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The BCL-2 family protein Bid is critical for pro-inflammatory signaling in astrocytes

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ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by the loss of motoneurons in the spinal cord, brainstem and motor cortex. Mutations in the superoxide dismutase 1 (*SOD1*) gene represent a frequent genetic determinant and recapitulate a disease phenotype similar to ALS when expressed in mice. Previous studies using *SOD1*^{G93A} transgenic mice have suggested a paracrine mechanism of neuronal loss, in which cytokines and other toxic factors released from astroglia or microglia trigger motoneuron degeneration. Several pro-inflammatory cytokines activate death receptors and may downstream from this activate the Bcl-2 family protein, Bid. We here sought to investigate the role of Bid in astrocyte activation and non-cell autonomous motoneuron degeneration. We found that spinal cord Bid protein levels increased significantly during disease progression in *SOD1*^{G93A} mice. Subsequent experiments *in vitro* indicated that Bid was expressed at relatively low levels in motoneurons, but was enriched in astrocytes and microglia. Bid was strongly induced in astrocytes in response to pro-inflammatory cytokines or exposure to lipopolysaccharide. Experiments in *bid*-deficient astrocytes or astrocytes treated with a small molecule Bid inhibitor demonstrated that Bid was required for the efficient activation of transcription factor nuclear factor-κB in response to these pro-inflammatory stimuli. Finally, we found that conditioned medium from wild-type astrocytes, but not from *bid*-deficient astrocytes, was toxic when applied to primary motoneuron cultures. Collectively, our data demonstrate a new role for the Bcl-2 family protein Bid as a mediator of astrocyte activation during neuroinflammation, and suggest that Bid activation may contribute to non-cell autonomous motoneuron degeneration in ALS.

Introduction

Amyotrophic Lateral Sclerosis (ALS) is an adult-onset, progressive motoneuron disease characterized by degeneration of motoneurons in the motor cortex, brainstem and spinal cord ventral horns. Mutations in the copper/zinc superoxide dismutase gene (*SOD1*) account for approximately 20–25% of familial ALS patients (Deng et al., 1993; Rosen, 1993). Over-expression of the human familial ALS-linked *SOD1*^{G93A} mutation in transgenic mouse models (*mtSOD1*) confers a phenotype similar to that of ALS patients with motor function deficits and a reduced lifespan (Gurney et al., 1994). Although the precise mechanisms of motoneuron degeneration in ALS remain largely unidentified, proteotoxicity, endoplasmic reticulum stress, glutamate excitotoxicity, oxidative stress, and activation of apoptosis have been shown to facilitate motoneuron death in *mtSOD1* mice and other ALS disease models (Bruijn et al., 2004; Pasinelli and Brown, 2006).

Apoptosis is a genetically controlled cell death process that is activated by multiple stress stimuli. Most forms of stress-induced apoptosis engage the so-called mitochondrial apoptosis pathway. This pathway is controlled by the Bcl-2 protein family (Youle and Strasser, 2008). The Bcl-2 homology domain 3 (BH3)-only proteins are pro-apoptotic members of this family. BH3-only proteins are transcriptionally and post-translationally activated in neurons in response to stress (Bruijn et al., 2004; Ward et al., 2004), and induce apoptosis due to their ability to bind and neutralize anti-apoptotic Bcl-2 family proteins (Youle and Strasser, 2008). Activation of BH3-only proteins leads to the mitochondrial membrane insertion and oligomerization of Bax and Bak (Lovell et al., 2008; Tait and Green, 2010). The channels formed by these oligomers constitute release

Abbreviations: ALS, Amyotrophic Lateral Sclerosis; APAF1, Apoptotic protease activating factor 1; BH3-only protein, Bcl-2 homology domain 3-only protein; *bid*, BH3 interacting domain death agonist; GFAP, glial fibrillary acidic protein; Iba-1, ionized calcium-binding adapter molecule 1; IFNγ, interferon-γ; IKK, inhibitor of kappaB kinase; IL-1β, interleukin-1β; mtSOD1, mutant superoxide dismutase 1; NEMO, NF-kappaB essential modifier; NF-κB, nuclear factor-kappaB; NOD1, nucleotide-binding oligomerization domain containing 1; SOD1, superoxide dismutase 1; PND, post-natal day; SMI-32, Sternberger monoclonal-incorporated antibody 32; tg, transgenic; wt, wild-type.

