AWARD NUMBER DAMD17-97-1-7247

TITLE: Exploiting bcl-2 Overexpression in the Chemotherapy of Breast Cancer

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REPORT DATE: June 1999

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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Exploiting bcl-2 Overexpression in the Chemotherapy of Breast Cancer

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In neural crest cells, bcl-2 overexpression results in altered handling of glutathione, and consequent increased cellular reducing potential. This change potentiates the induction of apoptosis by enediyne prodrugs in neural crest tumor cells. Our studies examined the applicability of these findings to MCF-7 breast cancer cells, known to overexpress bcl-2 in response to estradiol treatment. We have found that estradiol treatment of MCF-7 cells does indeed result in an increase in the expression of bcl-2. However, transfection of MCF-7 cells with an expression construct for bcl-2 does not result in potentiation of enediyne-induced apoptosis. Unlike the case for neural crest tumor cells, glutathione handling is not altered in MCF-7 cells induced by transfection to overexpress bcl-2. Our results to date suggest that determination of the effect of bcl-2 expression on glutathione handling may allow prediction of the response of a particular tumor cell to treatment with enediynes. In the one-year extension period of this grant, we will merge these two lines of investigation and determine the effects of estradiol treatment (rather than direct bcl-2 transfection) on glutathione handling in MCF-7 cells. Initial studies indicate that estradiol treatment does alter the native glutathione content of MCF-7 cells.
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INTRODUCTION

The proto-oncogene bcl-2 is expressed in 65-80% of human breast cancers (1,2). Approximately 50% of breast cancers are found to overexpress this gene (1,3). Overexpression of bcl-2, which results in overproduction of the 26 kD protein Bcl-2, has been shown to confer resistance to apoptotic cell death induced by a variety of stimuli, including chemotherapeutic-induced apoptosis in breast cancer (4,5). This is particularly important in the case of advanced, estrogen receptor-positive tumors, a circumstance in which Bcl-2 content correlates with clinical resistance to chemotherapy (5).

Recent studies in other cell lines have suggested that Bcl-2 exerts its protective effects by a mechanism that includes a shift in the redox potential of the cell to a more reduced state (6,7). Among other biochemical effects, these studies specifically implicate an increase in cellular reduced glutathione (GSH) content in this alteration in redox potential. This makes particular sense because other studies link the apoptotic process to exposure to reactive oxygen species (8-10). One potential strategy for overcoming Bcl-2-mediated chemotherapeutic resistance is to take advantage of the increase in cellular GSH and, therefore, free sulfhydryl content by using chemotherapeutic agents that require reduction by sulfhydryl compounds for their activity. We have recently explored in vitro the efficacy of one such group of agents, the enediyynes, in pheochromocytoma cells that were genetically engineered to overexpress bcl-2 (11).

Neocarzinostatin (NCS) is an enediyne DNA-cleaving natural product that induces apoptosis in some tumor cell lines in culture (12). Like other naturally-occurring enediyynes, NCS is actually a prodrug that requires sulfhydryl activation for efficacy. As such, the cytotoxicity of NCS has been demonstrated to vary directly with the sulfhydryl content of the cell (13-15). This information led to our prediction that, contrary to the case for all 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.

We have shown that, in PC12 rat pheochromocytoma cells that have been bcl-2- or control-transfected, bcl-2 overexpression does indeed potentiate the apoptosis-inducing activity of NCS by increasing cellular levels of GSH (11). The reduction-dependent enediyne prodrugs are therefore the only class of drug that has been demonstrated to work best in those tumor cells that have become resistant to other known chemotherapeutic agents.

Although the naturally-occurring enediyynes have been difficult to implement clinically because of toxicity issues (16), recent advances in pharmacology and pharmaceutics have begun to get around these problems. Modifying the NCS chromophore structure to produce other more efficacious and/or less toxic enediyynes (17), masking the protein component with inert polymers (18), using enediyynes adjunctively with cell-selective activating agents (13), and coupling the newer enediyynes to monoclonal antibodies directed at tumor-specific antigens (19) have all proved useful in producing enediyne candidates for human therapeutics. In fact, the last of these
approaches is now in Phase I/II clinical trials for ovarian carcinoma (L. Hinman, personal communication), and has shown promise in a breast cancer cell line in culture (19). The 4-fold gap in sensitivity to NCS that we observed between bcl-2- and control-transfected PC12 cells (11) has led us to speculate that the enediynes would be both more efficacious and safer in these patients than in those whose tumors do not overexpress bcl-2.

These interesting and promising preliminary results led us to predict that breast cancer cells that overexpress bcl-2 would have a higher GSH content and therefore be more susceptible to the effects of the enediynes than are breast cancer cells that do not overexpress this gene or normal cells. We further predicted that we could augment the difference in enediyne sensitivity between bcl-2-native normal and -overexpressing tumor cells by increasing the availability of cysteine, the rate-limiting substrate for GSH synthesis. Furthermore, we predicted that by increasing the Bcl-2 content of estrogen receptor-positive breast cancer cells with tamoxifen (20) or estrogen (4), we could increase the effectiveness of enediynes against these cells, raising the possibility of adjunctive treatment with enediynes and tamoxifen. This would translate clinically into an improved therapeutic index for treatment of breast cancer with the enediynes.
(6) BODY

(6)-1. Experimental Methods, Assumptions, and Procedures

All of the methods we proposed for use in these studies are currently established in our laboratory using cultured cell systems (11,12,21-29). We describe below the specific hypotheses that were tested and the methods that were used over the two years of Department of Army funding.

(6)-1.1: General Methods Applicable to All Tasks and Technical Objectives

MCF-7 (estrogen receptor-positive) cells were obtained from the American Type Culture Collection (Rockville, MD), and were maintained as stock cultures in α-MEM (GIBCO-BRL) containing phenol red and supplemented with nonessential amino acids, 0.3% glucose, 5% fetal bovine serum, and 2 μg/ml gentamicin sulfate (37°C, 5% CO₂). Where indicated, studies were conducted under four sets of conditions known to alter bcl-2 expression in this cell line in predictable ways: maintenance of the conditions of the stock cultures (E+; bcl-2-positive at “resting” levels; 4,5); addition to the stock culture conditions of 1 nM 17ß-estradiol and maintenance in the estradiol-enriched medium for 48 hr prior to study (E++; 3-fold enhancement of bcl-2 content over resting levels; 5); maintenance for 7 days prior to study in phenol red-free DMEM (GIBCO-BRL) containing 5% fetal bovine serum stripped of steroids by absorption to dextran-coated charcoal (Sigma Chemical Corp., St. Louis, MO) for 45 min at 45°C (E-; 6-fold reduction of bcl-2 levels relative to resting state; 4). In addition, E+ cells were studied after a four-day exposure to tamoxifen (10⁻⁸M; condition T+), a condition the in vivo analogue of which is associated with induction of bcl-2 (20). In all cases, the same lot of fetal bovine serum (GIBCO-BRL) was used for all conditions in each experiment, and to the extent possible, for all experiments.

In addition, clonal transfectants of MCF-7 human breast cancer cells were obtained from Drs. Charles Rudin and Craig Thompson (University of Chicago, Chicago, IL). These transfectants were produced by electroporation (300 mV, 960 mFD) with pSFFV-neo and pSFFV-bcl-2 (plasmids described in ref. 30), respectively.

(6)-1.2: Task 1 [Verification by Western blotting of effects of manipulations of estrogen exposure (i.e., conditions E++, E+, E-, T+) on bcl-2 content of MCF-7 cells]

For all studies, relative bcl-2 content of native MCF-7 cells maintained under each condition was assayed by Western blotting as we have previously described (11). In the case of MCF-7 transfectants, stably transfected clones were screened for Bcl-2 production by Western blot analysis using the N-19 anti-Bcl-2 antibody (Santa Cruz Biotechnology).
To determine the GSH and total glutathione (GSH+GSSG) contents of MCF-7 cells containing different amounts of bcl-2, E+, E++, E-, and T+ cells (10^7 of each) were washed free of medium, and suspended in 1 ml of phosphate-buffered saline (PBS). The suspension was homogenized and assayed for GSSG and GSH+GSSG by the method of Tietze (31) or for GSH by the Thio-Glo method (32). For the Tietze method, the rate of change in the OD_{412} was measured spectrophotometrically over a period of 3 min. This rate was converted to the total glutathione concentration by plotting on a simultaneously run standard curve for ΔOD_{412} versus total glutathione concentration (constructed using GSH standard solutions). The concentration of GSSG alone was determined by an identical procedure performed on samples that were treated with N-ethylmaleimide prior to assay, to eliminate the reaction of GSH with DTNB (31). The cellular contents of GSH and GSH+GSSG were then calculated for each condition. Each determination was performed in triplicate. Values so obtained for each experiment were compared between E+ and E++, E-, or T+ cells using Student’s t test, as we have done in our published work (24,25).

