ROLE FOR BCL-2 IN NEURODEGENERATIVE DISEASES. Young J. Oh, Department of Biology, Yonsei University College of Sciences, Seoul 120-749, Korea

Introduction: The proto-oncogene bcl-2 has been shown to prolong cellular survival in a variety of cell types including hematopoietic and neuronal lineages in the absence of growth factors as well as in the presence of various cell death-inducing stimuli (Reed, 1994; Thompson, 1995). Bcl-2 has also shown to suppress cell death in various neuronal cell types following the withdrawal of neurotrophic factor (Garcia et al., 1992; Allsopp et al., 1993) or axonal injury (Dubois-Dauphin et al., 1994). These data, as well as the finding that human Bcl-2 can prevent programmed cell death in the nematode C. elegans (Vaux et al., 1992) and is homologous to the nematode cell survival gene, ced-9 (Hengartner and Horvitz, 1994) have firmly established Bcl-2 as an integral regulator of cell mortality. While the exact mechanism by which Bcl-2 prevents cell death is unknown, several recent findings have suggested that Bcl-2 protects cells by attenuating the damaging effects of reactive oxygen species (Hockenberry et al., 1993; Kane et al., 1993) even though recent reports demonstrate that protective role for Bcl-2 may not be associated with its anti-oxidant activity (Jacobson and Raff, 1995; Shimizu et al., 1995). It has been also suggested that Bcl-2 counteracts aberrations in mitochondrial functions (Jacobson et al., 1993). Because both oxidative stress and abnormality of mitochondrial energy metabolism have been hypothesized to play a role in neurodegenerative disorders such as Parkinson's disease, we sought to evaluate the potential of Bcl-2 in sparing cells from the dopaminergic neurotoxins, N-methyl-4-phenylpyridinium (MPP^+) and 6-hydroxydopamine (6-OHDA). Both MPP^+ and 6-OHDA are transported into dopaminergic cell types via the high-affinity dopamine uptake system. Once in the cell, 6-OHDA is autooxidized within the cytoplasm to produce hydrogen peroxide, superoxide and hydroxyl radicals. MPP^+ is sequestered within mitochondria where it appears to block NADH-linked electron transport. This results in decreased ATP synthesis and possibly increased reactive oxygen species.

RESULTS: The MN9D cell line was established by fusion of the neuroblastoma cell line, N18TG2, with embryonic murine mesencephalic neurons (Choi et al., 1991; Tang et al., 1994). The resulting clonal cell line has been shown to synthesize and release dopamine and to be sensitive to MPP^+ treatment. Using these cells, initial attempt was made to characterize the type of cell death induced by two dopaminergic neuronal toxins.

MN9D/Neo cells exhibited unique morphological patterns of cell death in response to 6-OHDA or MPP^+. MN9D/Neo cells treated with 6-OHDA (10 - 100 uM) exhibited morphological features typical of apoptosis such as shrinkage of

cellular membranes and nuclei as well as transitional membrane blebbing. In contrast, MPP^+-mediated MN9D cell death (1 - 100 uM) was not accompanied by these classic apoptotic changes. Despite these morphological differences, cell death induced by either toxin was completely blocked by addition of 1 ug/ml of cycloheximide suggesting protein synthesis is involved in both types of cell death. Because one of the hallmarks of apoptosis is internucleosomal DNA fragmentation, DNA from MN9D/Neo cells treated with MPP^+ or 6-OHDA was analyzed. In correlation with the classic apoptotic morphological changes described above, DNA fragmentation was readily observed in 6-OHDA-treated cells whereas it was never seen in MPP^+-treated cells. Changes in nuclear chromatin were confirmed at the cellular level by incubating cells in the presence of nuclear dye Hoechst 33258. Nuclei of 6-OHDA-treated cells had shrunken nuclei with clear signs of chromatin condensation whereas MPP^+-treated cells exhibited homogeneously stained nuclei with no apparent sign of nuclear shrinkage. These distinct changes were also confirmed by TUNEL staining.

