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Bcl-x_L increases axonal numbers but not axonal elongation from rat retinal explants

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Abstract

The Bcl-2 family of proteins has been characterized as a key regulator of cell death programs. In addition, these proteins also play important roles in cellular differentiation, such as axonal growth. The role of Bcl-2 family members on axonal regeneration and neurite extension has been controversial so far. Here, we examine the influence of Bcl-x_L on axonal regeneration from adult retina explants in vitro. We delivered recombinant Bcl-x_L into retinal tissue, mediated by the Tat-protein transduction domain, and observed its effect on retinal axon extension. We found that Bcl-x_L increased the number of regenerating neurites, but did not increase their length. Our results indicate that Bcl-x_L stimulates axonal initiation but not axonal elongation after crush injury to retinal explants, without altering the number of surviving neurons.

Keywords: Nerve trauma; Cell penetrating peptide (CPP); Protein transduction domain (PTD); Regeneration; Optic nerve crush; Bcl-2 family; Apoptosis; Retinal ganglion cell

1. Introduction

About 80 years ago, Ramon y Cajal stated that in the central nervous system (CNS) "this defective capacity for regeneration ... [depends on] the absence in the surroundings of catalytic agents able to overcome the osmotic equilibria of the cones of growth, to provoke their vigorous nutrition, and to direct the path they must follow" [57]. Work in the following decades showed that the loss of adult CNS neurons' capability to regenerate when their axon is severed is due to extrinsic signals from glial cells and extracellular matrix molecules formed in the glial scar ([7], for a few recent examples [58,65]). However, part of the decline in regenerative capacity is also due to changes within the neurons themselves [24]. These intrinsic influences have also been shown for retinal ganglion cells [29,55], which are often used as a model system to examine axonal degeneration and regeneration [37,66]. It is not known how these internal changes may occur.

The role of Bcl-2 family members in promoting or inhibiting apoptosis is widely known [[49], for review]. However, less work has been published on other functions of these genes, for example on synaptic transmission [34], proliferation and cell cycle regulation [6,12,15], or sensory [50] or dopaminergic [47] neuronal differentiation. Of particular interest in our context is that experiments investigating *bcl-2* overexpression or null mutant on retinal ganglion cells [10,11,14], primary embryonic sensory neurons [31], a neural-crest-derived cell line [68], a dopaminergic cell line [53], or PC-12 cells [35,45,63] suggest that Bcl-2 promotes axonal or neurite growth or regeneration, independently of its ability to promote neuronal survival. However, after intracranial nerve crush, *bcl-2* overexpression did not improve axonal regeneration [13,48], and the earlier findings of Chen et al. could not be reproduced [48]. *Bcl-2* overexpression also did not enhance optic nerve regeneration stimulated by a peripheral nerve transplant [32]. Moreover, in highly purified postnatal RGC, adenoviral *bcl-2* overexpression itself did not induce axonal growth [28,29]. In the entorhinal-hippocampal formation, *bcl-2* overexpression did not increase the regenerative potential of axotomized neurons [61]. In other studies, the enhanced recovery of facial nerve injury in *bcl-2*-overexpressing mice might be

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attributed to an improved survival of the axotomized neurons [51].

The *bcl-x* gene has been isolated by low-stringency hybridization of a cDNA library with a *bcl-2* probe [5]. Bcl-2 and Bcl-x_L are similar in structure, share common pathways, and are partially redundant in their anti-apoptotic activity [9,62]. They are both expressed in the adult and in the developing brain [30]. A major difference, however, is their subcellular localization: while Bcl-x_L acts primarily on the mitochondrial membrane, Bcl-2 is localized to other membranes as well, such as the ER [36]. The *bcl-x_L*-deficient mouse is the only targeted deletion of a *bcl-2* family member that shows a profound neuronal phenotype [[46], for review], suggesting that Bcl-x_L plays a particularly important role in nervous system development. This role is not only to promote cellular survival, but also to influence neuronal differentiation, of dopaminergic neurons for example [47,60]. The results of some experiments using viral vectors suggest that Bcl-x_L inhibits neuritogenesis in retinal ganglion neurons [54], while others indicate that Bcl-x_L inhibits axonal growth [42].

To examine the function of Bcl-x_L [20,38] or neurotrophins [21,39,40] in more detail, we coupled it to the so-called protein transduction domain derived from the HIV Tat protein. This 11 amino acid sequence allows the delivery of proteins and other substances across cellular membranes and the blood-brain barrier [59]. We showed in earlier studies that Tat-Bcl-x_L prevents apoptosis in models for neurodegenerative diseases, e.g. potassium and serum deprivation [20]; nerve trauma [20]; ischemia [38]; a model for bacterial meningitis [22]; a model for multiple sclerosis [18], β-amyloid toxicity [56] and other models (Dietz et al., in preparation).

The only neurons that project their axon through the optic nerve are the ganglion cells of the retina. When retinal explants, preconditioned by crush lesion or axotomy before dissection, are placed on a permissive substrate like laminin, some retinal ganglion cells regenerate their axon [3]. This system makes it possible to test growth factors or conditioned medium [64] or other substances [2] for their ability to promote neurite extension. To clarify whether Bcl-x_L influences neurite extension, we delivered the protein via the Tat protein transduction domain into retinal tissue, and tested it for its effect in the in vitro assay for neurite outgrowth from retinal ganglion cells.