An NCS concentration-response study was conducted for E+, E++, E-, and T+ MCF-7 cells and MCF-7 transfectants, as we have previously described for pheochromocytoma cells (11). Cells plated in 6-well tissue culture plates were treated with a range of concentrations of NCS (0-0.5 μg/ml) for 1 hr at 37°C. Cultures were then washed free of NCS, and adherent cell number was determined daily in control- and NCS-treated E+, E++, E-, and T+, and MCF-7 transfectant cultures as we have previously described for neuroblastoma and pheochromocytoma cells (11,21,33). The statistical significance of differences between E+ and E++, E-, and T+ cells, in turn, was assessed for each concentration of NCS using Student’s t test, with p maximally 0.05 being considered significant.

A manuscript co-authored by Drs. Schor, Kagan and his laboratory colleagues, and Thompson and his laboratory colleagues has been submitted to *Molecular Pharmacology* (34). The BRCA IDEA award from the Department of the Army is acknowledged in this manuscript, and it is appended to this report.

Our finding that the alteration of bcl-2 expression by transfection of MCF-7 cells did not lead to changes in glutathione content led to our decision that completion of these Tasks was not likely to be fruitful. We have only recently overcome initial technical
difficulties examining the glutathione content of E-, E+, and E++ cells (see 1998 Annual Report), and have determined that E2 exposure, with its concomitant increase in Bcl-2, leads to an increase in cellular glutathione content that does not appear to be concentration-dependent (38). We have therefore applied for and been granted a one-year no-cost extension to this grant, so that we may complete these studies in this originally-planned model system.

(6)-1.7: Task 7 (Accomplishment of the loading of the membranes of E, E+, E++ cells with cis-parinaric acid)

The membranes of E-, E+, and E++ MCF-7 cells were loaded with cis-parinaric acid (PnA; Molecular Probes, Eugene, OR) as we have previously described (32), as an in situ probe for the peroxidation of membrane phospholipids. Upon peroxidation, PnA loses its fluorescence, making the fluorescence of each of the membrane phospholipids into which it has been incorporated a measure of the peroxidation of those phospholipids (35).

(6)-1.8: Task 8 (Determination of the susceptibility of E-, E+, and E++ MCF-7 cells to GSH oxidation membrane lipid peroxidation)

Our previous studies have demonstrated that the diazo radical initiator, 2,2'-azobis(2,4-dimethylvaleronitrile) (AMVN; Polysciences, Inc., Warrington, PA) induces the concentration-dependent incidence of apoptosis in PC12 pheochromocytoma cells, and that Bcl-2 abrogates apoptosis induction and membrane phospholipid oxidation in these cells (32). While such oxidation in general may accompany or be etiologic in the induction of and commitment of a cell to apoptosis, the specific oxidation and translocation of phosphatidylserine appear to accompany the enactment phase of the apoptotic process (36,37). The pattern of oxidation of individual phospholipids can therefore suggest the point at which reactive oxygen species and their modification play a role in the incidence and/or abrogation of apoptosis. In order to test the hypothesis that the mechanistic relationship between the antioxidant and anti-apoptosis effects of estradiol depends on the duration of estradiol exposure, we have examined the pattern of oxidation of phospholipids in the membranes of MCF-7 cells by AMVN after the addition of estradiol to the medium immediately or for 14 days preceding AMVN treatment.

In the case of immediate estradiol treatment, the medium was removed and cells were maintained for 24 days in estrogen-free, phenol-free DMEM (Biofluids) supplemented with 5% charcoal-treated FBS (Cocalico Biological, Inc., Reamstown, PA). Estradiol was then added at the time of AMVN treatment. In the case of long-term estradiol treatment, prior to each experiment, the medium was removed and replaced with estrogen-free, phenol-free DMEM. The cells were maintained in this estrogen-free medium for 10 days. Subsequently, this medium was replaced with estrogen-free medium supplemented with varying concentrations of 17ß-estradiol (E2; 0 - 5x10^{-7} M; Sigma, St. Louis, MO), and the cells were maintained in the supplemented medium for an additional 14 days.
PnA served as an *in situ* probe for phospholipid oxidation, as we have previously described (32,35). GSH was measured as detailed above [section (6)-1.3].

(6)-1.9: Task 9 (Preparation of a manuscript describing the findings relative to Tasks 5-8)

A manuscript describing these findings has been accepted for publication in *Biochemical Biophysical Research Communications* (38). The BRCA IDEA award from the Department of the Army is acknowledged in this manuscript, and it is appended to this report.

(6)-1.10: Task 10 (Determination of the effects of *bcl-2*-overexpression in MCF-7 cells on the increase in glutathione afforded by incubation with N-acetylcysteine)

N-acetylcysteine (10 mM) was added to the medium bathing the cells and the cells were then incubated at 37°C for 0.5 - 4 hr. Cells were washed free of extracellular N-acetylcysteine and assayed at various timepoints for total glutathione content by the spectrophotometric method of Tietze (31).

(6)-1.11: Task 11 (Determination of the effects of *bcl-2*-overexpression in MCF-7 cells on the change in sensitivity to NCS afforded by incubation with N-acetylcysteine)

N-acetylcysteine (10 mM) was added to the medium bathing the cells and the cells were then incubated at 37°C for 4 hr. Cells were washed free of extracellular N-acetylcysteine (to ensure that NCS did not get activated by this compound extracellularly), and an adherent cell number concentration-response curve to NCS was constructed for each transfectant as we have done in our previously published studies (11). The ED$_{50}$ for NCS was determined for each transfectant as we have previously done for apoptosis induction in SH-SY5Y cells (12,22). The ED$_{50}$s obtained with and without N-acetylcysteine were compared for each transfectant using Student’s t test, as we have previously described (22).

(6)-1.12: Task 12 (Preparation of a manuscript describing the findings relative to Tasks 10-11)

The results of these studies form an integral part of the abovementioned manuscript submitted to *Molecular Pharmacology* (34).

(6)-2. Results and Discussion

(6)-2.1: Task 1 [Verification by Western blotting of effects of manipulations of estrogen exposure (i.e., conditions E++, E+, E-, T+) on *bcl-2* content of MCF-7 cells]

We have measured the effects of addition to and subtraction from the medium of estradiol to the Bcl-2 content of MCF-7 cells in culture. Our first experiments involved using complete medium as the control, and adding various amounts of estradiol to the
medium, as originally described in the proposed Methods. These studies, all using the same batch of serum for constitution of the medium, demonstrated no change in the Bcl-2 content of the cells. Our assumption was that the serum-replete medium contained sufficient estradiol that neither our addition of small amounts of this compound nor our partial depletion of estradiol with charcoal treatment made a significant change in the Bcl-2 content. We surmounted this problem in two ways. First, we obtained estrogen-free medium commercially stripped (by column chromatography) of estrogen, and performed our studies by adding back various amounts of estradiol. This study conclusively demonstrated the estrogen concentration-dependent increase in concentration of Bcl-2 in the cells (See Figure 1). In addition, we have obtained from the laboratory of Dr. Craig Thompson (University of Chicago, Chicago, IL) two clones of MCF-7 cells that have been transfected with the bcl-2 gene, and their mock-transfected counterparts. We have confirmed overproduction of Bcl-2 in the bcl-2-transfected cells, and have maintained all of these lines in our laboratory.

(6)-2.2: Task 2 [Measurement of concentrations of GSH, GSSG, and total glutathione (GSH+GSSG) in E++, E+, E-, and T+ MCF-7 cells]

In the first year of funding, we measured the total glutathione content of the transfectants obtained from Dr. Thompson's laboratory. Accompanying the increase in Bcl-2 content afforded by the bcl-2 transfection, there was a small but significant increase in glutathione content (see Figure 2).

Recently, we have been able to maintain MCF-7 cells in estrogen-depleted (E-) medium, medium with a physiological estrogen concentration (E+), and media containing each of two estrogen-excess concentrations (E++). With our co-investigators in the laboratory of Dr. Valerian Kagan, we have demonstrated that long-term E2 incubation leads to an increase in total glutathione content of the cells (see Figure 3).

(6)-2.3: Task 3 [Performance of concentration-response studies for E++, E+, E-, and T+ MCF-7 cells and MCF-7 transfectants exposed to neocarzinostatin]

Our previous studies have demonstrated the paradoxical increase in sensitivity of PC12 rat pheochromocytoma cells to NCS afforded by bcl-2 transfection (11). This potentiation of NCS toxicity was abrogated by prior incubation of PC12 cells with BSO. To determine the generalizability of these findings to the overexpression of bcl-2 in different biological systems, similarly transfected MCF-7 human breast cancer cells were examined for the effects of bcl-2 overexpression on sensitivity to NCS. Unlike PC12 cells, bcl-2 overexpression protected MCF-7 cells from death induced by NCS (Figure 4). At each concentration, the survival of the bcl-2 transfectants exceeds that of the mock transfectants.

