Regulation of Bcl-2 Oncoprotein Levels with Differentiation of Human Neuroblastoma Cells

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ABSTRACT

When established in culture, human neuroblastoma cell lines typically are comprised of heterogeneous cellular subpopulations, including neuroblastic (N-type), substrate-adherent (S-type), and intermediate (I-type) cells that can be distinguished by their characteristic morphologies and expression of differentiation-associated antigens. Here we examined the relative levels of the Bcl-2 oncoprotein in 15 clones derived from four different neuroblastoma cell lines. Among six clones isolated from the SK-N-SH line, levels of p26-Bcl-2 correlated with morphology and differentiation. The expression of Bcl-2 was inversely correlated to relative levels of the S-type marker proteins vimentin and b-2-microglobulin. Furthermore, stimulation of one of the N-type clones, SH-SY5Y, with the phorbol ester, 12-O-tetradecanoylphorbol-13-acetate, induced differentiation toward a more neuronal-like phenotype and resulted in a 5- to 10-fold elevation in the relative levels of Bcl-2 protein. High relative amounts of p26-Bcl-2 protein were also found in an N-type clone derived from the SMS-KCN line. In two N-type clones derived from the LA-N-1 line, however, levels of Bcl-2 protein were only moderately elevated, and in one N-type clone from the SK-N-BE(2) line the levels of Bcl-2 protein were low. Thus, high relative levels of Bcl-2 oncoprotein are not a universal feature of N-type cells (three of six clones tested). In contrast, all 5 of the S-type clones evaluated contained relatively low levels of Bcl-2 protein, suggesting that these cells (which may represent embryonic precursors of Schwann, glial, and melanocytic cells) do not typically express the bcl-2 gene at high levels. Consistent with this inverse correlation between Bcl-2 protein levels and S-type characteristics, stimulation of an I-type clone close derived from the SK-N-BE(2) line with 5-bromodeoxyuridine was accompanied by an accumulation of S-type cells in these cultures, decreased Bcl-2 protein, dimininations in the neuronal markers neurofilament-M and neuron-specific enolase, and an increase in the relative levels of the S-type marker proteins vimentin and b-2-microglobulin. Conversely, stimulation of this I-type clone with retinoic acid resulted in an accumulation of N-type cells (which are thought to represent embryonic precursors of sympathetic neurons), decreased vimentin and b-2-microglobulin, increased neurofilament-M, and a marked elevation in p26-Bcl-2. To begin to explore the functional significance of variations in the relative levels of Bcl-2 protein among neuroblastomas, we used a cell line with I-type characteristics and relatively low levels of p26-Bcl-2 (IMR-5) was stably infected with either a control or Bcl-2-encoding retrovirus, thus producing lines IMR-5-BCL-2 and IMR-5-NEO. IMR-5-BCL-2 cells contained ~5-fold higher levels of Bcl-2 protein than IMR-NEO and were markedly more resistant to killing by several chemotherapeutic drugs as measured in short-term cytotoxicity assays. Taken together, these findings indicate that Bcl-2 protein levels are regulated during the differentiation of at least some neuroblastoma cell clones in culture, thus raising the possibility of pharmacologically altering bcl-2 gene expression in neuroblastomas in vivo and thereby modulating sensitivity to chemotherapeutic drugs.

INTRODUCTION

NB is the most common extracranial solid tumor of childhood (1). Patients with NB can be separated into low- and high-risk prognostic groups based usually on age and tumor histology. Despite attempted therapeutic interventions ranging from surgery and combination chemotherapy to ablative therapy with subsequent autologous bone marrow transplantation, the outlook for patients with high-risk disease remains bleak, with median survivals of 1–2 years being reported for most studies (2–4). Furthermore, although patient age, tumor histology, and clinical stage do often accurately predict clinical outcome and thus help to guide therapy, significant numbers of patients with “low-risk” tumors eventually succumb to their disease (5).

The molecular events responsible for the initiation and progression of NB are incompletely understood, but amplification of the N-myc gene is frequently found in tumors derived from high-risk patients and has been correlated with worse prognosis irrespective of other clinicopathological parameters (6). More than one-half of NB patients with aggressive disease, however, lack N-myc gene amplification, thus prompting a search for other genetic alterations that can help to predict clinical outcome for children with these tumors (7).

Recently we have found that about one-third of human NB cell lines contain high levels of the M, 26,000 protein encoded by the bcl-2 protooncogene (8). The bcl-2 gene was originally discovered because of its frequent involvement in non-Hodgkin's lymphomas, where chromosomal translocations cause transcriptional deregulation of this gene and thus result in aberrantly high levels of p26-Bcl-2 protein production (9). Although some NB cell lines contain levels of Bcl-2 protein approaching those found in lymphoma cell lines, the molecular basis for these high levels of bcl-2 gene expression remains unknown, as conventional Southern blotting analysis has failed to reveal structural alterations of the gene. The protein encoded by the bcl-2 gene is of interest both for its subcellular location and for its biological activity. At least a portion of the total cellular Bcl-2 protein has been reported to be associated with mitochondria (10–12); and high levels of Bcl-2 protein appear to contribute to neoplastic cell expansion primarily by extending cell survival rather than by accelerating the rate of cellular proliferation (13). Specifically, p26-Bcl-2 blocks programmed cell death (also termed “apoptosis”), an active form of cellular demise that typically requires new RNA and protein synthesis (14) and that may be of clinical importance in that the majority of drugs commonly used in the treatment of cancer ultimately kill cells by stimulating apoptosis (reviewed in Ref. 15).

