Overexpression of Bcl-2 in a murine dopaminergic neuronal cell line leads to neurite outgrowth

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Abstract

A dopaminergic neuronal cell line was established which stably expressed human Bcl-2 (MN9D/Bcl-2) or a control vector (MN9D/Neo). Overexpression of Bcl-2 cells led to robust neurite formation without cessation of cell division. Most markers of neuronal and/or dopaminergic maturation were unchanged although the synaptosomal associated protein, SNAP-25, was increased three-fold in MN9D/Bcl-2 cell lines. These data support a role for Bcl-2 in developmental processes at least during a certain stage(s) of neurogenesis.

Keywords: Bcl-2; Dopamine; Neurite extension; Differentiation; Mesencephalic cells

A role for the oncogene-derived protein, Bcl-2, in neural development has been suggested by studies investigating its temporal and spatial regulation in developing brain and peripheral neurons [10]. In the brain, high levels of Bcl-2 expression are observed in cells of the cortical plate as well as in cerebellar granular cells in the course of migration and extension of neurites [10]. Bcl-2 levels are also regulated during the differentiation of some types of neuroblastomas [8] and overexpression of Bcl-2 in the pheochromocytoma cell line, PC 12, results in a differentiated phenotype when cultured under restrictive growth conditions [14].

In order to determine what role, if any, Bcl-2 might play in CNS dopaminergic cells, we employed a dopaminergic cell line derived from embryonic murine mesencephalon (MN9D) [4]. Previous studies have shown that MN9D cells could synthesize, release and take up dopamine [4,16] as well as exhibit many features of their endogenous counterparts in target/non-target interactions [5]. Therefore, MN9D cells were transfected with either the human Bcl-2 cDNA or a control vector as described [11]. At least 14 monoclonal Bcl-2 transfected cell lines were established by multiple rounds of limiting dilution. Cell populations were judged to be homogeneous by immunofluorescent staining and Western blot analysis [11]. The resulting stable cell lines (MN9D/Bcl-2-1, MN9D/Bcl-2-2 and MN9D/Bcl-2-3), as well as cells expressing the control vector (MN9D/Neo), were used for these studies. Data reported below are derived from the MN9D/Bcl-2-2 cell line. Similar results were observed with the MN9D/Bcl-2-1 and MN9D/Bcl-2-3 cell lines in two to three independent experiments.

For the measurement of neurite outgrowth, cells were plated at $1 \times 10^{5}$ on 0.1 mg/ml poly-d-lysine-coated 6-well dishes (Costar), maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum and 500 μg/ml of the antibiotic G418 for 3–4 days, and then analyzed as described previously [15] using a computer-interfaced drawing system with a digitizing light pad (Bioquant). All statistical analyses were non-parametric, since the morphological features were not normally distributed.

Western blot analysis was performed exactly as described by Oh et al. [11] using various antibodies. These included mouse monoclonal anti-microtubule associated protein 2 (Boehringer Mannheim), affinity purified rabbit polyclonal anti-growth associated protein 43 (a generous gift from Dr. M. Willard, Washington University, St. Louis, MO) and mouse monoclonal anti-SNAP 25 (Sternberger Meyer).
The catecholamine biosynthetic enzyme, tyrosine hydroxylase (TH), was assayed as described in Owens et al. [13]; high-affinity dopamine uptake was measured as described by Tang et al. [16]. Reverse transcription-polymerase chain reaction (RT-PCR) was used to measure mRNA levels of TH [6]; the level of actin mRNA was used to normalize the amount of mRNA in each sample. The number of thermal cycles was empirically tested to ensure that PCR conditions were within a linear range. Values from each of the three MN9D/Bcl-2 cell lines were quantitated by using the PhosphorImager SF (Molecular Dynamics) and expressed as a percentage of the control, MN9D/Neo cells (100%).

Following transfection and several rounds of limiting dilution, all of the MN9D/Bcl-2 cell lines demonstrated high levels of Bcl-2 expression within their cytoplasm [11]. Because overexpression of Bcl-2 in most neuronal cell lines has not led to obvious changes in cell morphology, growth rates and/or differentiation (e.g. [1]), we were surprised to observe that 13 out of 14 MN9D/Bcl-2 stable cell lines exhibited very long neuritic processes as typified by Fig. 1A. In contrast, only a few, very short processes were observed in MN9D/Neo controls (15/15; Fig. 1B) or in the parental MN9D cells [15]. To quantify these morphological changes, we measured the number of neurites, total neuritic extent, and primary neurite length [15]. As shown in Table 1, increases in primary neurite length and total neuritic extent were highly significant in MN9D/Bcl-2 versus MN9D/Neo controls or in the MN9D parental cells transfected with a human BDNF cDNA.