Attempts to perform the same set of studies in cells exposed to E++, E+, E-, and T+ media, or E- medium and subsequent transfer to graded E concentrations were thwarted by the tendency of estrogen-deprived cells to lift off of the culture surface over the final 5-10 days of the experimental exposure. Although biochemical studies (e.g., glutathione
measurements) can be made on these detached cells, the serial assessment of cell culture
growth is technically not feasible using our current techniques.

(6)-2.4: Task 4 (Preparation of a manuscript describing the findings relative to Tasks 1-4)

A manuscript describing these findings has been submitted to Molecular
Pharmacology (34). This manuscript is co-authored by NF Schor, C Rudin, A-R
Hartmann, CB Thompson, Y Tyurina, and VE Kagan. The support of the Department
of the Army Breast Cancer Program has been acknowledged in this manuscript, and it is
appended to this report.

(6)-2.5: Tasks 5, 6 (Determination of glutathione metabolic enzyme activities and efflux
rate in MCF-7 cells with varying expression of bcl-2)

We plan to perform these studies in the originally described model system in the no-
cost extension year of grant funding (see above).

(6)-2.6: Task 7 (Accomplishment of the loading of the membranes of E, E+, E++ cells
with cis-parinaric acid)

Specific incorporation of PnA into membrane phospholipids of MCF-7 cells is shown
in Table 1. PnA served as an in situ probe for phospholipid oxidation, as we have
previously described (32,35).

(6)-2.7: Task 8 (Determination of the susceptibility of E-, E+, and E++ MCF-7 cells to
GSH oxidation membrane lipid peroxidation)

Treatment of control MCF-7 cells with AMVN (500 μM; 2 h at 37°C) resulted in
statistically significant peroxidation of all four phospholipid species examined (Table 2).
The fraction of membrane phospholipid peroxidized varied slightly from 3.8% [for
phosphatidylethanolamine (PE)] to approximately 2% [for phosphatidylinositol (PI)] of
the native total phospholipid content. Phosphatidylcholine (PC) and phosphatidylserine
(PS) demonstrated intermediate degrees of peroxidation. This oxidation occurred without
sufficient change in the overall phospholipid composition of the membranes to induce
necrotic death of the cells (Table 3).

Immediate E2 exposure of MCF-7 cells resulted in statistically significant protection
from AMVN-induced peroxidation of PI and PS, and a trend towards such protection of
PE and PC (i.e., decreased mean peroxidized fraction after AMVN exposure relative to
non-E2-exposed cells that did not reach statistical significance; Figure 5A). This
wholesale protection of all phospholipids studied from peroxidation suggests a direct
antioxidant role for estrogen in this system.

On the other hand, long-term E2 exposure resulted in the statistically significant
protection of PS and, at the highest E2 concentration only, PE (Figure 5B). This pattern
is suggestive of Bcl-2-mediated protection from enactment of apoptosis (36,37), rather
than direct protection from membrane phospholipid peroxidation per se. Protection of PS in this paradigm was accompanied by a 2-fold decrease in the percentage of the cells demonstrating apoptotic morphology after a 24 h exposure to AMVN (500 μM). Our previous studies demonstrated that apoptotic morphology peaks at 24 h in AMVN-treated PC12 cells (32). Apoptosis was seen in 40 ± 4 (SEM) percent of estrogen-deprived cells exposed to AMVN, and 22 ± 2 percent of long-term E2-treated (5x10^{-7} M) cells similarly exposed (p < 0.01, Student’s t test).

In light of the previous reports of glutathione-dependent (39) and independent (40-42) effects of E2 and Bcl-2 on apoptosis, we examined the effect of immediate and long-term E2 exposure on the glutathione content of MCF-7 cells. We also determined the effect of AMVN on the glutathione content of immediate and long-term E2-exposed cells. As is shown in Figure 3, immediate E2 exposure had no effect on the glutathione content of MCF-7 cells, while long-term exposure resulted in an increase in glutathione levels. At the concentrations of E2 used, this increase was not concentration-dependent. Regardless of the length of E2 exposure, however, AMVN treatment did not result in a decrement in glutathione levels, indicating that despite the increase in glutathione content that accompanies long-term E2 exposure, the protective effects of such exposure are likely glutathione-independent. This is in concert with previous reports of increased glutathione content related to bcl-2 overexpression, and the independence from glutathione of the effects of Bcl-2 on apoptosis (6).

These results indicate that E2 may act as an antioxidant and an antiapoptotic agent for breast cancer cells via both direct and indirect mechanisms. Direct and indirect (i.e., glutathione-mediated) radical scavenging activity of E2 is estrogen receptor-independent (39), but may be enhanced by induction of estrogen metabolism (43). In contrast, bcl-2 induction associated with long-term E2 exposure is estrogen receptor-dependent and does not occur in the presence of specific antiestrogens or in estrogen receptor-negative breast cancer cells (4). The present studies further indicate that this Bcl-2-associated effect is glutathione-independent. The multiplicity of mechanisms by which E2 may augment the survival of breast cancer cells implies that the efficacy of antiestrogens in breast cancer therapy may depend on the degree to which E2-mediated protection is estrogen receptor-dependent in that particular cell or tumor.

(6)-2.8: Task 9 (Preparation of a manuscript describing the findings relative to Tasks 5-8)

A manuscript describing these findings has been accepted for publication in *Biochemical Biophysical Research Communications* (38). The co-authors of this manuscript are Drs. Schor, Kagan, Tyurin, and Tyurina. The BRCA IDEA award from the Department of the Army is acknowledged in this manuscript, and it is appended to this report.
Treatment of mock- and bcl-2-transfected MCF-7 cells with N-acetylcysteine resulted in GSH content kinetics that resembled those seen with mock-transfected PC12 cells (Figure 2). There was no significant difference between the MCF-7 bcl-2- and mock-transfectants in this regard.

In contrast to the case for bcl-2-transfected PC12 cells, previously shown by our group to increase in sensitivity to NCS after incubation with N-acetylcysteine, the concentration-response curve of bcl-2-transfected MCF-7 cells to NCS is unaffected by N-acetylcysteine treatment (Figure 6). The sensitivity to NCS of mock-transfected cells is similarly unaffected by N-acetylcysteine exposure (data not shown). These results are in accord with our finding that bcl-2-transfected MCF-7 cells do not demonstrate altered handling of glutathione.

The results of these studies form an integral part of the abovementioned manuscript submitted to *Molecular Pharmacology* (34).
(7) KEY RESEARCH ACCOMPLISHMENTS

- Determination of the effects of bcl-2 overexpression on the GSH content of MCF-7 breast cancer cells.
- Determination of the effects of bcl-2 overexpression on the susceptibility of MCF-7 breast cancer cells to NCS-induced apoptosis.
- Determination of the mechanistic differences between MCF-7 and PC12 cells that determine the differential response to bcl-2 overexpression vis-à-vis GSH handling and susceptibility to NCS-induced apoptosis.
(8) REPORTABLE OUTCOMES

Manuscripts


Our results indicate that E2 may act as an antioxidant and an antiapoptotic agent for breast cancer cells via both direct and indirect mechanisms. Direct and indirect (i.e., glutathione-mediated) radical scavenging activity of E2 is estrogen receptor-independent (39), but may be enhanced by induction of estrogen metabolism (43). In contrast, \textit{bcl-2} induction associated with long-term E2 exposure is estrogen receptor-dependent and does not occur in the presence of specific antiestrogens or in estrogen receptor-negative breast cancer cells (4). Our studies further indicate that this Bcl-2-associated effect is glutathione-independent. The multiplicity of mechanisms by which E2 may augment the survival of breast cancer cells implies that the efficacy of antiestrogens in breast cancer therapy may depend on the degree to which E2-mediated protection is estrogen receptor-dependent in that particular cell or tumor.

Some studies of the effects of \textit{bcl-2} overexpression on ROS handling in neural cells have suggested a role for Bcl-2 in increasing the tolerance of such cells to oxidant stress (6,7,32). Conversely, some studies have proposed a pro-oxidant role for Bcl-2, and view the enrichment of the intracellular reducing potential as a compensatory, cell-generated phenomenon (44). Still others have pointed out the Bcl-2 overproduction is protective from apoptosis under near-anaerobic conditions (45), and that GSH depletion does not abolish the protective effects of Bcl-2 (43). The present results demonstrate the cell-dependence of the effects of Bcl-2 on GSH metabolism. The impact of Bcl-2 on the reducing potential of the cell is milieu-dependent. This may contribute to the variability of the involvement of ROS and their scavenging in the enactment and prevention of apoptosis.