2. Materials and methods

2.1. Purification of Tat fusion proteins

The *tat-bcl-x_L* construct was cloned and the protein purified as described [20,52]. The pTAT-HA vector [59] used to generate the Tat domain control protein, which had also been used to clone the *Bcl-x_L* expression construct, was kindly provided by S.F. Dowdy (San Diego, CA). *Tat-bcl-x_L* was expressed in *E. coli* strain BL21(DE3)pLysS (Novagen, Madison, WI). Inclusion bodies were disrupted by sonication in 8 M urea. Bacterial debris was pelleted and supernatant subjected to metal-affinity chromatography using a Ni–NTA matrix (Qiagen, Hilden, Germany). We removed salt by gel filtration on Sephadex G-25 M (Amersham Biosciences, Uppsala, Sweden). We confirmed the identity of proteins by PAGE and Western blotting (Fig. 2A). Anti-hemagglutinin antibodies were purchased from BAbCO (Richmond, CA).

2.2. Surgical procedure, tissue explants, and image recordings for neurite growth assay

Rats were treated according to German guidelines for the care and use of laboratory animals and in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC). Male Wistar rats (bred from rats purchased from Harlan Winkelmann GmbH, Borchen, Germany) weighing 200–250 g were anesthetized by ip injection of 420 mg/kg chloral hydrate. The eyes of the animals were carefully mobilized by slight pressure near the optic cavity. The optic nerve was crushed by tightly squeezing it between the (bent) prongs of watchmaker's tweezers for 5 s. Three days after this preconditioning crush, the retina was dissected and freed from connective, lens and vitreal tissue, spread on a cellulose nitrate filter, pore size 0.45 μm (Sartorius, Göttingen, Germany) using aspiration as described [1] and chopped into 500 μm strips (Fig. 1A). The 6 middle membrane-tissue strips were transferred onto poly-L-lysine and laminin-coated cell culture dishes with a gas-permeable base (petriPERM culture dish, VIVASCIENCE AG), with the tissue facing the substrate. To keep the strips in place, they were weighed down with small steel blocks (Fig. 1B). Three strips were explanted per 5.5 cm dish with 4 ml of medium.

The retinal explants were incubated in a medium as described [3] in 70% O_2 to enhance the number of outgrowing fibers, 5% CO_2, at 37 °C, with the test protein at 100, 300 nM, 1 or 3 μM concentrations. At day 0 and day 3, Vitamin C was added to a final concentration of 10 μg/ml. After 5 d in culture, the tissue was fixed and stained for neurofilament using the SMI 31 monoclonal antibody (1:1000 in TBS) against neurofilament (Sternberger Monoclonals Inc., Lutherville, Maryland).

To evaluate axonal growth, each tissue strip and the stained axons were digitally photographed in their entirety using a Zeiss Axioplan 2 fluorescent microscope and Zeiss Axiovision Software (Zeiss Jena, Germany) (Fig. 1 C). Images of whole strips (up to a few mm in length) were assembled from single pictures taken with a 10× objective (Fig. 1D). A total of 144 strips were analyzed. To measure strip circumference, neurite numbers and neurite length observed under different Tat protein concentrations, a macro was programmed using the KS400 image analysis package (Zeiss, Jena, Germany). To determine the length distribution shown in Fig. 3C, the sum of neurite length measurements of the Tat-HA control condition was set to 100. Neurite lengths of Tat-Bcl-x_L-treated cultures were normalized against the control condition according to their neurite density relative to Tat-HA control. Neurite lengths measured at different protein concentrations were divided into intervals of 0–50%; 50–100%; 100–150%; 150–200%; 200–250% and larger than 250% of the average of Tat-HA control condition. The total of the intervals determined in the Tat-HA control condition was set to 100%, and the sum of the interval counts for each Tat-Bcl-x_L concentration were normalized. Statistical significance was evaluated by one-way ANOVA followed by Bonferroni-Dunn's post hoc test.

2.3. Determination of retinal ganglion cell survival

RGC were pre-labeled 3 d before optic nerve crush by injection of the retrograde tracer FluoroGold (Biotium, Hayward, CA) into the superior colliculus. To that end, rats were anesthetized with chloral hydrate as above, an incision was made along the midline of the scalp, and two holes were drilled 6.04 mm posterior of the bregma and 1.4 mm lateral to the left side of the saggital suture. Two microlitre of a 5% FluoroGold solution in PBS were injected 4.2 mm deep from the skull surface over 5 min. Afterwards, the needle was left in place for another minute to avoid leakage of the dye along the needle track. Subsequently, the scalp was closed by a suture. Five days later, the optic nerve was crushed as described above. Three day after this surgery, the retinas were dissected and cultured for 5 d with recombinant proteins. Explants were fixed with 4% PFA and the FluoroGold-labeled retinal ganglion cells were counted in 9 fields of each explant.

2.4. Immunohistochemistry

To determine transduction of retinal explants in culture, they were treated with Tat-Bcl-x_L immediately after preparation, allowed to incubate over night, washed 5 times with PBS, fixed in 4% PFA, blocked with 2% BSA, and incubated with mouse monoclonal anti-HA antibody (BAbCO, Richmond, CA, 1:200 in