Potentiation of Enediyne-induced Apoptosis and Differentiation by Bcl-2

Megan Cortazzo and Nina Felice Schor

Abstract

Bcl-2 overexpression has been shown to be protective against apoptosis induced by a variety of mechanistically diverse chemotherapeutic drugs. Recently, oxygen radical species have been implicated in the process of apoptosis, and Bcl-2 has been proposed to exert its protective effect by altering the redox state of the cell. Unlike most other chemotherapeutic agents, naturally occurring enediyenes are rendered more cytotoxic in the presence of a higher reducing potential, because as prodrugs, they require reduction for activation. We demonstrate herein that induction of Bcl-2 expression in PC12 cells potentiates the induction of apoptosis and differentiation by the enediyne neocarzinostatin. In contradistinction, Bcl-2 abrogates the induction of apoptosis and differentiation by the autoactivating enediyne, enediyne-5, and the non-enediyne chemotherapeutic agent, cisplatin. We further demonstrate that enediyne potentiation by Bcl-2 is related to an increase in cellular glutathione. The present studies suggest that enediyenes that require reductive activation might be critically useful agents in the therapy of tumors such as neuroblastomas and estrogen-responsive breast cancers, the resistance of which is related to up-regulation of Bcl-2.

Introduction

The proto-oncogene, bcl-2, was first recognized in B-cell lymphoma/leukemia lines in which it had been overexpressed by virtue of a chromosomal translocation (1). Its expression, which results in production of the M, 26,000 protein Bcl-2, has been shown to confer resistance to cell death upon a wide variety of cell types. Recent studies have implicated Bcl-2 in resistance to apoptotic cell death in particular and have suggested that this protein exerts its protective effects by a mechanism that includes a shift in the redox potential of the cell to a more reduced state (2, 3). These studies specifically implicate an increase in the non-protein sulfhydryl content in this alteration in redox potential, but they do not rule out the existence of other mediators (e.g., relative increases in reduced pyridine nucleotide or protein sulfhydryl content) of this change. A reduction-related mechanism for Bcl-2 activity is particularly plausible in light of other studies that link both the initiating and subsequent steps involved in the apoptotic process to exposure of the cell to reactive oxygen species (4–6). NCS is a DNA strand-cleaving enediyne natural product that induces apoptosis and differentiation in neural crest tumor cells in culture (7). Unlike other chemotherapeutic agents, many of which are detoxified by glutathione and other free radical-scavenging reducing agents, NCS is a prodrug that requires reduction for activation. As such, the cytotoxicity of NCS has been demonstrated to vary directly with the sulfhydryl content of the cell (8–10).

This led to the hypothesis that, contrary to the case for other chemotherapeutic agents studied, overexpression of Bcl-2 and the resulting shift in redox potential of the cell would potentiate the induction of apoptosis by NCS. This hypothesis was based on the assumption that NCS activation would be potentiated and that, in the process, reducing equivalents and other species that would have impeded subsequent steps in the apoptotic pathway would be consumed and, therefore, be unavailable for apoptosis protection.

Materials and Methods

Cells and Cell Culture. Control-transfected (pBabe-puro) PC12 cells and PC12 cells transfected with the bcl-2 gene ligated into the retroviral vector, pBabe, containing a puromycin resistance gene (bcl-2-pBabe-puro; Ref. 2) were provided by Dale E. Bredesen (La Jolla Cancer Institute, La Jolla, CA). Cells were maintained as adherent monolayers in DMEM made with 10% in horse serum, 0.5% in fetal bovine serum, and 1.1% in penicillin/streptomycin (GIBCO). Cells were fed every 3–4 days, and biweekly, 1 µg/ml puromycin was added to the medium. Cells were examined for Bcl-2 expression by Western blotting every 10 passages. Bcl-2 expression did not vary in either cell line over the course of these studies.

Determination of Adherent Cell Number, Percentage of Differentiation, and Percentage of Apoptosis. Adherent cell number, percentage of differentiation, and percentage of apoptosis were determined daily in control and treated cultures, as we have described previously for neuroblastoma cells (7, 11, 12). The statistical significance of differences between control and treated cultures was assessed in all cases using Student's t test, with P ≤ 0.05 being considered significant.

Effects of BSO Treatment on the Differential Sensitivity of Mock- and bcl-2-transfected PC12 Cells. To determine the role of glutathione in the differential sensitivity of mock- and bcl-2-transfected PC12 cells, cells were continuously exposed to 0.5 mM BSO beginning 24 h prior to treatment with NCS and throughout the remainder of the experiment. Cell counts were performed as detailed above and as we have described previously (12).

Results

Treatment of control-transfected (retroviral vector pBabe linked to the puromycin resistance gene) PC12 rat pheochromocytoma cells with NCS (5–50 nm) for 1 h led to apoptosis of a dose-dependent fraction of the cells (Fig. 1, A–C). Morphological differentiation occurred in a dose-dependent fraction of the surviving cells (Fig. 1, D and E). Stable expression of Bcl-2, achieved by transfection of bcl-2 in the retroviral vector-puromycin resistance construct (2), potentiated induction of apoptosis and differentiation by NCS (Fig. 1, C and E), and lowered the ED50 for suppression of culture growth rate 4-fold (Fig. 2A; Table 1).