The determination by the intracellular environment of the redox activity of Bcl-2 and/or the species that are produced or altered as a result of its expression is not unique. The potential for other redox-active agents, including vitamins C and E, to either potentiate or inhibit oxidation is well documented (46).

Our studies of NCS treatment of PC 12 pheochromocytoma and MCF-7 breast cancer cells demonstrate the consequences of this biochemical variability and exemplify the potential therapeutic importance of our findings. We have previously reported that, unlike the case for other chemotherapeutic agents from which cells are protected by Bcl-2 (4,5,47-51), Bcl-2 potentiates the induction of apoptosis by the enediyne NCS (11). From the mechanistic standpoint, the present results suggest increased intracellular activation of NCS in \textit{bcl-2}-overexpressing cells, as evidenced by increased production of the glutathionyl radical. The relatively small concentration ratio of added NCS in the bathing medium to GSH intracellularly may be deceptive, since NCS is actively taken up into cells by endocytosis and may therefore be considerably more concentrated intracellularly than extracellularly. Additional increments in NCS concentration may arise from specific compartmentalization of this compound within cellular organelles (52). In addition, our finding that the rate of turnover of GSH is higher in Bcl-2-overproducing PC12 cells than in native producers exposed to NCS implies that not only the endogenous rate of production of ROS, but also the role of GSH in and ability to
compensate for consumption of reducing equivalents is aberrant in some *bcl-2-* overexpressing cells. This increased GSH turnover, as evidenced by increased formation of the glutathionyl radical, is not related to changes in GSH peroxidase activity, as such changes are not associated with glutathionyl radical formation (53). They are rather related to the non-enzymatic generation of glutathionyl radical via interaction with NCS (54,55). This finding, along with our previous observation that the downstream block in the apoptosis final common pathway produced by Bcl-2 is lifted in NCS-treated cells (37), explains the enhanced apoptotic rate in bcl-2-transfected PC12 cells treated with the reduction-dependent prodrug, NCS. The exploitation by NCS of the effects of Bcl-2 on GSH handling in some cells make NCS a potential chemotherapeutic drug for these chemoresistant tumors. However, the cell-dependent effects of Bcl-2 on GSH handling imply that Bcl-2 content alone could not be used as a criterion for predicting the efficacy of NCS against tumor cells.

That incubation with N-acetylcysteine accentuates the difference in GSH handling between *bcl-2-* and mock-transfected PC12 cells, but not MCF-7 cells, suggests that such incubation would augment the potentiation of apoptosis in *bcl-2-*overexpressing PC12 cells, but not in the analogous MCF-7 cells. Indeed, our results bear this out, suggesting that an in vitro assay of GSH accumulation after N-acetylcysteine incubation might be developed to predict the likely responsiveness of a particular tumor to NCS.
REFERENCES


FIGURE LEGENDS

Figure 1. Western blot for human Bcl-2 performed on Estradiol (E2)-deprived (10 d) MCF-7 Cells after 14 d Replacement with E2 (0 - 5x10^{-7} M). Western blotting was performed. A whole cell homogenate of 10^6 cells was applied to each lane. (A) Photograph of representative lanes of the Western blot depicting bcl-2-transfected PC12 cells as a positive control and MCF-7 cells initially estrogen-deprived and subsequently treated with 5x10^{-7} M E2. The numbers to the left of the gel photograph indicate the running position on the gel of various molecular weight standards. (B) Densitometric vertical scans of each lane of the Western blot stained for Bcl-2.

Figure 2. Effects of bcl-2 transfection on accumulation of GSH in tumor cells continuously incubated with N-acetylcysteine (NACys; 10 mM). The results at t = 0 represent the native GSH content of each of these cell lines. (A) PC12 pheochromocytoma cells. Four independent experiments were performed and gave comparable results. (B) MCF-7 breast cancer cells. The results of single determinations from each of two mock- and two bcl-2-transfected clones of MCF-7 cells are shown. A second independent experiment gave comparable results.

Figure 3. Effect of Immediate and Long-term Exposure to E2 and Treatment with AMVN on Glutathione Content of MCF-7 Breast Cancer Cells. Control, immediate, and long-term E2-exposed MCF-7 cells were incubated in the presence or absence of AMVN (500 μM) for 2 h at 37°C. After incubation, cells were washed twice with PBS, and reduced glutathione was determined by ThioGlo™ assay. Results shown are means of triplicate determinations and error bars signify SEM. Results for long-term E2-exposed cells treated in the absence of AMVN differ significantly from those for the corresponding control and immediate E2-exposed cells with p < 0.0001 (Student’s t test). However, treatment with AMVN did not alter the glutathione content of either immediate or long-term E-2-exposed cells.

Figure 4. Effects of bcl-2 transfection on sensitivity of cultured tumor cells to NCS. In all cases, cells were treated for 1 h with NCS on day 0 and counted daily. Points represent the mean of counts from three separate high power fields expressed as a percent of the count on day 0. Error bars signify the SEM. (A) PC12 pheochromocytoma cells; (B) MCF-7 breast cancer cells.

Figure 5. Effect of Treatment with Estradiol (E2) on Oxidation of cis-Parinaric Acid (PnA)-labeled Phospholipids Induced by AMVN in MCF-7 Breast Cancer Cells. MCF-7 cells were treated with E2 (0 - 5x10^{-7} M) concurrently with (immediate; A) or for 14 days preceding (long-term; B) a 2 h AMVN treatment (500 μM; 37°C). Immediately prior to AMVN treatment, cells were loaded with PnA. Immediate E2 treatment afforded statistically significant protection against oxidation of PI and PS (p < 0.02 relative to AMVN alone at all E2 concentrations; Student’s t test), and a trend towards protection against oxidation of PE at all E2 concentrations. In contrast, long-term E2 treatment
afforded statistically significant protection against oxidation of PS alone (p < 0.03 relative to AMVN alone at 10^{-8} and 5 \times 10^{-7} M E2; Student's t test).

Figure 6. Effect of N-acetylcysteine (10 mM; 4 h) pretreatment on NCS sensitivity of PC12 pheochromocytoma cells (A) and MCF-7 cells (B). NCS treatment (1 h) was performed on sister cultures immediately after washout of N-acetylcysteine (NACys) or vehicle (no NAcCys) from the medium. Results shown represent the mean cell counts of three determinations from one of two independent and comparable experiments. SEMs of the triplicate determinations are plotted but in some cases are too small to be resolved on the plot. Adherent cell counts are shown during mid-log phase growth (day 5 for PC12 cells and day 3 for MCF-7 cells).
Figure 1A

- 106.0 KDa
- 80.0 KDa
- 49.5 KDa
- 32.5 KDa
- 27.5 KDa
- 18.5 KDa

bcl-2-transfected
PO12 Cells (control)

5 x 10^{-7} M E2
10^{-7} M E2
10^{-8} M E2
0 M E2
Figure 1B

bcl-2-transfected PC12 Cells (control)
5x10^{-7} M E2
10^{-7} M E2
10^{-8} M E2
0 M E2
GSH+GSSG (nmoles x 10^-6/cell)
Figure 2B

GSH+GSSG (nmol x 10^-6/cell)

Hours Incubation with NACys
Day 7 Cell Count (% Day 0 Count)

Figure 4A

PC12 Transfectant
+ bcl-2
+ control
Day 7 Cell Count (% Day 0 Count)

Figure 4B

MCF-7 Transfectant

Bel-2.3
Neo.2-control
Bel-2.1
Neo.1-control
Day 5 Adherent Cell Count
(% Day 0 Count)

Figure 6A
Day 3 Adherent Cell Count
(% Day 0 Count)

![Graph showing data points and lines representing different conditions.

Legend:
- NCS
- NCS+NaCys
- NCS+NaCys-Bal-2.3
- NCS+Bal-2.1

Figure 6B

Page 36
TABLE 1: Specific Incorporation of cis-Parinaric Acid (PnA) Into Membrane Phospholipids of MCF-7 Breast Cancer Cells. MCF-7 cells were membrane-loaded with PnA and the steady-state specific incorporation of PnA into the various membrane phospholipids was determined.

<table>
<thead>
<tr>
<th>Phospholipids</th>
<th>Specific Incorporation (mol PnA : mol phospholipid)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphatidylcholine</td>
<td>1 : 4</td>
</tr>
<tr>
<td>Phosphatidylethanolamine</td>
<td>1 : 10</td>
</tr>
<tr>
<td>Phosphatidyserine</td>
<td>1 : 13</td>
</tr>
<tr>
<td>Phosphatidylinositol</td>
<td>1 : 31</td>
</tr>
</tbody>
</table>
TABLE 2: Effect of AMVN on Membrane Phospholipids as Reflected by Fluorescence-detected Levels of cis-Parinaric Acid (PnA). MCF-7 cells were membrane-loaded with PnA and treated for 2 h (37°C) with vehicle or an equivalent volume of AMVN (500 μM). Peroxidation of PnA in the various membrane phospholipids was determined.