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channels in the mitochondrial outer membrane, enabling the release of pro-apoptotic factors that trigger caspase-dependent and caspase-independent apoptosis. Bax was shown to accumulate in mitochondria in animal models of ALS (Guegan et al., 2001), and deletion of the *bax* gene in *mtSOD1* transgenic mice inhibited motoneuron death (Gould et al., 2006). Recently it was shown that conditional, combined deletion of *bax* and *bak* potently delayed disease onset and progression in the $SOD1^{G93A}$ mouse model of ALS (Reyes et al., 2010). BH3-only proteins responsible for the activation of Bax (and potentially Bak) in animal models of ALS were also recently identified. The BH3-only protein Bim was shown to be transcriptionally up-regulated in response to *mtSOD1* overexpression, and deletion of *bim* protected against motoneuron loss in *mtSOD1* mice *in vivo* (Hetz et al., 2007). We showed recently that endoplasmic reticulum stress was able to activate the BH3-only protein Puma in motoneurons, and that deletion of *puma* protected motoneurons against cell death *in vitro* and in *mtSOD1* mice (Kieran et al., 2007).

Interestingly, several studies also suggest a non-cell autonomous mechanism of motoneuron loss in ALS (Ilieva et al., 2009). One possible explanation for a paracrine mechanism of motoneuron death is the release of pro-inflammatory or cell death-inducing cytokines from non-neuronal cells. Interleukin-1β (IL-1β) and interferon-γ (IFNγ) have been implicated in ALS disease progression (Friedlander et al., 1997; Meissner et al., 2010; Wang et al., 2011). Motoneuron apoptosis in ALS was also shown to involve Fas ligand up-regulation and activation of Fas death receptors (Locatelli et al., 2007; Raoul et al., 2002). Release of pro-inflammatory cytokines may lead to death receptor and caspase-8 activation, which is able to directly activate executioner caspases such as caspase-3 (Locatelli et al., 2007; Raoul et al., 2002). However, in most cell types, this direct activation pathway is not sufficient to activate apoptosis, and an amplification loop is required for cell death execution that involves the BH3-only protein Bid, and hence engages the mitochondrial apoptosis pathway (Lovell et al., 2008; Luo et al., 1998). Indeed, increased expression of Bid in both neurons and astrocytes as well as increased levels of Bid cleavage were observed in symptomatic and late stage $SOD1^{G93A}$ mouse spinal cords (Guegan et al., 2002). Interestingly, Bid was recently implicated in the production of pro-inflammatory cytokines in macrophages and microglia (Mayo et al., 2011) and was reported to be involved in the activation of the transcription factor nuclear factor-κB (NF-κB) in response to stimulation of pattern recognition receptors in intestinal epithelial cells, independent of its direct apoptosis-regulating function (Yeretssian et al., 2011). Therefore, in the present study we sought to clarify the role of Bid in the context of neurodegeneration and neuroinflammation relevant to ALS.

Materials and methods

Animals

All experiments described in this study were performed under a license from the Department of Health and Children in Ireland (B100/3985) in accordance with the European Communities regulations 2010 (2010/63/EU). All procedures were previously approved by the Research Ethics Committee of the Royal College of Surgeons in Ireland. Transgenic SOD1 mice (*Mus musculus*), IMSR: B6.Cg-Tg ($SOD1^{*G93A}$) 1Gur/J, with the incorporation of the G93A mutant form of human superoxide dismutase (*SOD1*), were purchased from The Jackson Laboratories (JAX, Bar Harbor, Maine, USA). $bid^{-/-}$ mice (*M. musculus*) were generated in the laboratory of Prof. Andreas Strasser, WEHI, Melbourne, Australia (Kaufmann et al., 2007). After weaning on postnatal day (PND) 28, all pups from litters of the same generation and colony were housed in groups of three to five per cage and maintained at 21 ± 1 °C on a 12 h light/dark cycle, (07:00 h on; 19:00 h off) with *ad libitum* access to food and water.