Although the function of bcl-2 in NB remains to be established, data derived from other types of cells and cancers suggest at least two reasons why it may be important. First, gene transfer studies have shown that the combination of c-myc and bcl-2 oncogenes is very synergistic for inducing growth factor independence and enhancing...
tumorigenicity in mice (11, 16). Furthermore, in patients with indolent lymphomas that contain a translocation involving bcl-2, and in transgenic mice that contain the equivalent of a t(14;18), spontaneous acquisition of c-myc gene activation is associated with tumor progression and the onset of aggressive disease (17, 18). The molecular basis for this synergy between bcl-2 and c-myc has recently been revealed, showing that c-myc has both mitogenic and apoptotic activities, the latter of which can be nullified by bcl-2 (19, 20). Given the functional equivalence of c-myc and N-myc in other settings (21), therefore, it seems likely that the combination of N-myc gene amplification and high levels of bcl-2 gene expression could also be complementary events in NB. Second, overproduction of the Bcl-2 protein has been shown to increase the relative resistance of cells to killing by all chemotherapeutic drugs that have been tested to date (22, 23). Thus, the possibility exists that NBs which contain higher levels of Bcl-2 protein may respond poorly to therapeutic interventions.

Established neuroblastoma cell lines typically contain as many as three morphologically distinct cell populations. These include (a) neuroblastic cells (often referred to as “N-type”) cells that have small refractile cell bodies, long neuritic processes, and dense core vesicles; (b) a population of nonneuronal cells that grow tightly adherent to the substratum (thus, the term “S-type”) cells, exhibit a flat epithelial-like cell morphology, and have abundant cytoplasm without cell processes; and (c) cells with intermediate morphological characteristics (“I-type”) cells (24–26). In addition to morphological criteria, these three populations of cells can be distinguished on immunocytochemical and biochemical grounds. Consistent with the usual origin of NBs in the paraspinal sympathetic ganglia or adrenal medulla, the N-type cells express genes associated with neuronal differentiation such as NSE and neurofilament proteins, and contain enzymes involved in the synthesis of catecholamines. The S-type cells, in contrast, lack these and neurofilament proteins, and contain enzymes involved in the synthesis of catecholamines. The I-type, or intermediate, cells express genes associated with neuronal differentiation, such as NSE and neurofilament proteins, and contain enzymes involved in the synthesis of catecholamines.

MATERIALS AND METHODS

Chemical Inducers of Differentiation. TPA and 4-α-PDD (Calbiochem, Inc.; La Jolla, CA) were dissolved in ethanol to a concentration of 1 mg/ml and stored at −20°C. Just before use, these drugs were diluted 1:100 into medium containing 10–15% (v/v) fetal bovine serum in polypropylene tubes and then added to cultures at a final concentration of 100 ng/ml. All-trans-RA (Sigma, Inc.; St. Louis, MO) was dissolved in high-performance liquid chromatography-grade dimethyl sulfoxide (Aldrich, Inc.) at a concentration of 30 mM and stored at −20°C. RA and DMSO were diluted 1:3000 with culture medium and filter sterilized by filtration through 0.22 μm cellulose acetate disks (Millipore, Inc.; St. Louis, MO) was dissolved in high-performance liquid chromatography-grade dimethyl sulfoxide (Aldrich, Inc.) at a concentration of 30 mM and stored at −20°C. RA and DMSO were diluted 1:3000 with culture medium and filter sterilized by filtration through 0.22 μm cellulose acetate disks (Millipore, Inc.; St. Louis, MO) was dissolved in high-performance liquid chromatography-grade dimethyl sulfoxide (Aldrich, Inc.) at a concentration of 30 mM and stored at −20°C. RA and DMSO were diluted 1:3000 with culture medium and filter sterilized by filtration through 0.22 μm cellulose acetate disks (Millipore, Inc.; St. Louis, MO) was dissolved in high-performance liquid chromatography-grade dimethyl sulfoxide (Aldrich, Inc.) at a concentration of 30 mM and stored at −20°C. RA and DMSO were diluted 1:3000 with culture medium and filter sterilized by filtration through 0.22 μm cellulose acetate disks (Millipore, Inc.; St. Louis, MO) was dissolved in high-performance liquid chromatography-grade dimethyl sulfoxide (Aldrich, Inc.) at a concentration of 30 mM and stored at −20°C. RA and DMSO were diluted 1:3000 with culture medium and filter sterilized by filtration through 0.22 μm cellulose acetate disks (Millipore, Inc.; St. Louis, MO) was dissolved in high-performance liquid chromatography-grade dimethyl sulfoxide (Aldrich, Inc.) at a concentration of 30 mM and stored at −20°C. RA and DMSO were diluted 1:3000 with culture medium and filter sterilized by filtration through 0.22 μm cellulose acetate disks (Millipore, Inc.; St. Louis, MO) was dissolved in high-performance liquid chromatography-grade dimethyl sulfoxide (Aldrich, Inc.) at a concentration of 30 mM and stored at −20°C. RA and DMSO were diluted 1:3000 with culture medium and filter sterilized by filtration through 0.22 μm cellulose acetate disks (Millipore, Inc.; St. Louis, MO) was dissolved in high-performance liquid chromatography-grade dimethyl sulfoxide (Aldrich, Inc.) at a concentration of 30 mM and stored at −20°C.
purified rabbit anti-mouse IgG antiserum (Cappel/Organon-Teknika, Inc.) as described previously (8), prior to treatment with 125I-protein A. Affinity-purified, biotin-conjugated secondary antibodies for immunocytochemistry included horse anti-mouse IgG and goat anti-rabbit IgG (purchased from Vector Labs, Inc.) and were used at 2.0 or 2.8 μg/ml, respectively.