Because previous studies have shown that neuroblastoma cells can undergo extensive differentiation in response to various agents, one possible interpretation of these experiments is that the overexpression of Bcl-2 is affecting the neuroblastoma fusion partner versus the mesencephalic partner. In order to directly test this hypothesis, N18TG2 cells, the neuroblastoma fusion partner, were transfected with the human Bcl-2 expression clone. Five independent homogeneous clones were selected by multiple rounds of limiting dilution and then tested for morphological changes. In contrast to results with the MN9D/Bcl-2 cells, stable transfection of human Bcl-2 in N18TG2 cells did not result in Bcl-2-specific neurite extension (not shown). In accordance with its widespread expression pattern in the developing CNS, these data suggest that Bcl-2 may exert a neurite-

<table>
<thead>
<tr>
<th>Measurement</th>
<th>MN9D/Neo</th>
<th>MN9D/BDNF</th>
<th>MN9D/Bcl-2-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of cells</td>
<td>90</td>
<td>61</td>
<td>90</td>
</tr>
<tr>
<td>Area of cell body (μM²)</td>
<td>294.68 ± 13.69</td>
<td>246.40 ± 17.05</td>
<td>249.41 ± 15.73</td>
</tr>
<tr>
<td>Number of neurites/cell</td>
<td>1.63 ± 0.14</td>
<td>1.54 ± 0.16</td>
<td>2.60 ± 0.12²⁺</td>
</tr>
<tr>
<td>Primary neurite (μM)</td>
<td>13.08 ± 1.81</td>
<td>11.13 ± 1.05</td>
<td>57.51 ± 6.22²⁺</td>
</tr>
<tr>
<td>Total neuritic extent (μM)</td>
<td>20.81 ± 2.56</td>
<td>16.76 ± 2.07</td>
<td>92.89 ± 9.42²⁺</td>
</tr>
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</table>

Cells were plated on poly-D-lysine-coated 35 mm dishes at a density of 1 x 10⁵ for 3–4 days. Randomly selected areas were photographed and morphological characteristics were quantitated as described [15]. All measurements were made without knowledge of the cell line and are expressed as mean ± SEM. MN9D/BDNF refers to a homogeneous clonal cell line of MN9D cells transfected with a human BDNF cDNA. Multivariate ANOVA posthoc comparisons of morphological characteristics between cell lines demonstrated that MN9D/Bcl-2 cells had significantly more neurites, longer primary neurites and greater total neurite extents than MN9D/Neo or MN9D/BDNF cells (*MN9D/Bcl-2 versus MN9D/Neo, P < 10⁻⁴; †MN9D/Bcl-2 versus MN9D/BDNF, P < 10⁻⁴; ‡MN9D/Bcl-2 versus MN9D/BDNF, P < 10⁻⁴; §MN9D/Bcl-2 versus MN9D/BDNF, P < 10⁻⁶). Similar results were observed with MN9D/Bcl-2-1 and MN9D/Bcl-2-3.
SNAP 25

GAP 43

MAP 2

Fig. 2. Western blot analysis of SNAP-25, GAP-43 and Map-2 proteins in MN9D/Neo (1) and MN9D/Bcl-2-2 (2) cell lines. Similar results were observed in the two other MN9D/Bcl-2 cell lines.

promoting action at either a particular developmental stage or on a particular cell type.

Despite the appearance of a differentiated cell type, MN9D/Bcl-2 cells continued to proliferate at a slightly slower rate of doubling time (32 h versus 38 h for MN9D/Neo). Preliminary studies using time-lapse video of individual cells demonstrated that neurites were retracted during the process of cell division. Although we have not yet determined whether a maximum neurite length can be attained, increased time in culture (>4 days) led to increased neurite length. These cells also tended to aggregate during proliferation which resulted in an underestimation of neuritic parameters. When completely isolated MN9D/Bcl-2 cells could be measured, primary neuritic length and total neurite extent were 93.8 ± 6.4 and 137.3 ± 9.0 μm, respectively (n = 85), versus 13.23 ± 2.3 and 22.5 ± 4.1 μm for MN9D/Neo controls (n = 90).

Because the expression of Bcl-2 appears to be associated with cellular maturation and differentiation processes in hemopoietic cell lines and certain non-hemopoietic tissues [7], we investigated whether other hallmarks of neuronal and/or dopaminergic maturation accompanied the morphological differentiation observed in the MN9D/Bcl-2 cell lines. Immunocytochemical studies using anti-growth cone-associated protein 43 (GAP-43) and microtubule-associated protein 2 (MAP-2) antibodies were indistinguishable, suggesting the neurites in MN9D/Bcl-2 cells had not polarized into either dendritic or axonal processes (not shown). Western blot analysis revealed no significant differences in the expression of these two neuronal markers when equivalent amounts of protein from MN9D/Bcl-2 or MN9D/Neo were analyzed (Fig. 2). Neither were differences in the pattern of neurofilament protein expression found (Fig. 2). Thus, at least these markers of neuronal maturation appeared unchanged.