To be certain that this potentiation was related to the need for reductive activation of NCS and not just a property of the enediyenes in general, the effects of increased Bcl-2 expression on the culture growth suppression induced by the autoactivating enediyne, enediyne-5, were determined. Einediyne-5 is a synthetic enediyne that does not require exogenous activation (reductive or otherwise) for induction of apoptosis and differentiation in neural crest cells (11, 13, 14). Contrary to the case for NCS, overexpression of Bcl-2 shifts the ED50 for enediyne-5 8-fold in the direction of protection from culture growth suppression (Fig. 2B; Table 1). In addition, consistent with

1 Received 11/14/95; accepted 1/24/96.
2 The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
3 These studies were supported by Grant DHP-128 from the American Cancer Society and by NRSA Senior Fellowship Grant CA67421 (to N. F. S.) from the NIH. M. C. was the recipient of an Undergraduate Summer Research Fellowship from Children's Hospital of Pittsburgh.
4 To whom requests for reprints should be addressed, at Division of Child Neurology, Children's Hospital of Pittsburgh, 3705 Fifth Avenue, Pittsburgh, PA 15213. Phone: (412) 692-6471; Fax: (412) 692-5723; E-mail: nfschor@vms.cis.pitt.edu.
5 The abbreviations used are: NCS, neocarzinostatin; BSO, buthionine sulfoximine.
Fig. 1. Induction by NCS of apoptosis and morphological differentiation in PC12 cells. PC12 cells were treated with NCS for 1 h at 37°C and washed free of the drug on day 0. A, fluorescence micrograph of control-transfected PC12 cells stained with Hoechst dye #33342 (Sigma; Ref. 7) on day 7 after treatment with 0.05 μM NCS (×200), demonstrating the margination, fragmentation, and condensation of chromatin used as the criteria for quantitating the incidence of apoptosis (graphed in C) in cultures of PC12 cells. B, computer reconstruction of the field shown in A highlighting the fragmented nuclei with some fragments in the process of extrusion (darker shaded areas). Once extruded, these fragments are known as "apoptotic bodies." C, NCS concentration dependence of the incidence of apoptosis in control- and bcl-2-transfected PC12 cells on day 7 after treatment. The percentage of apoptosis was determined as described (7). Bars, the SEM for three determinations. Where bars are not apparent, the SEM was smaller than the height of the symbol. D, light micrographs of control-transfected PC12 cells on day 7 after treatment with 0.05 nM NCS (×200). E, NCS concentration dependence of the incidence of differentiated morphology on day 7 after treatment. The percentage of differentiation was determined as described for NB41A3 neuroblastoma cells (12). Bars, the SEM for three determinations. Where bars are not apparent, the SEM was smaller than the height of the symbol.
Fig. 2. A-C, drug concentration dependence of adherent cell number. Bars, the SEM for three determinations. Where bars are not apparent, the SEM was smaller than the height of the symbol. A, day 7 after NCS treatment. The difference between control- and bcl-2-transfected cells first attained statistical significance on day 7 and was maintained throughout the remainder of the experiment. B, day 5 after enediyne-5 treatment. The difference between control- and bcl-2-transfected cells first attained statistical significance on day 3 and was maintained throughout the remainder of the experiment. C, day 7 after cisplatin treatment. The difference between control- and bcl-2-transfected cells first attained statistical significance on day 3 and was maintained throughout the remainder of the experiment. D, Bcl-2-mediated abrogation of the incidence of morphological differentiation on day 7 in PC12 cells treated with OCS (0.05 μM), enediyne-5 (0.05 μM), or cisplatin (25 μM). Control- and bcl-2-transfected PC12 cells were treated with NCS, enediyne-5, or cisplatin at 37°C (1 h, enediyne; 4 h, cisplatin) and washed free of the drug on day 0. Daily determinations of the percentage of the cells that exhibited differentiated morphology were performed as described previously (12), and the data plotted indicate the percentage of differentiation for the drug-treated culture minus that for a simultaneously sham-treated sister culture. The incidence of differentiation was maximal by day 7 in all cases, and data are shown for this day. Bars, the SEM for three determinations.

Discussion

We have documented previously that NCS, an antineoplastic antimitotic agent, induces apoptosis in neural-type neural crest tumor cells in culture (7, 11). Protection by Bcl-2 from chemotherapeutic agent-

previous reports in other cell lines (15, 16), Bcl-2 overexpression was protective of PC-12 cells treated with cisplatin (Fig. 2C; Table 1). Bcl-2 overexpression also abrogated the induction of differentiation by enediyne-5 or cisplatin (Fig. 2D).