**Retrovirus-mediated Gene Transfer.** The retroviral vectors pBC140 and pBC140-bcl-2-α (16) were packaged as amphotropic helper-free retroviruses by the method of Miller et al. (29), where plasmid DNAs were transfected into Psi-2 cells by a calcium phosphate precipitation method, and transiently produced ecotropic retrovirus was used to stably infect PA317 cells. Clones of G418-resistant PA317 cells were then isolated with the aid of cloning cylinders and clones producing virus at high titers (~10^7 cfu/ml) were identified. Restriction enzyme mapping in combination with Southern blotting was used to confirm that the integrated proviruses had not undergone rearrangements and had retained proper structure.

The NB cell line IMR-5 was stably infected with BC140 parental and BC140-bcl-2-α retroviruses by standard methods (29). Briefly, the medium was removed from dishes containing IMR-5 cells at 50% confluence and replaced with 2.5 ml of virus containing PA317 culture supernatants that had been supplemented with 4 μg/ml Polybrene and passed through 0.45 μm sterile filters. After 3 h, the viral supernatants were removed and the cells were washed once with medium before adding 10 ml of fresh medium. Two days later, the infected cells were split 1:5 into fresh 100-mm dishes and grown for 2–3 weeks in the presence of 250 μg/ml G418 (active drug). Multiple clones (>200) of G418-resistant cells were obtained and these were pooled together to create the cell lines IMR-NEO and IMR-5-BCL-2.

**Cell Viability Assay.** Relative proportions of viable cells were estimated by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye reduction assay (30). NB cells were cultured in 96-well flat-bottomed microtiter plates by adding 5 × 10^5 cells in 200 μl of medium with 10% FBS. Cytotoxic drugs were added at various concentrations as indicated, and dye reduction was measured 2 days later by using a Titertek Multiscan Plus microplate reader set at A570 nm. Pilot experiments verified that the assay was operating within its linear range under the conditions described here.

## RESULTS

**Comparisons of Bcl-2 Protein Levels and Differentiation Markers among NB Cell Clones.** Among the 15 NB cell clones derived from 4 cell lines, 6 were previously reported to be of the N-type, 2 of the N/I-type, 2 were I-type, and 5 were S-type (see Table 1). To explore the relative levels of p26-Bcl-2 protein in these cells and compare this with various differentiation markers, the clones were analyzed at least once with each of the two different anti-Bcl-2 antibodies and were transferred to nitrocellulose. Blots were cut into sections for incubation with various antibodies specific for p26-Bcl-2, p46-NSE, p12-β2M, or p57-vimentin. Antibody detection was by 125I-protein A and autoradiography. The number above each lane refers to the number of the clone as designated in Table 1.

### Table 1. Characteristics of neuroblastoma sublines

<table>
<thead>
<tr>
<th>Parent cell line</th>
<th>Subclone designation</th>
<th>Cell type</th>
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<tbody>
<tr>
<td>SK-N-SH</td>
<td>SH-SY5</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>SH-EP5</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>SH-EP17</td>
<td>N/I</td>
</tr>
<tr>
<td></td>
<td>SH-INh</td>
<td>N/I</td>
</tr>
<tr>
<td></td>
<td>SH-EF1</td>
<td>S</td>
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<tr>
<td></td>
<td>SH-FE</td>
<td>S</td>
</tr>
<tr>
<td>LA-N-1</td>
<td>LA1–19n</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>LA1–55n</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>LA1–226n</td>
<td>N/I</td>
</tr>
<tr>
<td></td>
<td>LA1-5s</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td>LA1–19Bs</td>
<td>S/I</td>
</tr>
<tr>
<td>SK-N-BE(2)</td>
<td>BE(2)-M17</td>
<td>S/I</td>
</tr>
<tr>
<td></td>
<td>BE(2)-C</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>BE(2)-B</td>
<td>S</td>
</tr>
<tr>
<td>SMS-KCN</td>
<td>KCN-60n</td>
<td>N</td>
</tr>
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* N, neuroblastic; S, substrate-adherent ("Schwann/glial/melanocytic"); I, intermediate.

A population of S-type cells purified from the parental SK-N-BE(2) cell line.