To assess the potential role of Bcl-2 in a dopaminergic neuronal cell line, MN9D, cells were transfected with either CMV/Bcl-2 or CMV/Neo control expression vector. Stable transfection followed by limiting dilution resulted in homogeneous populations of cells expressing high levels of the full length 26 kDa protein. Immunocytochemical localization of Bcl-2 demonstrated that at least three MN9D/Bcl-2 clonal cell lines express high level of Bcl-2 within their cytoplasm. Exposure of MN9D/Neo cells to MPP^+ led to cell death within 48 hours with a mean lethal dose (LD50) of ~10 uM. Cell death was significantly attenuated in MN9D/Bcl-2 cells (LD50 = ~40 uM). At a given concentration, MN9D/Bcl-2 delayed the cell death for at least 1-2 days (not shown). Reagents known to reduce reactive oxygen species such as ascorbic acid (10-1000 uM), Trolox (soluble form of vitamin E; 10-1000 uM), and the iron chelator, desferrioxamine mesylate (1-100 uM) did not alter MPP^+-induced cell death implying that reactive oxygen species are not directly involved in MPP^+-induced cell death in our system (not shown). In contrast, Bcl-2 overexpression did not block the cytotoxic effects of 6-OHDA. In fact, MN9D/Bcl-2 cells were significantly more sensitive to the action of 6-OHDA (LD50=35 uM versus LD50=75 uM, MN9D/Neo). In these experiments, inclusion of the antioxidant reagents or the iron chelator did substantially attenuate cell death caused by 6-OHDA. Furthermore, treatment of MN9D/Neo and MN9D/Bcl-2 with oxidative stress inducers such as hydrogen peroxide or menadione led to cell death in both cell lines with morphological characteristics which were indistinguishable from those observed in 6-OHDA-treated cells demonstrating that Bcl-2 did not block cell death induced by oxidative stress in a dopaminergic neuronal cells.

A direct role for MPP^+ in free radical formation has been controversial; evidence suggests oxidative stress occurs secondarily to inhibition of

mitochondrial respiration. To test the effectiveness of Bcl-2 in repressing cell death via these pathways more directly, MN9D/Bcl-2 and MN9D/Neo cells were incubated with known inhibitors of mitochondrial electron transport system. Overexpression of Bcl-2 substantially inhibits cell death induced by rotenone (a specific inhibitor of Complex I) or 3-nitropropionic acid (a specific inhibitor of Complex II). Both rotenone and 3-nitropropionic acid induced morphological changes associated with cell death that could not be readily differentiated from those observed in MPP+-treated cells. Taken together, these data suggest that in the MN9D cells, Bcl-2 did not appear to directly protect cells from the consequences of reactive oxygen species but rather protected cells from altered mitochondrial function.

Increases in mitochondrial membrane potential have been linked with the enhanced survival of fibrosarcoid cells overexpressing Bcl-2 in response to tumor necrosis factor treatment. Thus, it has been sought to determine whether the protective effect of Bcl-2 after MPP^+-treatment is mediated by affecting mitochondrial membrane potential. Mitochondrial membrane potential in MN9D/Neo cells decreased in a time-dependent manner upto 12 hr after incubation with 50 uM MPP^+ and reached a plateau at 12-24 hr. The mitochondrial membrane potential for both MN9D/Bcl-2 and MN9D/Neo cells decreased at the same rate in the presence of MPP^+. Furthermore, basal level of mitochondrial membrane potential between these two cell lines prior to MPP^+-treatment was the same. This suggests that increases in mitochondrial membrane potential can not account for the enhanced survival of MN9D/Bcl-2 cells after MPP^+ treatment.

Both in vivo and in vitro studies have shown that the cytotoxic effects of MPP^+ exposure are preceded by the increased rate of glucose utilization. The ability of cells to use glycolysis as a primary energy source presumably represents a compensatory mechanism in response to MPP^+-induced ATP depletion. To explore potential mechanisms by which Bcl-2 attenuates MPP^+-induced cell death, the level of glucose uptake in MN9D/Bcl-2 and MN9D/Neo cells was measured at various time intervals up to 12 hr after 50 uM MPP^+ treatment. During this time period, no sign of cell death were observed in either cell type as measured by trypan blue exclusion (not shown). In preparatory experiments, it has been found that the basal level of glucose uptake between these two cell line was identical (not shown). Following MPP^+ treatment, the rate of glucose uptake in MN9D/Bcl-2 cells was significantly diminished. At all time points tested, the rate of glucose uptake in MN9D/Neo was higher than that in MN9D/Bcl-2 although both declined over time. If overexpression of Bcl-2 in MN9D cells was simply blocking access of the metabolic inhibitors MPP^+, rotenone and/or 3-nitropropionic acid to the electron transport complexes, then it might be expected that the rate of glucose utilization in these cells would remain at basal levels particularly at early MPP^+

treatment time points. The divergence of responses in this assay, therefore, suggests that Bcl-2 is not just counteracting MPP^+-mediated inhibition of respiration but rather is utilizing some other mechanism(s) or energy source to prevent cytotoxicity.