<table>
<thead>
<tr>
<th></th>
<th>PI (ngPnA/mg Pi)</th>
<th>PE (ngPnA/mg Pi)</th>
<th>PS (ngPnA/mg Pi)</th>
<th>PC (ngPnA/mg Pi)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>35.5±14.9</td>
<td>242.3±25.6</td>
<td>37.7±6.5</td>
<td>1105.4±117.6</td>
</tr>
<tr>
<td>AMVN</td>
<td>13.9±2.5</td>
<td>149.4±9.5</td>
<td>27.6±3.1</td>
<td>948.6±120.5</td>
</tr>
<tr>
<td>&quot;p&quot; vs. Control</td>
<td>0.006</td>
<td>&lt; 0.001</td>
<td>0.005</td>
<td>0.023</td>
</tr>
</tbody>
</table>
TABLE 3: Effect of AMVN on Membrane Phospholipid Composition of MCF-7 Breast Cancer Cells. MCF-7 cells were incubated in phenol red-free DMEM in the absence or presence of AMVN (500 μM; 2 h; 37°C). Phospholipid composition was determined by HPTLC. All data are expressed as mean ± SEM, and n = 6 for each value. *p < 0.05 vs. control cells (Student's t test).

<table>
<thead>
<tr>
<th>Phospholipid</th>
<th>Percent of Total Phospholipids</th>
<th>Control</th>
<th>AMVN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diphosphatidylglycerol</td>
<td>2.33 ± 0.48</td>
<td>3.07 ± 0.41</td>
<td></td>
</tr>
<tr>
<td>Phosphatidylethanolamine</td>
<td>27.85 ± 0.28</td>
<td>25.61 ± 0.92</td>
<td></td>
</tr>
<tr>
<td>Phosphatidylcholine</td>
<td>47.62 ± 0.24</td>
<td>48.94 ± 0.88</td>
<td></td>
</tr>
<tr>
<td>Phosphatidylserine</td>
<td>5.63 ± 0.15</td>
<td>5.76 ± 0.33</td>
<td></td>
</tr>
<tr>
<td>Phosphatidylinositol</td>
<td>7.89 ± 0.27</td>
<td>6.90 ± 0.37</td>
<td></td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td>8.33 ± 0.03</td>
<td>8.14 ± 0.30</td>
<td></td>
</tr>
<tr>
<td>Lysophosphatidylcholine</td>
<td>0.48 ± 0.11</td>
<td>1.14 ± 0.25*</td>
<td></td>
</tr>
</tbody>
</table>
SELECTIVE OXIDATION AND EXTERNALIZATION OF MEMBRANE PHOSPHATIDYLSERINE: Bcl-2-INDUCED POTENTIATION OF THE FINAL COMMON PATHWAY FOR APOPTOSIS

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ABSTRACT

The induction of apoptosis in PC12 cells by the enediyne neocarzinostatin (NCS) is paradoxically potentiated by overexpression of bcl-2. The enhanced activation of NCS seen in bcl-2-overexpressing cells cannot by itself be responsible for the potentiation of apoptosis, since Bcl-2 would be expected to block apoptosis at a point distal to NCS activation (e.g., in the apoptosis final common pathway). We now report that overexpression of bcl-2 in PC12 cells does not protect the cells from NCS-induced oxidation of membrane phosphatidylserine (PS), and results in potentiation of NCS-induced externalization of membrane PS, two events associated with the apoptosis final common pathway. The mechanism of potentiation of apoptosis by Bcl-2 is related to the enhanced reducing potential of bcl-2-overexpressing PC12 cells.

CLASSIFICATION TERMS
Theme: Development and Regeneration
Topic: Neuronal Death
Key Words: apoptosis; membrane peroxidation; phospholipids; phosphatidylserine
INTRODUCTION

The induction of apoptosis in neural cells has been hypothesized to play a role in the morphological and physiological changes associated with a variety of developmental and pathological states [7]. For many of these, reactive oxygen species (ROS) have been implicated in the disease pathogenesis itself and/or as final common mediators of apoptosis [18]. Similarly, species known to influence the induction and enactment of apoptosis have been proposed as modulators of development and disease, and cellular handling of ROS. Studies of the effects of bcl-2 overexpression on ROS handling in neural cells have suggested a role for Bcl-2 in increasing the tolerance of such cells to oxidant stress [1,5,15]. Similarly, there is mounting evidence that the resistance of neural crest tumor cells to chemotherapeutic agent-induced apoptosis is related to the expression by these cells of bcl-2 [3,4]. The mechanism by which these phenomena occur is not entirely clear, but some studies have indicated that the cellular content of reducing species is increased in neural cells that overexpress bcl-2 [5]. We have previously demonstrated that apoptosis induced in neural crest tumor cells by the enediyne antineoplastic prodrug, neocarzinostatin (NCS), is potentiated by overexpression of bcl-2 [2]. We hypothesized that this paradoxical potentiation of apoptosis is related to increased reductive activation of NCS in bcl-2 overexpressing cells. Other studies have suggested, however, that the antioxidant and anti-apoptosis activities of Bcl-2 are related to its action downstream of the initial activity of chemotherapeutic agents and other apoptosis-inciting factors [9,19]. This would suggest that, even if a chemotherapeutic prodrug were activated to a greater extent in bcl-2-overexpressing cells, Bcl-2 should theoretically block the apoptotic pathway distal to that inciting event (i.e., at a point in the final common apoptosis pathway). We have therefore attempted to resolve this seeming paradox by examining the effects of NCS on changes associated specifically with the common apoptosis pathway in mock- and bcl-2-transfected
PC12 cells. Previous studies in our laboratory have demonstrated the role of peroxidation and externalization of membrane-bound phosphatidylserine (PS) early in the enactment phase of the apoptosis pathway [6]. They have further shown the activity of Bcl-2 in preventing peroxidation of PS and other membrane phospholipids by paraquat or the azo-initiator of lipid peroxyl radicals, 2,2'-azobis(2,4-dimethylvaleronitrile) (AMVN; [6,15]). We now report the effects of Bcl-2 on membrane phospholipid peroxidation and PS externalization in PC12 cells treated with NCS.

**MATERIALS AND METHODS**

**Cell lines and transfectants:** Mock- and *bcl-2*-transfected PC12 cells, both selected as polyclonal populations [5], were the kind gift of Dr. Dale E. Bredesen (Burnham Institute, La Jolla, CA). These cells were maintained as adherent monolayers as we have previously described [2].

**Incorporation of parinaric acid (PnA) into membrane phospholipids:** Membrane phospholipids of both transfectants were labeled with PnA as we have described previously [15]. Briefly, mock- and *bcl-2*-transfected PC12 cells were incubated with a PnA-human serum albumin complex for 2 h at 37°C in L1210 medium. After incubation, lipids were extracted and resolved by HPLC.

**Fluorescent labeling and analysis of external membrane phospholipids:** Derivitization of intact cells with fluorescamine was performed as we have previously described [12]. Two-dimensional HPTLC was then performed on a total lipid extract of $10^6$ logarithmically-growing cells using chloroform:methanol:28% ammonium hydroxide::65:25:5 in the first dimension and chloroform:acetone:methanol:glacial acetic acid:water::50:20:10:10:5 in the second dimension.
**Visualization of externalized PS:** Because of the specific propensity of annexin V to bind to PS only on the extracellular face of the cell membrane, this compound has been used to “stain” cells for the presence of this translocated PS [6,10,11,14,16,17]. Dual staining of mock- and bcl-2-transfected cells with propidium iodide and fluorescein-labeled annexin V [6] was performed at 24 h after completion of a 1 h exposure to NCS.

**Dose-response studies of enediyynes:** Dose-response studies of the enediyynes NCS and C1027 in apoptosis induction in mock- and bcl-2-transfected PC12 cells were performed as we have described previously [2]. In each case and for each drug, the ED$_{50}$ for apoptosis induction was defined and determined as detailed by Hartsell et al. [8].

