Reprint Series
9 October 1992, Volume 258, pp. 302–304

SCIENCE

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Approximately half of the neurons produced during embryogenesis normally die before adulthood. Although target-derived neurotrophic factors are known to be major determinants of programmed cell death—apoptosis—the molecular mechanisms by which trophic factors interfere with cell death regulation are largely unknown. Overexpression of the *bcl-2* proto-oncogene in cultured sympathetic neurons has now been shown to prevent apoptosis normally induced by deprivation of nerve growth factor. This finding, together with the previous demonstration of *bcl-2* expression in the nervous system, suggests that the Bcl-2 protein may be a major mediator of the effects of neurotrophic factors on neuronal survival.

Programmed cell death—apoptosis—is an active process of self-destruction that occurs in normal vertebrate development (*1*). Although RNA and protein synthesis seem to be required for many cells to die (*2*), the molecular pathways that regulate programmed cell death are unknown. Evidence suggests that, in neurons, this process is initiated under conditions in which the concentration of target-derived neurotrophic factors is reduced (*3*). The *bcl-2* proto-oncogene product (*4*) delays the onset of apoptotic cell death in B cells (*5*) and in T cells (*6*), and we now show that *bcl-2* overexpression prevents neuronal death induced by trophic factor deprivation.

In the presence of nerve growth factor (NGF), sympathetic neurons can be maintained in culture for several weeks; NGF deprivation induces neuronal death by an apoptosis-like mechanism that requires both mRNA and protein synthesis (*7*) and which is accompanied by nuclear DNA fragmentation (*8*). To assess the effect of *bcl-2* on neuronal death, we constructed the expression vector EB-2, consisting of 1.8 kb of 5′ flanking DNA of the rat neuron-specific enolase promoter linked to a DNA fragment encoding human *bcl-2* (*9*), and microinjected this construct into the nucleus of cultured rat sympathetic neurons (Fig. 1A). Approximately 80 to 90% of injected neurons survived the stress caused by the injection; damaged cells died less than 3 hours after injection and were not included in the results.

The percentage of injected neurons that expressed *bcl-2* was determined in cultures of neurons growing in an NGF-rich medium by means of a species-specific monoclonal antibody to human Bcl-2 (*10*). Twenty-four hours after injection, approximately 80% of the neurons that received a solution containing DNA reacted with anti–Bcl-2. [Coinjection of DNA with fluorescein isothiocyanate (FITC)–conjugated dextran in some cells demonstrated the efficacy of the injection procedure (Fig. 1, B and C).] This percentage decreased to 43 ± 2% (mean ± SEM, $n = 4$) (Fig. 1D) 3 days after injection and to 10% by day 10.

In lymphocytes, the Bcl-2 protein has been localized to the inner mitochondrial membrane (*11*). Observation of neurons expressing *bcl-2* with a confocal microscope revealed a punctate cytoplasmic immunostaining (Fig. 1E), resembling that of rhodamine 123, which specifically targets mitochondria (*12*) (Fig. 1F). This observation suggests that the protein is targeted to mitochondria in both neurons and lymphocytes. However, we cannot exclude another subcellular localization of Bcl-2 in neurons.

The effects of *bcl-2* on neuronal survival were investigated in low-density cultures (500 neurons per square centimeter) in which cells are prevented from clustering in small groups, thus facilitating cell injection as well as cell counting. Non-neuronal cells, a possible source of NGF, were virtually eliminated by the presence of 10 μM cytosine arabinoside C in the culture medium. Experiments were performed on 7-day-old cultures because the NGF dependency of sympathetic neurons in vitro has been shown to decrease with time in culture (*7*, *13*). NGF-containing medium was replaced with NGF-free medium 3 hours after injection. In some experiments, antibodies to NGF were added to the NGF-free medium to accelerate neuronal degeneration.

In control conditions (no injection or injection with control pNSE-LacZ vector), neuronal death—phase-dark cell body and neurite disintegration—was first apparent within 48 hours after NGF deprivation. By 72 hours, only 10% of the initial neuronal population had survived (Fig. 2E); these neurons may have survived because of nearby remaining non-neuronal cells. A more marked degeneration was observed in the presence of anti-NGF. At 72 hours, almost all neuronal cells had disappeared (Fig. 2, A and E). In contrast, 40 to 50% of the initial neuronal population injected with EB-2 (depending on the absence or presence of anti-NGF (Fig. 2E) displayed phase-bright cell bodies with thick neurites that adhered to the collagen substratum (Fig. 2B). The viability of these neurons was confirmed by staining with the vital marker acridine orange (*14*) (Fig. 2C). They also reacted with anti–Bcl-2 (Fig. 2D). EB-2–injected neurons were capable of surviving for more than 1 week in the absence of NGF (Fig. 2F). By day 10, the number of surviving neurons decreased to about 20%, possibly because of reduced synthesis of Bcl-2 with time. There was a clear correlation between the percentage of neurons containing Bcl-2 (in terms of efficacy and duration of transfection) and the percentage of neurons protected from death, suggesting that every neuron expressing *bcl-2* was capable of surviving in the absence of NGF. At day 12, the rescued