The densitometry results. Of the 6 N-type clones, 3 contained relatively high levels of p26-Bcl-2: SH-SY5Y, SH-EP5, and KCN-69n. Levels of Bcl-2 protein in these 3 clones ranged from 30 to 40% of the levels found in a B-cell lymphoma cell line that contains a t(14;18) translocation involving bcl-2. The cell viability results. Of the 6 clones, SH-EP17, all other NB cell clones contained relative levels of Bcl-2 protein that were <15% of the levels present in the same t(14;18)-containing lymphoma line RS11846 that was included on every blot as a control for normalization of data. Every cell line was analyzed at least once with each of the two different anti-Bcl-2 antibodies directed against non-overlapping epitopes in p26-Bcl-2, with comparable results.

All clones were immunophenotyped by using antibodies specific for the neuronal marker, NSE, and the S-type cell markers, β2M and vimentin. The highest levels of the neuronal marker NSE were found in SH-SY5Y cells. Comparable levels of NSE (50–100% of SY-SY5Y) were found in 4 of the 5 N-type clones, both of the N/I-type clones, and one of the I-type type clones. One of the clones that had previously been described as N-type, LA1-19n, contained only modest relative levels of NSE, suggesting that these cells might be less differentiated toward the neuronal lineage than the other N- and N/I-type clones.

The highest levels of β2M were found in one of the S-type clones of the SK-N-SH line, SH-EP1. Comparable β2M levels (50–100% of SH-EP1) were detected in 2 of 4 of the other S-type clones, SH-FE and LA1-5s. Slightly lower levels of β2M were present in the S-type line SK-N-BE(2)s. Very low levels of this nonneuronal marker were found in LA1-19Brs which contains some cells with I-type morphology amid the majority of flat, epithelial-like, S-type cells. All other NB cell clones contained levels of β2M that were <30% of the levels found in SH-EP1, with the lowest levels (<10%) tending to occur in the N- and N/I-type clones, as expected. Vimentin, another marker of S-type cells, was highest in SH-FE cells, with comparable levels (50–100%) being found in another of the S-type clones SH-EP1 and in the I-type clone SH-INh. Two of the S-type clones, however, contained levels of β2M that were only 20–30% of those found in SH-FE cells. With the exception of LA1-19n, all of the N-type clones contained low levels of vimentin (<10% of SH-FE).

Thus, the highest relative levels of p26-Bcl-2 were found in N-type clones. Bcl-2 protein levels however were not uniformly elevated in the N-type cells, even though some clones such as BE(2)-M17 contained very high levels of NSE.
Bcl-2 ONCOPROTEIN IN HUMAN NEUROBLASTOMAS

Fig. 2. Summary of immunoblot analysis of NB cell clones. NB cell clones were analyzed by immunoblotting at least 3 times as described in Fig. 1, and the relative intensities of bands corresponding to (A) Bcl-2 protein; (B) NSE; (C) β2M; and (D) vimentin were quantified by scanning densitometry analysis of the autoradiograms. Data represent mean ± SD. The results for Bcl-2, NSE, β2M, and vimentin are expressed as a percentage relative to the results obtained for the RSI 1846 lymphoma cell line. SH-SY5Y cells, SH-EP1 cells, and SH-FE cells, respectively. These cell lines and clones used for normalization of the data contained the highest levels of the Bcl-2, NSE, β2M, and vimentin, respectively, and were included on every blot as an internal control.

Fig. 3. Time course of differentiation-associated changes in Bcl-2 protein levels in NB cell clones. In (A), SH-SY5Y cells were treated with 100 ng/ml TPA or its inactive congener 4-α-PDD. In (B), BE(2)-C cells were treated with 10^{-5} M RA or an equivalent concentration of its diluent DMSO (0.03%, v/v). At various times thereafter, NB cells were collected from cultures and protein-containing cell lysates were prepared for immunoblot analysis of relative levels of p26-Bcl-2 and p46-NSE, as described for Fig. 1.

Comparison of Time Courses of Differentiation-associated Changes in Bcl-2 and Other Proteins in BE(2)-C Cells. To compare the kinetics of changes in Bcl-2 protein levels with various differentiation marker proteins, we performed experiments with the use of the BE(2)-C clone. These I-type cells can be induced to undergo conversion to S-type cells by treatment with BrdUrd (32), in addition to differentiating toward N-type cells in response to RA. BE(2)-C cells were therefore treated for 0, 3, 6, or 13 days with either RA or BrdUrd, and then the relative levels of p26-Bcl-2, p46-NSE, β2M, and vimentin were measured by immunoblotting. Fig. 4 shows data from an experiment where these immunoblot data were quantified by scanning densitometry. RA induced an approximately 50% increase in the relative levels of p26-Bcl-2 protein within 3 days of treatment, with levels of this oncoprotein accumulating to 3 times that found in unstimulated BE(2)-C cells by 6 days. Consistent with previous investigations showing that RA induces conversion of these I-type cells to N-type