Treatment with 0.5 mM BSO for 24 h, a condition that results in reduction of glutathione levels to below detectable levels (Ref. 2; data not shown), eliminates the differential sensitivity between mock- and bcl-2-transfected cells (Fig. 3), implying that increased glutathione levels in Bcl-2-overexpressing cells (2) are responsible for potentiation of the effects of NCS.

Discussion

We have documented previously that NCS, an antineoplastic antimitotic agent, induces apoptosis in neural-type neural crest tumor cells in culture (7, 11). Protection by Bcl-2 from chemotherapeutic agent-
induced apoptosis has been shown in several cell lines treated with cisplatin or etoposide. Both of these agents are proposed to work by mechanisms involving free radical intermediates. Reaction of these intermediates with cellular reducing compounds such as glutathione results in a decrement in the cytotoxicity of each of the agents (15, 16). It is tempting, therefore, to speculate that the increase in reducing potential that results from Bcl-2 overexpression prevents apoptosis at a step close to the initial action of each of the drugs that induce this pathway. However, studies of etoposide-induced apoptosis in cultured cells indicate that Bcl-2 prevents apoptosis without influencing the direct production of DNA topoisomerase II cross-links or single- and double-strand breaks by this drug. The formation of DNA “ladders” was, however, impeded by Bcl-2 overexpression (17). This would imply that Bcl-2 acts downstream of the initial activity of etoposide but upstream of the endonucleolytic fragmentation of DNA and that endogenous free radical generation is involved in these intermediate elements of the apoptosis pathway. The present finding that Bcl-2 abrogates both apoptosis and differentiation in PC12 cells treated with ene diyne-5 or cisplatin suggests that the step at which Bcl-2 exerts its apoptosis-protective action precedes temporally the branch point between apoptosis and differentiation. Similarly, the finding that Bcl-2 overexpression potentiates the induction by NCS of both apoptosis and differentiation in PC12 cells is consistent with the notion that, as is the case for the apoptosis-protective activity of Bcl-2 for conventional chemotherapeutic agents, the potentiation by Bcl-2 of the activity of NCS is due to effects on a part of the cellular pathway that precedes temporally the decision branch point between the two response types.

We hypothesized that the ability of Bcl-2 to potentiate the activity of NCS is related to the elevation of basal glutathione content reported for these transfected PC12 cells (2, 3). Potentiation of NCS cytotoxicity by cellular sulfhydryl loading and abrogation of drug activity by glutathione depletion are well documented (8–10). The present studies demonstrate that glutathione depletion completely eliminates the potentiation by Bcl-2 of the activity of NCS. This implies that increased glutathione levels constitute the major reason for increased activation of NCS in this system. The previous observation that glutathione depletion does not interfere with the anti-apoptosis activity of Bcl-2 (2, 3) suggests that glutathione excess is not responsible for the protective effect of Bcl-2. It is, therefore, curious that apoptosis proceeds unimpeded in Bcl-2-overexpressing PC12 cells treated with NCS. This finding could imply that the pathway leading from NCS treatment to apoptosis bypasses the step at which Bcl-2 exerts its protective activity. This is unlikely since autoactivated ene diyne-5 is mechanistically similar to reduction-activated NCS (11, 13, 14), and apoptosis due to the former drug is abrogated by Bcl-2 overexpression. It is much more likely that a shift in the redox potential of the cell results from the consumption by NCS of reducing species including, but not exclusively, glutathione. These nonglutathione species may in fact be the mediators of the protective effects of Bcl-2 on the cell.

Table 1 Potency of NCS, enediyne-5, and cisplatin for suppression of adherent cell number in PC12 cells with and without stably increased expression of Bcl-2

<table>
<thead>
<tr>
<th>Drug</th>
<th>Control-transfected PC12 cells</th>
<th>bcl-2-transfected PC12 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCS</td>
<td>0.02</td>
<td>0.005</td>
</tr>
<tr>
<td>Ene diyne-5</td>
<td>0.005</td>
<td>0.04</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>1</td>
<td>25</td>
</tr>
</tbody>
</table>

Finally, the potentiation by Bcl-2 of the activity of NCS suggests that reduction-activated cytotoxic drugs may be useful chemotherapeutic agents in the therapy of tumors with Bcl-2-related chemotherapeutic resistance. This is consistent with the previous reports that Bcl-2 expressing N-type neuroblastoma cells, notoriously resistant to conventional chemotherapeutic agents, are more sensitive to NCS than are Bcl-2-deficient, traditional chemotherapy-responsive neuroblastoma cells (11, 16). In addition, in view of recent reports of the role of Bcl-2 in the drug resistance of estrogen-responsive breast cancer (18), the present study suggests that the use of sulfhydryl-triggered enediyynes might be a rational chemotherapeutic approach to these tumors as well.

Acknowledgments

We thank Karen D. Nylander for expert technical assistance and Dr. Simon Watkins and the staff of the University of Pittsburgh Structural Biology Center for help with fluorescence microscopy.

References

Potentiation of Enediyne-induced Apoptosis and Differentiation by Bcl-2

Megan Cortazzo and Nina Felice Schor

Cancer Res 1996;56:1199-1203.