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neurons displayed a cell body apparently unchanged compared to day 3, but their neurites appeared thinner. NGF treatment of those neurons reversed neuritic atrophy (not shown) and allowed them to survive for an additional 10 days in culture (Fig. 2F), suggesting that neurons deprived of NGF for as long as 12 days still expressed NGF receptors. On the other hand, these results also suggest that, although *bcl-2* can rescue neurons from death, this protein cannot mimic all the trophic effects of NGF.

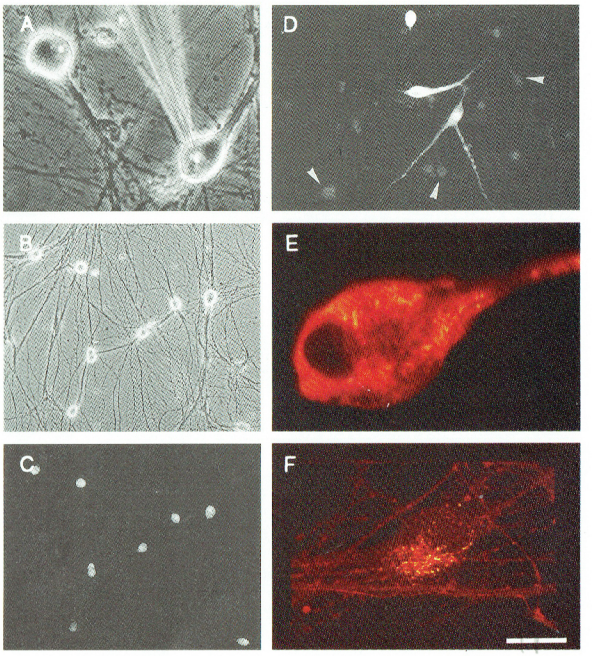

Fig. 1. Expression and localization of Bcl-2 in microinjected sympathetic neurons. (**A**) The EB-2 expression vector (*9*) was microinjected into the nucleus of sympathetic neurons from superior cervical ganglia of newborn rats (*22*). (**B** and **C**) Phase contrast (B) and fluorescence (C) images of neurons injected with DNA solution plus FITC-dextran. (**D**) Three days after injection, neurons were immunostained for Bcl-2 (*23*). Approximately 40% of microinjected neurons were positive for Bcl-2. Arrowheads indicate neurons that were microinjected but were negative for Bcl-2. The photograph was deliberately overexposed to allow visualization of these neurons. (**E** and F) Confocal fluorescence images with artificial colors of neurons immunostained for Bcl-2 (E) or stained with rhodamine 123 (F) (*24*). Bar, (A) 40 μm; (B and C) 120 μm; (D) 60 μm; (E) 12 μm; and (F) 20 μm.

In conclusion, our results demonstrate that *bcl-2* can save NGF-deprived sympathetic neurons from apoptosis. The *bcl-2* gene is expressed in the nervous system (*15*), particularly in sympathetic neurons and other neuronal types that also depend on trophic factors for their survival (*16*). Our findings suggest that Bcl-2 may be an important mediator of trophic factor effects on neuronal survival.

Certain features of apoptosis vary among different cell types (*7*, *17*), not only in the morphological aspects of dying cells but also in the molecular mechanisms required for activating the cell death program. For example, an influx of Ca^{2+} is required for thymocyte cell death, whereas NGF-deprived sympathetic neurons do not require Ca^{2+} influx to die (*18*). This variability suggests that distinct events may induce cell death through different mechanisms. Our finding that Bcl-2 blocks apoptosis in neurons as it does in thymocytes and certain lymphocytes (*5*, *6*, *19*) suggests that these different mechanisms share common effectors. Such a situation exists in *Caenorhabditis elegans*, in which a single protein, the *ced-9* gene product, acts to protect different cell