Induction of Differentiation of SH-SY5Y and BE(2)-C Cells Is Accompanied by Modulations in Bcl-2 Protein Levels. SH-SY5Y cells display typical N-type characteristics but have been reported to undergo further neuronal differentiation by treatment with the phorbol ester, TPA (31). We therefore examined the effects of TPA on the relative levels of Bcl-2 protein in SH-SY5Y cells. TPA induced elevations in the relative levels of p26-Bcl-2 protein that were detectable within 1 day after addition of the phorbol ester to cultures and that reached levels at least 10-fold above those found in unstimulated cells (Fig. 3A). In contrast to the rapid increase in Bcl-2 protein levels, differentiation of these cells (as defined by increases in the length of neuritic processes) was not evident until 4 days after stimulation (not shown). As expected, the inactive phorbol ester 4-α-PDD induced neither neuronal differentiation nor elevations in p26-Bcl-2. Analysis of NSE levels on the same blots demonstrated that approximately equal amounts of protein were loaded in all lanes, and suggested that the TPA-induced increase in p26-Bcl-2 protein was specific.

In addition to SH-SY5Y cells, the I-type cell clone BE(2)-C has been reported to undergo conversion to N-type cells and to extend neuritic processes when stimulated with RA (32). As shown in Fig. 3B, treatment of BE(2)-C cells with 10^{-5} M RA induced elevations in p26-Bcl-2 levels that were detectable within 3 days and reached maximal levels by 6 days. Addition of an equivalent amount of DMSO, the solvent in which RA was dissolved, resulted in no detectable changes in Bcl-2 protein levels and no alterations in BE(2)-C morphology (Fig. 3B; data not shown). Analysis of the relative levels of NSE on the same blots again demonstrated that equal amounts of protein were loaded in all lanes, and suggested that the RA-induced increases in p26-Bcl-2 were specific.

Comparison of Time Courses of Differentiation-associated Changes in Bcl-2 and Other Proteins in BE(2)-C Cells. To compare the kinetics of changes in Bcl-2 protein levels with various differentiation marker proteins, we performed experiments with the use of the BE(2)-C clone. These I-type cells can be induced to undergo conversion to S-type cells by treatment with BrdUrd (32), in addition to differentiating toward N-type cells in response to RA. BE(2)-C cells were therefore treated for 0, 3, 6, or 13 days with either RA or BrdUrd, and then the relative levels of p26-Bcl-2, p46-NSE, β2M, and vimentin were measured by immunoblotting. Fig. 4 shows data from an experiment where these immunoblot data were quantified by scanning densitometry. RA induced an approximately 50% increase in the relative levels of p26-Bcl-2 protein within 3 days of treatment, with levels of this oncoprotein accumulating to 3 times that found in unstimulated BE(2)-C cells by 6 days. Consistent with previous investigations showing that RA induces conversion of these I-type cells to N-type
Fig. 4. Comparison of kinetics of modulations in Bcl-2 and other proteins during drug-induced differentiation of BE(2)-C cells. BE(2)-C cells were stimulated either with 10^{-5} M RA (•) or with 10^{-5} M BrdUrd (○). At various times thereafter, cells were recovered from cultures and relative levels of (A) Bcl-2; (B) NSE; (C) β2M; and (D) vimentin were analyzed by immunoblot assay. The relative intensities of the bands were quantified by scanning densitometry and the data were expressed as a percentage relative to unstimulated BE(2)-C cells. Data are representative of two experiments.

Relative levels of β2M and vimentin decreased in RA-treated BE(2)-C cells. The decline in β2M and vimentin, however, appeared to begin later in these cultures than the increase in p26-Bcl-2, with clear reductions (approximately 60% for both proteins) occurring at 6 days after addition of RA to the cells. NSE levels were unchanged (Fig. 4).

In contrast to RA, treatment of BE(2)-C cells with 10^{-5} M BrdUrd induced an approximately 50% decline in Bcl-2 protein levels within 3 days after addition of drug to the cultures. Again, this change in p26-Bcl-2 levels occurred earlier than alterations in the relative levels of NSE, β2M, and vimentin, all of which were not evident until 6 days after treatment with BrdUrd (Fig. 4). Consistent with previous work showing that BrdUrd induces conversion to S-type cells in cultures of BE(2)-C cells (32), treatment of these cells with BrdUrd resulted in reductions in the N-type marker NSE, in addition to 2- to 3-fold elevations in the relative levels of the S-type markers β2M and vimentin.

Fig. 5 shows examples of the morphological changes that occurred in BE(2)-C cells following treatment with RA or BrdUrd. RA induced elongation of neuritic processes and formation of tightly packed cell aggregates ("pseudoganglia") (Fig. 5B) within 6 days after treatment. In contrast, BrdUrd stimulated the accumulation in cultures of flat epithelial-like cells that exhibited increased cell substratum adhesion and less evidence of cell-cell association (Fig. 5C). Appearance of a few of these S-type cells began within 6 days after treatment of BE(2)-C cells with BrdUrd, but the majority of the cells in these cultures did not assume an S-type morphology until 10–13 days.

