. In agreement with this hypothesis, transient expression of BIM_{EL} induced high levels of apoptosis when compared with BAX, indicating that motoneurons are highly susceptible to changes in BIM expression (Figure 1c).

In order to evaluate the role of BIM in FALS *in vivo*, we generated a BIM-deficient mouse and crossed it with a transgenic mouse overexpressing human SOD1^{G93A}. Deletion of BIM drastically decreased the number of TUNEL-positive cells in the ventral horn of SOD1^{G93A} mice (Figure 1d), similar to the effects described by manipulating the levels of BCL-2 or BAX via BCL-2 overexpression or BAX deletion.^{5,9} These results suggest that the induction of BIM is upstream of BAX-mediated apoptosis in FALS. In contrast to BAX-deficient mice,⁹ only minor changes were observed in motoneurons populating the ventral horn of BIM-deficient mice when compared with littermate controls (Supplementary Figure S2d). This suggests that developmental apoptosis in the spinal cord is not considerably altered in the absence of BIM. Neither the expression levels of human mutant SOD1^{G93A} nor the generation of high molecular weight SOD1 aggregates were drastically affected by abrogation of BIM expression (Figure 1e). Finally, we assessed the possible effects of BIM deletion on the disease onset and survival of SOD1^{G93A} transgenic mice. As shown in Figure 1f, BIM deficiency extended the lifespan of human SOD1^{G93A} transgenic mice when compared with BIM heterozygous mice. The increased survival of BIM-deficient mice was comparable with the effects described for BAX-deficient or BCL-2-over-expressing mice.^{5,9} In addition, disease onset was significantly delayed in BIM-deficient mice, supporting a role for this proapoptotic BH3-only protein in motoneuron death in FALS (Figure 1g).

We have provided evidence supporting a role of the proapoptotic protein BIM in FALS. Quantitative determination of the mRNA levels of different proapoptotic BCL-2 family members indicated that BIM is upregulated in a FALS mouse model during the symptomatic stage. Analysis of the role of BIM *in vitro* demonstrated that its expression is required to trigger cell death induced by SOD1 mutants, and that BIM expression *per se* is highly neurotoxic to the well-characterized motoneuron cell line NSC34.¹⁴ In agreement with these observations, ablation of BIM *in vivo* reduced cellular apoptosis in the ventral horn of a transgenic mouse model of FALS, increasing lifespan. These results are novel, as the upstream signals leading to mitochondrial-dependent apoptosis in FALS are not known.

A recent report characterized the hierarchical organization of the BCL-2 family of proteins.¹⁵ Two classes of BH3-only proteins were defined, where BID, BIM and PUMA are strong activators of BAX- and BAK-dependent apoptosis (killers), whereas other BH3-only proteins, such as BAD and NOXA, modulate the pathway by sequestering antiapoptotic proteins (sensitizers). We did not observe an activation of BID (associated with caspase-dependent cleavage; tBID) in SOD1 transgenic mice. PUMA expression levels were low and a slight but reproducible increase of its expression was detected in our FALS mouse model, suggesting that BIM may be a specific activator of motoneuron death in FALS. The

precise contribution of PUMA to ALS remains to be established. In addition to mRNA upregulation, BH3-only proteins are activated by post-translational modifications. Phosphorylation of BIM by the c-Jun N-terminal kinase (JNK) pathway increases its proapoptotic activity, which is known to trigger neuronal death in models of ischemia-reperfusion.¹⁶ BIM resides in an inactive form by binding to the microtubule-associated dynein motor complex and, when released by phosphorylation, promotes BAX-dependent apoptosis.¹⁷ Activation of JNK has been described in astrocytes in models of FALS¹⁸ and disruption of dynein/dynactin interaction inhibits axonal transport in motoneurons causing progressive motor degeneration. It remains to be determined whether or not post-translational modifications of BIM play a role in motoneuron loss in FALS.

Potential therapeutic benefits of targeting the mitochondrial-apoptosis pathway were suggested when enhanced motoneuron survival and improved neuromuscular function of a FALS model was observed after intraspinal injection of an adeno-associated virus encoding BCL-2.¹⁹ BAX and BCL-2 are components of the core proapoptotic pathway, where many different death signals converge to induce apoptosis. Thus, their targeting may have considerable side effects in the long term (i.e. cancer or autoimmunity) by affecting the physiology of other organs. Therefore, it is crucial to identify specific upstream regulators of BAX/BCL-2 in motoneurons as potential therapeutic targets. Pharmacological inhibition of proapoptotic molecules, such as BAX, prevent neuronal death in models of brain ischemia,²⁰ suggesting that a similar strategy may be feasible to inhibit the activity of BIM. Alternatively, lentiviral delivery of shRNA against *bim* mRNA may be feasible *in vivo* based on the proven success of the method in FALS models. The discovery of specific molecules contributing to motoneuron dysfunction may represent beneficial targets to treat ALS and other fatal motoneuron disorders.

Acknowledgements. We thank Nika Danial for providing support with animal housing. We thank Dorothy Zhang for performing histological analysis of spinal cord tissue. We thank B Turner and J Atkin (University of Melbourne) for providing us expression vectors for SOD1 mutants, N Cashman (McGill University) for providing NSC34 cells and the Broad Institute for kindly providing us shRNA vectors to target BIM. This work was supported by NIH grants P01 CA92625 and R37 CA50239 (SJK); R01 AI32412 and the V Harold and Leila Y Mathers Charitable Foundation (LHG); and FONDECYT no. 1070444 (CH) and FONDAP grant no. 15010006, and a Post Doctoral Fellowship from the Damon Runyon Cancer Research Foundation (CH). R Brown and P Pasinelli receive support for ALS studies from the N.I.N.D.S. the N.I.A., the Al-Athel ALS Research Foundation, the ALS Association, the ALS Therapy Alliance, the Angel Fund, Project ALS and the Pierre L de Bourgnecht ALS Research Foundation.

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