DISCUSSION: In this report, it is demonstrated that i) Bcl-2 attenuates cell death induced by inhibitors of mitochondrial electron transport; ii) Bcl-2 fails to prevent cell death induced by 6-OHDA or other prooxidant treatment; and iii) Bcl-2 expressing cells exhibit reduced rates of glucose uptake following MPP^+ treatment. Thus, Bcl-2 may also act to alter metabolic pathways in response to impaired mitochondrial function. Although the exact mechanisms associated with this process remain to be elucidated, inasmuch as previous studies have shown that cells overexpressing Bcl-2 can survive for at least 24 hours in medium lacking glucose (Zhong et al., 1993) and that this is also true in the MN9D/Bcl-2 cells (not shown), it seems likely that the decreased rate of glucose utilization in MN9D/Bcl-2 cells represents an active, cell survival response. These data would suggest that the MN9D/Bcl-2 cells do not switch to an anaerobic pathway to maintain their energy need. Rather, these cells either have access to alternate energy sources or they minimize their energy needs in response to MPP^+. However, these data do not exclude the possibility that Bcl-2 may recruit other protective mechanism(s). In our preliminary studies, it has been shown that Bcl-2 protects staurosporine-induced cell death without differentially affecting the rate of glucose uptake. Therefore, it is plausible to assume that Bcl-2 may adapt the different strategies to prevent cell death depending on the types of death stimuli.

Given the previous evidence demonstrating Bcl-2 blocks cell death induced by reactive oxygen species, it is somewhat surprising that its overexpression did not do so in MN9D cells. If anything, cell death in MN9D/Bcl-2 cells was moderately enhanced in response to 6-OHDA. In contrast, cell death mediated by MPP^+ was significantly attenuated by Bcl-2 overexpression despite its non-canonical apoptotic apprearance. These results imply that, by itself, overexpression of Bcl-2 is unable to block apoptotic cell death induced by oxidative stress-inducing stimuli in all cell types.

Bcl-2 is a member of a family of related proteins, at least some of which can form homo- or heterodimers (Reed, 1994). Many of these proteins can function as cell death suppressors whereas others such as Bax, Bad, Bak and Bcl-Xs promote cell death. The particular combination of Bcl-2 family members expressed in a given cell type presumably determines the response of a cell to environmental challenges stimulating apoptosis (Boise et al., 1993; Oltvai et al., 1993). The dissimilarity in responses between MN9D cells and the various hemopoietic cell lines or even the neuronal cell lines studied to date might suggest that a unique combination of Bcl-2 counterparts are expressed in the MN9D cells resulting in the

observed functional outcome. Other possible explanations for these discrepancies include: i) the absence or presence of possitive effector molecules or signalling pathways within these cells which are located either upstream or downstream of Bcl-2's site of action; ii) the absence or presence of a negative regulator(s) which antagonizes upstream or downstream effectors of the Bcl-2 pathway. Although it has not been determined whether these same responses occur in authentic mesencephalic, dopaminergic neurons, at the very least, results from the immortalized MN9D cells expand the repertoire of Bcl-2 functions and may serve to unmask signal transduction pathways essential to particular combinations of Bcl-2-related proteins.

Recently, Selvakumaran et al. (1994) reported that overexpression of Bcl-2 in a myeloid leukemic cell line could suppress TGF beta-1-induced cell death but not that mediated by the tumor suppressor, p53. The p53-mediated apoptosis appeared to be due to the upregulation of the cell death enhancer, Bax. Thus, differential regulation of Bax by these two stimuli was a key factor in the cell's response. In other studies, it is also shown that increase in Bax expression are very important determinants during cell death processes (Oltvai et al., 1993; Yin et al., 1994). In preliminary experiments, however, it has been observed that discernable changes in Bax mRNA or protein level is not seen in response to various drug treatments (not shown). Recently, Sato et al. (1994) suggested that the cell death enhancer Bcl-Xs, functioned by heterodimerizing with Bcl-2 which then left Bax free to promote cell death. Although, we have not fully tested the latter hypothesis, to date we have not found any differences in mRNA levels of death-related genes in response to 6-OHDA or MPP^+. Taken together, these data emphasize the diverse roles that Bcl-2 mat subserve in different cell types presumably due to its interaction with pre-existing or newly synthesized Bcl-2 family members ot other effectors which contribute to its overall response.

In summary, overexpression of Bcl-2 in this immortalized CNS cell line protected cells from aberrations in mitochondrial electron transfer processes. Impaired energy metabolism has been associated with a number of neurodegenerative disorders (Tipton and Singer, 1993). In Parkinson's disease, several studies have implicated defects in Complex I of the electron transport chain in the etiology of this disorder. Inasmuch as MPP^+ induces parkinsonism in human and non-human primates, further studies delineating the role of impaired energy metabolism in cell death as well as the protective effect engendered by Bcl-2 may enhance our understanding of these disorders. In preliminary studies in our laboratory, it has been demonstrated that overexpression of Bcl-2 reduced the beta-amyloid-induced cell death. Taken together, these and above mentioned data indicate that Bcl-2 may play an important role in regulating neuronal cell death in neurodegenerative diseases.

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