Reagents and chemicals

Unless otherwise stated, chemicals were purchased from Sigma-Aldrich (Arklow, Ireland) or Merck Chemicals (Nottingham, UK). Cell culture media were purchased from Gibco-Life Technologies (Dun Laoghaire, Ireland). BI-6C9 was from Sigma-Aldrich (Pubchem: ID 24724408).

Primary motoneuron cultures

Primary motoneuron cultures (mixed cultures enriched for motoneurons) were prepared from wild-type and *bid* gene deficient ($bid^{-/-}$) E12 mouse embryos as described previously (Sebastia et al., 2009). Briefly, the spinal cord ventral horns were dissected and the tissue was removed and incubated for 10 min in 0.025% trypsin in Neurobasal® media. Cells were transferred into a 0.1 mg/ml DNase1 solution, and gently dissociated. Dissociated motoneurons were counted using a hemocytometer and seeded at a density of 10^5 cells/ml onto poly-D,L-ornithine/laminin-coated cell culture wells and maintained at 37 °C and 5% CO_2. Motoneurons were maintained in complete Neurobasal® media supplemented with 2% horse serum, 2% B27, 2 mM GlutaMAX™, GDNF (Promega, Cat#2781; 2 ng/ml), CNTF (R&D Systems, Cat#557-NT-10, 1 ng/ml), 100 U/ml penicillin and 100 μg/ml streptomycin. Medium was changed after 1, 3 and 6 days *in vitro* (DIV). Cells were cultured up to 14 days. Primary motoneuron cultures yielded mixed populations of cells with glial (ca. 40–60%), neuronal (ca. 30–40%) and non-neuronal morphology (5–10%); approximately 30–50% of the neuronal population were motoneurons positive for the motoneuron marker SMI-32 (Suppl. Figs. 3A–A″).

Isolation and culturing of primary astrocytes

Mixed glial cultures were prepared from the cortices of P2 $bid^{-/-}$ and wild-type mice. In brief, the cortices were dissected and the meninges were removed before incubation in Minimum Essential Medium containing 0.025% trypsin and 0.1 mg/ml DNase I for 15 min at 37 °C. The tissue was triturated mechanically in DMEM to dissociate the cells, passed through a 40 μm nylon cell strainer (BD Falcon, Oxford, UK), and spun at 2000 rpm for 5 min. The pellet was resuspended and plated in T75 flasks in DMEM-F12/L-glutamine containing 1% penicillin/streptomycin and 10% Fetal Bovine Serum (Sigma-Aldrich). Cells were cultured for a minimum of 14 days before being passaged and cultured as astrocytes. For astrocyte-enriched cultures, astrocyte flasks were placed on the orbital shaker at 600 rpm to shake off microglia; the remaining adherent cells were trypsinized and replated for the experiment at 5×10^5 cells per 6 well. Motoneuron media were used to generate astrocyte conditioned media (ACM) for motoneuron toxicity experiments (Neurobasal media with the abovementioned supplements).

Isolation of primary microglia

Microglia were isolated from mixed glial cultures (as above). Following two weeks of cell culture, the T75 flasks of confluent mixed glial cultures were shaken in DMEM/F12 at 600 rpm on a plate shaker for 8 h at room temperature. The supernatant was collected and spun down at 2000 rpm for 5 min. The resuspended pellet was plated at a density of 1×10^5 cells/well of a 24 well plate. The isolated microglia were cultured for 2 days in well plates or Millicell-CM inserts (0.4 μm pore size, Fisher Scientific, Dublin, Ireland) in DMEM-F12/L-glutamine, 10% Fetal Bovine Serum and 1% Pen/Strep (Sigma-Aldrich) before treatment.

Real-time quantitative PCR (qPCR)

RNA was extracted using Qiazol extraction and/or RLT-buffer lysis and RNeasy processing according to the manufacturer's guidelines (Qiagen, Sussex, UK). cDNA synthesis was performed based on equal amounts of RNA using the Superscript™ II Reverse Transcriptase