**RESULTS**

**Membrane lipid peroxidation by NCS in mock- and bcl-2-transfected PC12 cells:** The membranes of mock- and bcl-2-transfected PC12 cells were saturated with PnA as a probe for membrane phospholipid oxidation [12,15]. The time course and pattern of incorporation of PnA into membrane phospholipids did not differ significantly between mock- and bcl-2-transfected PC12 cells (data not shown). Similar to the case for AMVN treatment, a 1 h exposure of mock-transfected PC12 cells to NCS (37°C; 1-5 μg/10⁶ cells) resulted in statistically significant (20-30%) peroxidation of phosphatidylinositol (PI), phosphatidylethanolamine (PEA), phosphatidylcholine (PC), and PS (Fig. 1). However, unlike the case for AMVN treatment, overproduction of Bcl-2 did not protect PC12 cells from PS oxidation induced by exposure to NCS. The selectivity of this phenomenon is demonstrated by the statistically significant protection by Bcl-2 of all other membrane phospholipids studied from NCS-induced peroxidation.
Externalization of PS in mock- and bcl-2-transfected PC12 cells treated with NCS:

Immediately after a 1 h exposure to NCS, both PEA and PS are externalized in an NCS concentration-dependent fashion, as determined by direct fluorescamine staining of external membrane phospholipids (Fig. 2). Externalization of PS is approximately 2-fold greater in bcl-2-transfected than in mock-transfected PC12 cells ($p < 0.01$). In contrast, externalization of PEA did not differ significantly between the transfectants at any NCS concentration.

Neither transfectant exposed to control conditions demonstrated staining with annexin V (Figs. 3a,d), indicating that membrane PS normally faces intracellularly in these cells. Treatment with NCS resulted in bcl-2-transfected cells that stained with annexin V, even at 0.5 $\mu$g/ml, the lowest NCS concentration examined (Figs. 2e,f). In contrast, no mock-transfected cells stained with annexin V at 0.5 $\mu$g/ml NCS (Fig. 3b), and exposure to 2.5 $\mu$g/ml NCS resulted scant staining of an occasional cell (Fig. 3c). These studies suggest that, unlike the case for treatment with AMVN or paraquat, Bcl-2 does not abrogate, but rather potentiates, PS oxidation and externalization in PC12 cells.

Role of reductive activation in potentiation of NCS-induced apoptosis by Bcl-2: From a mechanistic standpoint, potentiation by Bcl-2 of NCS-induced apoptosis ($ED_{50}$: PC12, 0.02 $\mu$M; PC12/Bcl-2, 0.005 $\mu$M) is dependent on the requirement for reductive activation of this prodrug. Mock- and bcl-2-transfected PC12 cells treated with the reduction-independent, autoactivating enediyne, C1027, demonstrated bcl-2-induced protection from, rather than potentiation of apoptosis ($ED_{50}$: PC12, 1x10^{-5} $\mu$M; PC12/Bcl-2, 2x10^{-5} $\mu$M).
DISCUSSION

Recent studies have demonstrated the protective effect of Bcl-2 against apoptosis produced by direct triggering of the final common apoptosis pathway [13,20]. Our previous observation that Bcl-2 overproduction potentiates the induction of apoptosis in PC12 cells by NCS would appear to contradict this notion. If indeed Bcl-2 acts only at a distal common point in the induction of apoptosis, then NCS-induced apoptosis should be abrogated by Bcl-2, even if Bcl-2 directly or indirectly enhances the activation of NCS. The present studies demonstrate potentiation by Bcl-2 of events in the final common apoptosis pathway.

In T lymphocytes, initiation of apoptosis is accompanied by down-regulation of the ATP-dependent amino-phospholipid translocase and activation of a nonspecific phospholipid scramblase [6,10]. Both of these changes result in transfer of PS from the inner to the outer surface of the cell membrane. The effects of Bcl-2 on PS externalization induced by NCS was studied both by direct fluorescamine staining of external membrane phospholipids and annexin V staining of external PS in intact cells. The results of these studies indicate that potentiation by Bcl-2 of NCS-induced apoptosis is accompanied by selective maintenance of vulnerability to oxidation of PS and potentiation of early events in the common pathway for apoptosis.

The potentiation of NCS-induced apoptosis has been hypothesized to be related to increased activation of this enediyne in the highly reducing intracellular milieu of bcl-2-transfected cells [2,5]. The present studies comparing autoactivating and reduction-requiring enediynes definitively demonstrate the role of reductive activation in this model. While it is possible that NCS-induced apoptosis is not susceptible to the regulatory influence of Bcl-2, it is perhaps more likely that increased activation of NCS results in consumption of species critical to the anti-apoptosis effects of Bcl-2, or in the quantitative overriding of the protective activity of Bcl-2. In its most specific application, the
potentiation, by overexpression of bcl-2 in PC12 cells, of NCS activity and PS oxidation and externalization suggests that in vitro screening for such potentiation could be used to identify chemo-resistant tumors that might be responsive to treatment with enediynes. In a more general sense, this potentiation raises the caveat that, in neurodegenerative disorders for which gene therapy with bcl-2 or its relatives has been suggested, such therapy must be carefully assessed in light of other aspects of the pharmacological and physiological environment in which the cells in question sit.
REFERENCES


ACKNOWLEDGEMENTS

This work was presented in preliminary form at the 1998 Child Neurology Society Meeting, Montreal, Canada.

The authors wish to thank Drs. Dale E. Bredesen and Irving Goldberg, and Prof. John Hickman for helpful discussions during the preparation of this paper. This work was funded by grants CA74289 from the National Institutes of Health and DAMD17-97-1-7247 from the Department of the Army, grant ES-09387 from the NIEHS, the International Neurological Science Fellowship Program (F05 NS10669) administered by NIH/NINDS in collaboration with WHO, Unit of Neuroscience, Division of Mental Health and Prevention of Substance Abuse (Y.Y.T.), and the NCI Oncology Research Faculty Development Program (V.A.T.).
FIGURE LEGENDS

Fig. 1. Neocarzinostatin-induced oxidation of cis-parinaric acid-labeled [12,15] phospholipids in mock- and bcl-2-transfected PC12 cells. Each point represents the mean ± SEM of five determinations made in two independent experiments. Abbreviations: PnA, cis-parinaric acid; NCS, neocarzinostatin; PC12, mock-transfected PC12 cells; PC12/bcl-2, bcl-2-transfected PC12 cells; PI, phosphatidylinositol; PEA, phosphatidylethanolamine; PS, phosphatidylserine; PC, phosphatidylcholine. Student’s t test analysis: *p < 0.05 vs. vehicle-treated PC12 cells; **p < 0.05 vs. NCS-treated PC12 cells; ***p < 0.05 vs. vehicle-treated PC12/bcl-2 cells.

Fig. 2. Neocarzinostatin- (NCS-) induced externalization of phosphatidylethanolamine (PEA; panel A) and phosphatidylserine (PS; panel B). Mock- and bcl-2-transfected PC12 cells were incubated with NCS (0 - 4 μg/10⁶ cells) for 1 h at 37°C in the dark in L1210 medium. The cells were then rinsed twice and external phospholipids were derivatized with fluorescamine. The total cell lipids were then extracted and analyzed by two-dimensional HPTLC [12]. Open bars, mock-transfected cells; hatched bars, bcl-2-transfected cells. Data shown represent the mean ± SEM of 5 determinations in 3 independent experiments. *p < 0.05 vs. vehicle-treated cells; **p < 0.01 vs. vehicle-treated cells; *p < 0.01 vs. bcl-2-transfected cells treated with the same concentration of NCS.

Fig. 3. Representative fluorescence micrographs of control and NCS-treated mock- and bcl-2-transfected PC12 cells dual-stained with propidium iodide and fluorescein conjugated annexin V [6]. PC12 cells transfected with bcl-2 (d-f) and mock-transfected PC12 cells (a-c) following 24 h exposure to 0.5 μg/ml (0.05 μM; b,e) or 2.5 μg/ml (0.25 μM; c,f) NCS. Vehicle-treated cells are shown in panels a and d. Each of the panels shown is representative of 5 different high-power fields (400 X).
cis-Parinaric acid - labeled phospholipids, % of control

Figure 1
Figure 2

% Total PEA Derivatized by Fluorescamine

Neocarzinostatin, μg/10^6 cells

Neocarzinostatin, μg/10^6 cells
Figure 3

PC-12

<table>
<thead>
<tr>
<th>Control</th>
<th>PC-12/bcl-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>d</td>
</tr>
<tr>
<td>b</td>
<td>e</td>
</tr>
<tr>
<td>c</td>
<td>f</td>
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</tbody>
</table>

NCS 0.5 µg/ml

NCS 2.5 µg/ml
DIFFERENTIAL MEMBRANE ANTIOXIDANT EFFECTS OF IMMEDIATE AND LONG-TERM ESTRADIOL TREATMENT OF MCF-7 BREAST CANCER CELLS

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ABSTRACT

Previous studies have documented the direct antioxidant effects of estradiol, and it is tempting to ascribe the antiapoptosis effects of estradiol to its scavenging of reactive oxygen species. However, recent reports have also demonstrated that long-term exposure of MCF-7 human breast cancer cells to estradiol results in estrogen receptor- and estradiol dose-dependent overexpression of the antiapoptosis gene, bcl-2. We have used the pattern of protection of membrane phospholipids from oxidation as a probe to separate these direct and indirect effects of estradiol from one another. Immediate exposure to estradiol non-specifically protects all membrane phospholipids from oxidation by the diazo radical initiator, AMVN. This implies the direct antioxidant activity of estradiol in this system. In contrast, long-term exposure, with associated increased expression of bcl-2, protects only phosphatidylserine, the oxidation of which is a critical component of the final common pathway for apoptosis. This bcl-2-mediated indirect effect of estradiol is accompanied by prevention of apoptosis in MCF-7 cells.
Direct, estrogen receptor-independent antioxidant effects have been identified for estrogen in a number of systems (1-3). Synergy with glutathione has been demonstrated in some, leading to the proposal of combined estrogen-antioxidant therapy for neurodegenerative disorders thought to have their origins in the generation of reactive oxygen species (4). This direct effect of estrogen is not exposure time-dependent; that is, it is observed immediately upon addition of estrogen to the system.