Since the synaptosomal-associated protein of 25 kDa (SNAP-25) has been implicated with the extension of processes [12], we examined the level of SNAP-25 in MN9D/Neo and MN9D/Bcl-2 cell lines. As shown in Figure 2, the level of SNAP-25 in MN9D/Bcl-2 increased by at least 3 fold. This represents a specific increase in this protein since in the same samples the level of GAP-43 and MAP-2 did not change between the two cell lines. Previously, we have shown that the MN9D/Bcl-2-3 cell line expressed about 50% less Bcl-2 than did MN9D/Bcl-2-2 or -1 [11]. Since the MN9D/Bcl-2-3 cells also expressed half as much SNAP-25 (not shown), this suggests that the expression of SNAP-25 is correlated with the level of Bcl-2.

Parental MN9D cells express a variety of dopaminergic characteristics, including the rate-limiting enzyme in dopamine biosynthesis, TH, as well as a high-affinity dopamine uptake system [4,16]. As shown in Table 2, overexpression of Bcl-2 did not affect high-affinity dopamine uptake. However, TH mRNA and enzyme activity were significantly increased as compared with MN9D/Neo controls. In the latter case we did not see a correlation with Bcl-2 expression levels, although this may be because TH is already expressed at very high levels in these cells (O’Malley, unpublished observation).

The finding that overexpression of Bcl-2 in the CNS-derived, dopaminergic cell line, MN9D, led to highly significant neurite outgrowth suggests a dual role for this protein not only as a negative regulator of cell death but as a positive regulator of differentiation processes. To date, most studies have emphasized the role of Bcl-2 in hemopoietic model systems. However, high levels of Bcl-2 expression have been demonstrated in the developing nervous system, including the midbrain [10]. Since the MN9D cells were derived from embryonic day 14 mouse mesencephalon, they may represent a stage- or cell type-specific model for elucidating the biochemical mechanisms associated with neuronal Bcl-2 expression. Such an interpretation would be consistent with reports correlating

<table>
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<tr>
<th>Measurement</th>
<th>MN9D/Bcl-2-2</th>
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<tr>
<td>High-affinity dopamine uptake (n = 3)</td>
<td>100.7 ± 13.5</td>
</tr>
<tr>
<td>TH enzyme activity (n = 3)</td>
<td>190.3 ± 41.3*</td>
</tr>
<tr>
<td>TH mRNA (n = 2)</td>
<td>317.5 ± 19.4†</td>
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</table>

Cells plated at 1 × 10^5 on poly-D-lysine-coated 35 mm wells were maintained for 5 days and then incubated with 25 nM [3H]dopamine (Amersham; 45 Ci/mmol) at 37°C for 20 min. Assays were performed as described in the text. Values from triplicated wells were normalized by equal amounts of protein and were expressed as percent control MN9D/Neo. Values from three independent experiments were expressed as mean ± SEM. *P < 10^-4, †P < 10^-3. Student’s unpaired t-test. Similar results were observed with MN9D/Bcl-2-1 and MN9D/Bcl-2-3 in 2–3 independent experiments.
Bcl-2 expression with morphological differentiation in certain neuroblastoma cell lines [8], but not in N18TG2 cells (this work) or in other neuronal cell lines (e.g. [11]). Recently, Sato et al. [14] reported that overexpression of Bcl-2 in PC12 cells could lead to neurite outgrowth, but only when cells were grown without serum on collagen-coated substrates. MN9D/Bcl-2 cells appear to have bypassed such a requirement for restricted growth conditions.

In contrast to the highly significant morphological changes apparent in overexpressing Bcl-2 cells, few differences were observed between MN9D/Neo and MN9D/Bcl-2 cell lines when selected protein markers were examined (Fig. 2). Interestingly, however, SNAP-25 was increased at least three-fold in MN9D/Bcl-2 cell lines (Fig. 2). Because its expression and subcellular distribution is highly correlated with the transformation of growth cones to synaptic terminals, SNAP-25 has been used as a marker for synaptogenesis [2]. Moreover, SNAP-25 antisense oligonucleotides block neurite elongation in vitro and in vivo [12]. Recently, SNAP-25 has been shown to be an integral part of the docking-fusion complex involved in vesicular exocytosis (e.g. [3]). Whether up-regulation of SNAP-25 protein in MN9D/Bcl-2 directly results in neurite extension or is secondary to other signal transduction pathways triggered by Bcl-2 is not known at present. The MN9D/Bcl-2 cell line may prove to be a good model system with which to explore these interactions.

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