Immunocytochemical Analysis of Differentiation-associated Changes in Bcl-2 Protein Levels. The immunoblotting approach provides information only about the average net changes in Bcl-2 protein levels that occur during the in vitro differentiation of NB cells. To evaluate at the single cell level the changes in Bcl-2 protein accumulation that occur during drug-induced differentiation, BE(2)-C cells were grown on coverslips and then Bcl-2 protein as well as the differentiation markers NF-M and vimentin were immunocytochemically detected by using specific antibodies. As shown in Fig. 6, the majority of unstimulated BE(2)-C cells exhibited moderate intensity immunostaining with antibodies directed against Bcl-2 (Fig. 6A) or the intermediate filament proteins NF-M (Fig. 6D) and vimentin (Fig. 6G). After treatment for 6 days with RA, however, intense cytosolic immunostaining with antibodies to Bcl-2 and NF-M was noted for nearly all cells (Fig. 6, B and E). In contrast, many of the RA-treated
cells displayed less immunoreactivity with an antibody directed against vimentin (Fig. 6H).

Treatment of BE(2)-C cells for 13 days with BrdUrd led to the accumulation in cultures of large, flat, epithelial-like cells, essentially all of which contained relatively little Bcl-2 protein (Fig. 6C) as determined by this immunocytochemical assay. Most of these BrdUrd-treated cells also exhibited little or no immunostaining with antibodies specific for NF-M (Fig. 6F). In contrast, relative levels of vimentin were markedly elevated in nearly all of these cells, as revealed by their intense immunostaining with vimentin-specific antibodies (Fig. 6I).

Bcl-2 Protein Influences Sensitivity of a NB Cell Line to Chemotherapeutic Drugs. Previously we observed that lymphoid cells containing high relative levels of Bcl-2 protein either as a result of gene transfer manipulations or because of chromosomal translocations that involve the bcl-2 gene are markedly more resistant to the acute toxic effects of a wide variety of drugs commonly used in the treatment of cancer (22, 23). To extend these studies to NB cells, we arbitrarily chose a NB cell line IMR-5 for analysis that is comprised primarily of I-type cells and that contains relatively low levels of Bcl-2 protein. IMR-5 cells were stably infected with recombinant retroviruses that contain a neomycin phosphotransferase gene (neo-+) alone or in combination with a complementary DNA encoding the p26-Bcl-2 protein (see Fig. 7). Analysis of the resulting G418-resistant cells by immunoblotting revealed a ~5-fold elevation in the levels of Bcl-2 protein present in IMR-5-BCL-2 cells. These levels of Bcl-2 protein were comparable to those found in lymphoma and leukemia cell lines that contain a t(14;18) translocation and to some NB cell lines with N-type features (Fig. 7; not shown). The introduction of viral vectors into these cells had no effect on their morphological characteristics.

IMR-5-BCL-2, IMR-5-NEO, and the parental IMR-5 cell lines were next evaluated with regard to their relative sensitivities to killing

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Fig. 6. Immunocytochemical analysis of BE(2)-C cells before and after differentiation. BE(2)-C cells were grown on glass coverslips either without addition of drugs to the media (A) or with stimulation with RA for 6 days or BrdUrd for 13 days, as described for Fig. 4. Cells were fixed and subjected to immunostaining by using antibodies specific for Bcl-2 (A-C; ×100); NF-M (D-F; ×100); or vimentin (G-I; ×100 for G and H, ×200 for I). Color development was with 3,3'-diaminobenzidine (brown) and counterstaining was with hematoxylin (blue).

Fig. 7. Immunoblot analysis of retrovirus-infected IMR-5 cells. IMR-5-NEO and IMR-BCL-2 cells were analyzed by immunoblot analysis (100 μg protein/lane) essentially as described for Fig. 1. The top portion of the blot was stained with Ponceau-S to verify loading of approximately equal amounts of protein for all samples (not shown). The bottom portion was incubated with anti-Bcl-2 antisera followed by 125I-protein A. 380 is a lymphocytic leukemia line that contains a t(14;18). Below the autoradiogram is shown a diagram of the recombinant bcl-2 retrovirus used to create the IMR-5-BCL-2 cell line.

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induced by a 2-day exposure to various anticancer drugs, including Adriamycin, etoposide, vincristine, and methotrexate. Preliminary experiments demonstrated that the rates of DNA synthesis and cell doubling times were essentially identical for all 3 of these lines (not shown), thus obviating concerns that differences in cell cycle kinetics could influence the results of these experiments. As shown in Fig. 8, cultures of IMR-5-NEO and the parental IMR-5 cell lines experienced similar, dose-dependent declines in the relative proportions of viable cells when treated with Adriamycin, etoposide, vincristine, and methotrexate. In contrast, IMR-BCL-2 cells were markedly more resistant to the acute cytotoxic effects of these drugs. The diminished drug sensitivity observed for IMR-5-BCL-2 cells in 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays was a reflection of improved cell survival in the presence of drugs as opposed to more rapid cell proliferation, because measurements of \[^{3}H\]thymidine incorporation into DNA demonstrated similar dose-response curves for drug-mediated inhibition in all 3 cell lines (not shown).