Similarly, the antiapoptosis protein, Bcl-2, has been hypothesized to play direct (5) and indirect (6) roles in altering the redox state of the cell. Studies demonstrating an estradiol concentration- and estrogen receptor-dependent induction of the bcl-2 gene in MCF-7 human breast cancer cells (7) have led us to predict that estradiol would also have an indirect antioxidant and antiapoptotic effect on these cells. Unlike its direct effects, the induction of bcl-2 by estradiol requires long-term (i.e., days to weeks) estradiol exposure.

Our previous studies have demonstrated that the diazo radical initiator, 2,2'-azobis(2,4-dimethylvaleronitrile) (AMVN; Polysciences, Inc., Warrington, PA) induces the concentration-dependent incidence of apoptosis in PC12 pheochromocytoma cells, and that Bcl-2 abrogates apoptosis induction and membrane phospholipid oxidation in these cells (8). While such oxidation in general may accompany or be etiologic in the induction of and commitment of a cell to apoptosis, the specific oxidation and translocation of phosphatidylserine appear to accompany the enactment phase of the apoptotic process (9,10). The pattern of oxidation of individual phospholipids can therefore suggest the point at which reactive oxygen species and their modification play a role in the incidence and/or abrogation of apoptosis. In order to test the hypothesis that the mechanistic relationship between the antioxidant and anti-apoptosis effects of estradiol depends on the duration of estradiol exposure, we have examined the pattern of oxidation of phospholipids in the membranes of MCF-7 cells by AMVN after the addition of estradiol to the medium immediately or for 14 days preceding AMVN treatment.
MATERIALS AND METHODS

MCF-7 cells (ATCC, Rockville, MD) were maintained as adherent monolayers in 75mm² tissue culture flasks (Life Technologies, Grand Island, NY) fed twice weekly with α-MEM (Mediatech, Herndon, VA) supplemented with 5% FBS (Atlanta Biologicals, Norcross, GA), 0.3% glucose, 2 mM L-glutamine (Life Technologies, Grand Island, NY), and 2 µg/ml gentamicin sulfate (Biofluids, Rockville, MD). In the case of immediate estradiol treatment, the medium was removed and cells were maintained for 24 days in estrogen-free, phenol-free DMEM (Biofluids) supplemented with 5% charcoal-treated FBS (Cocalico Biological, Inc., Reamstown, PA). Estradiol was then added at the time of AMVN treatment. In the case of long-term estradiol treatment, prior to each experiment, the medium was removed and replaced with estrogen-free, phenol-free DMEM. The cells were maintained in this estrogen-free medium for 10 days. Subsequently, this medium was replaced with estrogen-free medium supplemented with varying concentrations of 17β-estradiol (E2; 0 - 5x10⁻⁷ M; Sigma, St. Louis, MO), and the cells were maintained in the supplemented medium for an additional 14 days.

The membranes of the cells were loaded with cis-parinaric acid (PnA; Molecular Probes, Eugene, OR) as we have previously described (8), as an in situ probe for the peroxidation of membrane phospholipids. Upon peroxidation, PnA loses its fluorescence, making the fluorescence of each of the membrane phospholipids into which it has been incorporated a measure of the peroxidation of those phospholipids (11).

RESULTS AND DISCUSSION

As is shown in Fig. 1, long-term E2 supplementation resulted in a concentration-dependent increase in the Bcl-2 content of the cells. Immediate E2 supplementation did not affect cellular Bcl-2 content (data not shown).
Specific incorporation of PnA into membrane phospholipids of MCF-7 cells is shown in Table 1. PnA served as an in situ probe for phospholipid oxidation, as we have previously described (8,11). Treatment of control MCF-7 cells with AMVN (500 μM; 2 h at 37°C) resulted in statistically significant peroxidation of all four phospholipid species examined (Table 2). The fraction of membrane phospholipid peroxidized varied slightly from 3.8% [for phosphatidylethanolamine (PE)] to approximately 2% [for phosphatidylinositol (PI)] of the native total phospholipid content. Phosphatidylcholine (PC) and phosphatidylserine (PS) demonstrated intermediate degrees of peroxidation. This oxidation occurred without sufficient change in the overall phospholipid composition of the membranes to induce necrotic death of the cells (Table 3).

Immediate E2 exposure of MCF-7 cells resulted in statistically significant protection from AMVN-induced peroxidation of PI and PS, and a trend towards such protection of PE and PC (i.e., decreased mean peroxidized fraction after AMVN exposure relative to non-E2-exposed cells that did not reach statistical significance; Fig. 2A). This wholesale protection of all phospholipids studied from peroxidation suggests a direct antioxidant role for estrogen in this system.

On the other hand, long-term E2 exposure resulted in the statistically significant protection of PS and, at the highest E2 concentration only, PE (Fig. 2B). This pattern is suggestive of Bcl-2-mediated protection from enactment of apoptosis (9,10), rather than direct protection from membrane phospholipid peroxidation per se. Protection of PS in this paradigm was accompanied by a 2-fold decrease in the percentage of the cells demonstrating apoptotic morphology after a 24 h exposure to AMVN (500 μM). Our previous studies demonstrated that apoptotic morphology peaks at 24 h in AMVN-treated PC12 cells (8). Apoptosis was seen in 40 ± 4 (SEM) percent of estrogen-deprived cells exposed to AMVN, and 22 ± 2 percent of long-term E2-treated (5x10^7 M) cells similarly exposed (p < 0.01, Student’s t test).
In light of the previous reports of glutathione-dependent (4) and independent (12-14) effects of E2 and Bcl-2 on apoptosis, we examined the effect of immediate and long-term E2 exposure on the glutathione content of MCF-7 cells. We also determined the effect of AMVN on the glutathione content of immediate and long-term E2-exposed cells. As is shown in Figure 3, immediate E2 exposure had no effect on the glutathione content of MCF-7 cells, while long-term exposure resulted in an increase in glutathione levels. At the concentrations of E2 used, this increase was not concentration-dependent. Regardless of the length of E2 exposure, however, AMVN treatment did not result in a decrement in glutathione levels, indicating that despite the increase in glutathione content that accompanies long-term E2 exposure, the protective effects of such exposure are likely glutathione-independent. This is in concert with previous reports of increased glutathione content related to bcl-2 overexpression, and the independence from glutathione of the effects of Bcl-2 on apoptosis (15).

These results indicate that E2 may act as an antioxidant and an antiapoptotic agent for breast cancer cells via both direct and indirect mechanisms. Direct and indirect (i.e., glutathione-mediated) radical scavenging activity of E2 is estrogen receptor-independent (4), but may be enhanced by induction of estrogen metabolism (1). In contrast, bcl-2 induction associated with long-term E2 exposure is estrogen receptor-dependent and does not occur in the presence of specific antiestrogens or in estrogen receptor-negative breast cancer cells (7). The present studies further indicate that this Bcl-2-associated effect is glutathione-independent. The multiplicity of mechanisms by which E2 may augment the survival of breast cancer cells implies that the efficacy of antiestrogens in breast cancer therapy may depend on the degree to which E2-mediated protection is estrogen receptor-dependent in that particular cell or tumor.
REFERENCES


ACKNOWLEDGEMENTS

The authors thank Karen D. Nylander and Patricia Will for expert technical assistance.
This work was funded by grants CA74289 from the National Institutes of Health and DAMD 17-97-1-7247 from the Department of the Army, by a Fellowship from the International Neurological Science Fellowship Program (F05 NS 10669) administered by NIH/NINDS in collaboration with WHO (YYT), and by a Fellowship from the NCI Oncology Research Faculty Development Program (VAT).
**TABLE 1: Specific Incorporation of cis-Parinaric Acid (PnA) Into Membrane Phospholipids of MCF-7 Breast Cancer Cells.** MCF-7 cells were membrane-loaded with PnA and the steady-state specific incorporation of PnA into the various membrane phospholipids was determined as we have previously described (11).