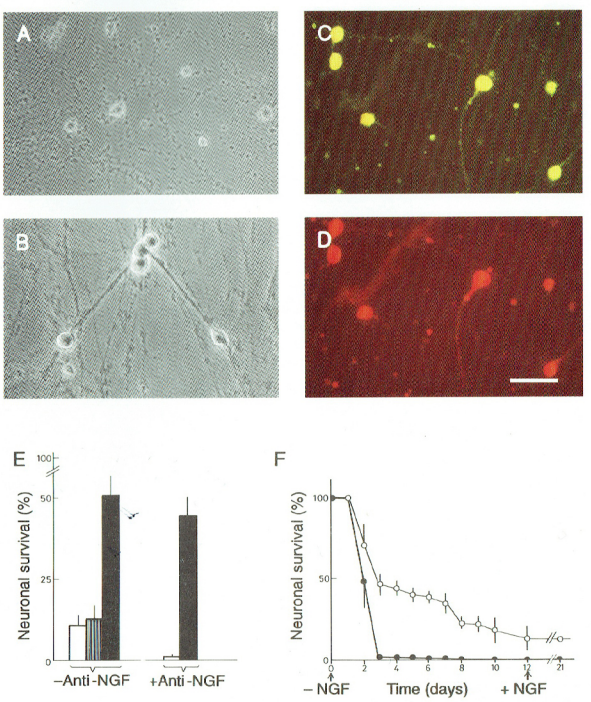

Fig. 2. Effects of Bcl-2 on the survival of NGF-deprived neurons. Neurons were cultured in 3.5-cm diameter dishes for 7 days. Rectangles (10 by 3 mm) containing 150 to 200 neurons were drawn on the bottom of each dish to allow localization of injected neurons. Neurons either were not injected or were injected with pNSE-LacZ or EB-2. Three hours after injection, the NGF-containing medium was replaced with NGF-free medium. In some experiments anti-NGF (Boehringer) (100 ng/ml) was added to the NGF-free medium to accelerate neuronal degeneration. The medium was changed daily. Three days after NGF deprivation, the cultures were stained with the vital marker acridine orange (Sigma) (1 μg/ml) in phosphate-buffered saline for 5 min. (**A**) Non-injected or pNSE-LacZ–injected neurons 3 days after NGF deprivation. (**B**) Neurons injected with EB-2 3 days after NGF deprivation. (**C**) Acridine orange fluorescence and (**D**) Bcl-2 immunostaining of surviving EB-2–injected neurons 3 days after NGF deprivation. Bar, 60 μm. (**E**) Cells displaying acridine orange staining 3 days after NGF deprivation, expressed as the percentage of neurons present 3 hours after injection, which is time zero. Open bars, non-injected cells; striped bars, cells injected with pNSE-LacZ; solid bars, cells injected with EB-2. Results are means ± SEM of six (~1200 injected neurons) and three experiments (~500 injected neurons) performed in the absence or presence of anti-NGF, respectively. (**F**) Neuronal survival for non-injected (●) and EB-2–injected (○) neurons. Time zero is 3 hours after injection. Anti-NGF was added when the culture medium was changed, every 2 days. Results are means ± SEM of four experiments (~750 microinjected neurons).

types that normally undergo apoptosis (*20*).

The finding that Bcl-2 is an inner mitochondrial membrane protein (*11*) raises the possibility that mitochondria may play an important role in apoptosis. Understanding the mode of action of Bcl-2 might provide insights into the nature of degenerative diseases (*21*).

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22. Sympathetic neurons from superior cervical ganglia were cultured as previously described [E. Hawrot and P. H. Patterson, *Methods Enzymol.* **53**, 574 (1979)]. Circular plasmids were dissolved in tris-EDTA buffer at a concentration of 0.1 mg/ml. Approximately 500 DNA molecules were introduced per cell with a low-pressure microinjection system (automatic injector Inject+Matic, Geneva) to ensure high neuronal survival [I. Garcia *et al.*, *Mol. Cell Biol.* **51**, 294 (1986)].
23. Mouse monoclonal antibodies used in this study were Bcl-2-100 and Bcl-2-124 (*10*). After microinjection, neurons were kept in NGF-rich medium for 3 days before measuring Bcl-2 immunoreactivity. Neurons were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min at room temperature, permeabilized in 0.1% Triton X-100 in PBS, and incubated with anti–Bcl-2 and then rhodamine-conjugated rat antibodies to mouse immunoglobulin G (Boehringer).
24. Living cells were incubated with rhodamine 123 (Sigma) (1 μg/ml PBS for 30 min at 37°C, rinsed three times with PBS, and fixed in 4% paraformaldehyde and 0.5% glutaraldehyde.

25. We thank S. Forss-Petter for neuron-specific enolase promoter; D. Y. Mason for Bcl-2 monoclonal antibodies; P. Schwarb (Zeiss, Zurich) for the confocal microscopy; R. Zufferey for SV40 T intron; F. Pillonel for technical assistance; and S. Catsicas, A. C. Kato, J. Knowles, J. Tschopp, P. Vassalli, and M. J. Weber for reading the manuscript. Supported by grants from the Swiss National Foundation (to A. C. Kato and P. Vassalli), the Association Française contre les Myopathies, and by grants CA-50551 and CA-51864 (to Y.T.) from the National Cancer Institute. Y.T. is a Leukemia Society of America Scholar.

8 June 1992; accepted 12 August 1992.