DISCUSSION

Previously, we compared the relative levels of p26-Bcl-2 protein in 9 NB cell lines and found that some lines contained levels of this oncoprotein that approached those found in lymphoid cell lines harboring a t(14;18), whereas some other NB cell lines contained low, barely detectable levels of p26-Bcl-2 (8). When these data regarding Bcl-2 protein levels were correlated with other characteristics of the cell lines, including morphology, N-myc gene amplification status, and p75-NGF-receptor expression, a clear association was noted between Bcl-2 and cellular morphology. Specifically, NB cell lines that were comprised mostly of S-type cells uniformly contained little or no detectable Bcl-2 protein, and lines characterized by large proportions of N-type cells tended to contain relatively high levels of Bcl-2 protein. Because NB cell lines are often heterogeneous and contain mixtures of N-, I-, and S-type cells in proportions that can vary widely depending on how the cells are maintained in culture, we sought to extend these initial observations by determining the relative levels of p26-Bcl-2 protein in 15 well-characterized NB cell clones (24, 25). For the most part, these clones are morphologically and immunophenotypically homogeneous. To control for any instability in the phenotype of these clones that might occur with time in culture, however, we simultaneously assessed the relative levels of other proteins that have traditionally served as markers for neuronal (NSE, NF-M) or for Schwann/glial/melanocytic cells (\(\beta_2\)M, vimentin) during previous studies of cultured human NB cells. Consistent with our previous investigations (8), S-type NB cell clones without exception contained low relative levels of p26-Bcl-2 protein (5 of 5 clones). The highest levels of Bcl-2 protein were found in clones having N-type characteristics, where 3 of 6 clones examined contained levels of this oncoprotein that were at least 30-40% of that seen in t(14;18)-containing lymphoma cell lines. Clones with N/I- or I-type features tended to contain intermediate levels of Bcl-2 protein (Figs. 1 and 2).

The most consistent correlation between Bcl-2 protein levels and cell morphological characteristics was noted for the 6 subclones of the SK-N-SH line, where Bcl-2 protein levels were highest in the clones classified as N-type (SH-SY5Y; SH-EP5), lower in a clone with N/I-type morphology (SH-EP17), lower still in an I-type clone (SH-IN9I), and lowest in the S-type clones SH-EP1 and SH-PE (see Fig. 2). In contrast, strict correlation between Bcl-2 levels and cell morphology was not evident for the subclones of the LA-N-1 and SK-N-BE(2) lines. These clones, however, also did not exhibit as firm a correlation between their morphological phenotype and the differentiation marker proteins NSE, vimentin, and \(\beta_2\)M.

Because the normal patterns of Bcl-2 protein production have yet to be delineated for the developing sympathetic ganglia and adrenal medulla (the presumed normal counterparts of NBs), it is difficult to speculate whether the high levels of Bcl-2 protein seen in some NB clones with N-type characteristics is normal for their particular stage of differentiation, or alternatively, an indication that only some NB tumor cells acquire the genetic abnormalities necessary to produce high levels of this oncoprotein. The experiments described here, where NB cell differentiation was induced with the use of pharmacological reagents, however, argue in favor of the former possibility. For example, stimulation of the N-type cell clone SH-SY5Y cells with TPA induced a 5- to 10-fold elevation in Bcl-2 protein levels and extension of longer neurite processes, suggesting that bcl-2 gene expression is up-regulated as these neoplastic cells undergo further neuronal differentiation. Similarly, treatment of the I-type clone BE-(2)-C with RA resulted in conversion to an N-type morphology and extension of longer neurite processes, suggesting that bcl-2 gene expression is up-regulated as these neoplastic cells undergo further neuronal differentiation. Similarly, treatment of the I-type clone BE-(2)-C with RA resulted in conversion to an N-type morphology and extension of longer neurite processes, in association with a 3-fold increase in Bcl-2 protein levels, a rise in the neuron-specific intermediate filament protein NF-M, and a decrease in the Schwannian/glial cell markers \(\beta_2\)M and vimentin. Conversely, stimulation of this I-type clone with BrdUrd resulted in the accumulation of large, flat, epithelial-like cells in these cultures, in association with a ~60% reduction in Bcl-2 protein levels, a decrease in NF-M immunoreactivity, and a 2- to 4-fold rise in the relative levels of \(\beta_2\)M and vimentin.

These data are thus consistent with a model wherein a multipotential precursor cell with I-type characteristics can be induced to differentiate either toward a neuronal phenotype with an increase in Bcl-2 protein levels, or toward a nonneuronal phenotype [proposed to represent a precursor of Schwann, glial, or melanocytic cells (32)], where relatively little Bcl-2 protein accumulation occurs. Whether the differentiation events and changes in Bcl-2 expression seen in these NB cells in culture are reflective of normal differentiation cannot be addressed from the data presented here.

![Fig. 8. Elevated levels of Bcl-2 protein increase resistance of IMR-5 cells to chemotherapeutic drugs. IMR-5 (□), IMR-5-NEO (○), and IMR-5-BCL-2 (●) were cultured at 5 X 10^4 cells/well in 200 μl of medium in microtiter plates with or without ("C") various concentrations of drugs as shown, including A, Adriamycin (ADR); B, etoposide (VP-16); C, vincristine (VCR); and D, methotrexate (MTX). Relative proportions of viable cells were assessed 2 days later by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and the results were expressed as a percentage relative to control cultures which received no drugs. Data represent mean ± SD for 3 determinations. Similar results were obtained when cell viability was assessed by trypan blue dye exclusion instead of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dye reduction (not shown).](https://cancerres.aacrjournals.org/content/57/6/4979/suppl/DC1/5701640417-00577.png)
Although it could be argued that the drug-induced changes in Bcl-2 protein levels seen in NB cells represent a direct effect of the drugs on the expression of the bcl-2 gene rather than a true association of bcl-2 expression with neuronal differentiation, this seems unlikely given that similar results were achieved by using drugs that are thought to act through different mechanisms (e.g., RA and TPA). Furthermore, the drug-induced modulations in p26-Bcl-2 are not limited to one or two particular clones of NB cells, as we have observed similar associations between Bcl-2 protein levels and NB cell morphology, using other NB clones and lines (not shown). Many of these other NB lines, however, are less homogeneous in their differentiation responses to various drugs, and thus are less suitable models for demonstrating an association between Bcl-2 protein levels and NB cell morphology or immunophenotype.

Admittedly, the studies described here have not proven that treatment of NB cells with RA, TPA, or BrdUrd induces differentiation, as opposed to allowing for the selective expansion of rare cells having either N- or S-type characteristics. Permanent conversion of I-type BE(2)-C cells to either N-type cells by treatment with RA or to S-type cells by BrdUrd has been reported recently (33), arguing that the responses we observed reflect differentiation. The cytostatic effect of these drugs on NB cells also favors the possibility of differentiation (34). Moreover, at least for the drugs that induced a neuronal phenotype, there was a clear increase in the length of the neuritic processes of these cells, consistent with more extensive differentiation. Although the time course of changes in Bcl-2 protein levels in NB cells treated with drugs occurred earlier than detectable alterations in the various differentiation marker proteins examined here, it seems unlikely that p26-Bcl-2 directly regulates the differentiation process based on current understanding of its function as a regulator of cell survival. It is possible, however, that Bcl-2 confers a selective survival advantage to cells that up-regulate expression of the bcl-2 gene during differentiation. Nevertheless, the findings with the various S-type clones examined here and with the I-type clone [BE(2)-C] that was converted to S-type upon treatment with BrdUrd demonstrate that bcl-2 gene expression is not required for the survival of at least some types of cells derived from NB tumors. It is interesting in this regard that S-type cells tend to express N-myc at much lower levels than N- and I-type cells, even when derived from the same line and even when the N-myc locus is amplified (35). Given that c-myc has been shown to accelerate apoptosis of growth factor-deprived cells and that bcl-2 can counteract this effect of c-myc (19, 20), it is possible that the lower levels of N-myc expression in the S-type cells renders bcl-2 expression unnecessary for maintenance of survival or at least allows these cells to survive with only small amounts of the Bcl-2 protein.

The finding that drugs such as RA can modulate Bcl-2 protein levels in NB cells may have important implications for the treatment of children with these tumors, given that high levels of p26-Bcl-2 have been associated with increased resistance to cell killing by chemotherapeutic drugs and X-irradiation (22, 23, 36, 37). For example, induction of terminal differentiation of NBs in vivo through use of retinoids, if incomplete, might push the cells to a slightly more differentiated state where cell proliferation is reduced but not completely stopped and where levels of Bcl-2 protein are higher. As a result, these residual neoplastic cells, although having a slower proliferative rate, might also be more resistant to the cytotoxic effects of drugs and irradiation. The higher levels of Bcl-2 protein in RA-treated cells might also allow occasional neoplastic cells to survive the damage induced by antineoplastic reagents and develop additional secondary genetic changes that contribute to tumor progression and treatment failure.

In support of a possible link between p26-Bcl-2 levels and drug sensitivity in NB cells, previous studies of clones derived from the SK-N-SH cell line have demonstrated a correlation between morphology and resistance to antimetabolites such as methotrexate and methotrexate, as well as cytokines and cytotoxic agents such as vincristine, vinblastine, and colchicine (38). Specifically, the N-type clone S14 (which has high levels of Bcl-2 protein) has been shown to be 4- to 8-fold more resistant to these drugs than an S-type clone SH-EP1 derived from the same cell line (which we have shown has very low levels of Bcl-2 protein). Of course, comparisons of the relative sensitivity to drugs of various NB subclones can be tainted by differences in their growth rates, stage of differentiation, rates of DNA repair, rates of drug uptake and efflux, nucleotide pools, and other parameters that can influence drug responses. For this reason we used gene transfer approaches to more directly examine the effects of Bcl-2 on the sensitivity of a NB cell line to killing by various cytotoxic drugs used commonly in the treatment of cancer. These experiments demonstrated that the relative levels of Bcl-2 protein can have a profound influence on the sensitivity of NB cells to chemotherapeutic drugs (Fig. 8), at least where acute toxicity of these drugs is concerned. Although highly speculative, therefore, the data presented here and elsewhere (22, 23, 36, 37), raise the hope that novel reagents could be developed that would down-regulate levels of Bcl-2 protein in NBs, thereby leaving the cells in a more vulnerable state for killing by conventional chemotherapeutic drugs and radiation.

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Regulation of Bcl-2 Oncoprotein Levels with Differentiation of Human Neuroblastoma Cells

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