<table>
<thead>
<tr>
<th>Phospholipids</th>
<th>Specific Incorporation (mol PnA : mol phospholipid)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphatidylcholine</td>
<td>1 : 4</td>
</tr>
<tr>
<td>Phosphatidylethanolamine</td>
<td>1 : 10</td>
</tr>
<tr>
<td>Phosphatidylserine</td>
<td>1 : 13</td>
</tr>
<tr>
<td>Phosphatidylinositol</td>
<td>1 : 31</td>
</tr>
</tbody>
</table>
TABLE 2: Effect of AMVN on Membrane Phospholipids as Reflected by Fluorescence-detected Levels of \textit{cis}-Parinaric Acid (PnA). MCF-7 cells were membrane-loaded with PnA (11) and treated for 2 h (37°C) with vehicle or an equivalent volume of AMVN (500 μM). Peroxidation of PnA in the various membrane phospholipids was determined as we have previously described (11).

<table>
<thead>
<tr>
<th></th>
<th>PI</th>
<th>PE</th>
<th>PS</th>
<th>PC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td>35.5±14.9</td>
<td>242.3±25.6</td>
<td>37.7±6.5</td>
<td>1105.4±117.6</td>
</tr>
<tr>
<td>(n=13)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>AMVN</strong></td>
<td>13.9±2.5</td>
<td>149.4±9.5</td>
<td>27.6±3.1</td>
<td>948.6±120.5</td>
</tr>
<tr>
<td>(n=5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>“p” vs. Control</strong></td>
<td>0.006</td>
<td>&lt; 0.001</td>
<td>0.005</td>
<td>0.023</td>
</tr>
</tbody>
</table>

Peroxidation levels are expressed as ngPnA/mg Pi.
TABLE 3: Effect of AMVN on Membrane Phospholipid Composition of MCF-7 Breast Cancer Cells. MCF-7 cells were incubated in phenol red-free DMEM in the absence or presence of AMVN (500 μM; 2 h; 37°C). Phospholipid composition was determined by HPTLC as we have previously described (8). All data are expressed as mean ± SEM, and n = 6 for each value. *p < 0.05 vs. control cells (Student's t test).

<table>
<thead>
<tr>
<th>Phospholipid</th>
<th>Control</th>
<th>AMVN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diphosphatidylglycerol</td>
<td>2.33 ± 0.48</td>
<td>± 0.41</td>
</tr>
<tr>
<td>Phosphatidylethanolamine</td>
<td>27.85 ± 0.28</td>
<td>25.61 ± 0.92</td>
</tr>
<tr>
<td>Phosphatidylcholine</td>
<td>47.62 ± 0.24</td>
<td>48.94 ± 0.88</td>
</tr>
<tr>
<td>Phosphatidylserine</td>
<td>5.63 ± 0.15</td>
<td>5.76 ± 0.33</td>
</tr>
<tr>
<td>Phosphatidylinositol</td>
<td>7.89 ± 0.27</td>
<td>6.90 ± 0.37</td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td>8.33 ± 0.03</td>
<td>8.14 ± 0.30</td>
</tr>
<tr>
<td>Lysophosphatidylcholine</td>
<td>0.48 ± 0.11</td>
<td>1.14 ± 0.25*</td>
</tr>
</tbody>
</table>
FIGURE LEGENDS

Figure 1. Western blot for human Bcl-2 Performed on Estradiol (E2) -deprived (10 d) MCF-7 Cells After 14 d Replacement with E2 (0 - 5x10^\textsuperscript{-7} M). Western blotting was performed as we have previously described (8). A whole cell homogenate of 10^6 cells was applied to each lane.

Figure 2. Effect of Treatment with Estradiol (E2) on Oxidation of \textit{cis}-Parinaric Acid (PnA) -labeled Phospholipids Induced by AMVN in MCF-7 Breast Cancer Cells. MCF-7 cells were treated with E2 (0 - 5x10^\textsuperscript{-7} M) concurrently with (immediate; A) or for 14 days preceding (long-term; B) a 2 h AMVN treatment (500 \textmu M; 37°C). Immediately prior to AMVN treatment, cells were loaded with PnA as we have previously described (8,11). Immediate E2 treatment afforded statistically significant protection against oxidation of PI and PS (p < 0.02 relative to AMVN alone at all E2 concentrations; Student's t test), and a trend towards protection against oxidation of PE at all E2 concentrations. In contrast, long-term E2 treatment afforded statistically significant protection against oxidation of PS alone (p < 0.03 relative to AMVN alone at 10^8 and 5x10^\textsuperscript{-7} M E2; Student's t test).

Figure 3. Effect of Immediate and Long-term Exposure to E2 and Treatment with AMVN on Glutathione Content of MCF-7 Breast Cancer Cells. Control, immediate, and long-term E2-exposed MCF-7 cells were incubated in the presence or absence of AMVN (500 \textmu M) for 2 h at 37°C. After incubation, cells were washed twice with PBS, and reduced glutathione was determined by ThioGlo™ assay (8). Results shown are means of triplicate determinations and error bars signify SEM. Results for long-term E2-exposed cells treated in the absence of AMVN differ significantly from those for the corresponding control and immediate E2-exposed cells with p < 0.0001 (Student's t test). However, treatment with AMVN did not alter the glutathione content of either immediate or long-term E-2-exposed cells.
Figure 1

- 106.0 KDa
- 80.0 KDa
- 49.5 KDa
- 32.5 KDa
- 27.5 KDa
- 18.5 KDa
Figure 2A

Bar charts showing the percentage of various compounds (P) compared to controls (C) for PHOSPHATIDYLINOSITOL, PHOSPHATIDYLCHOLINE, PHOSPHATIDYLSERINE, and PHOSPHATIDYLETHANOLAMINE. The x-axis represents the percentage of compounds, and the y-axis represents the different compounds.
Figure 3

Glutathione (nmol/mg protein)

Control
- Control
- AMVN

Immediate E2
- $10^{-8}$ M E2
- $10^{-8}$ M E2 + AMVN
- $10^{-7}$ M E2
- $10^{-7}$ M E2 + AMVN
- 5x$10^{-7}$ M E2
- 5x$10^{-7}$ M E2 + AMVN

Long-term E2
- $10^{-8}$ M E2
- $10^{-7}$ M E2
- $10^{-7}$ M E2 + AMVN
- 5x$10^{-7}$ M E2
- 5x$10^{-7}$ M E2 + AMVN
Bcl-2-INDUCED ALTERATION OF GLUTATHIONE HANDLING AND ITS EXPLOITATION IN TARGETED CANCER CHEMOTHERAPY


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RUNNING TITLE: GSH Handling and bcl-2 Overexpression

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24 Text Pages
0 Tables
6 Figures
180 Words/Abstract
188 Words/Introduction
754 Words/Discussion
35 References
Bcl-2 has been associated with both oxidative and antioxidative effects in vivo. Moreover, despite evidence that Bcl-2 is antiapoptotic by virtue of its effect on reactive oxygen species and their scavengers, Bcl-2 exerts its antiapoptotic effects even under anaerobic conditions. The reasons for the variable relationship between Bcl-2 and reactive oxygen species are not clear. The present studies demonstrate that the impact of Bcl-2 on glutathione (GSH) metabolism is cell line-dependent. Bcl-2 overproduction in PC12 cells is associated with increased functional thiol reserves, increased reductive activation of chemotherapeutic prodrugs, and GSH accumulation after treatment with N-acetylcysteine. In contrast, Bcl-2-overproducing MCF-7 breast cancer cells demonstrate neither altered GSH handling nor potentiation of chemotherapeutic prodrug reduction. These findings indicate that the effects of Bcl-2 on GSH handling are milieu-dependent. This could account for the variable effects of Bcl-2 in vivo systems. Furthermore, since our previous studies have demonstrated that reduction-dependent prodrugs may be useful chemotherapeutic agents against tumors that demonstrate altered GSH handling, screening in vitro for alteration of GSH handling may predict responsiveness of such tumors to these reduction-dependent agents.
INTRODUCTION

The induction and enactment of apoptosis have been associated with the generation of reactive oxygen species. In keeping with this association, overproduction of the antiapoptosis protein, Bcl-2, is associated in some systems with increased tolerance to oxidant stress (Kane et al., 1993; Albrecht et al., 1994; Tyurina et al., 1997), an increase in the cellular content of reducing species (Kane et al., 1993), and prevention of the release of cytochrome c from the mitochondrion (Yang et al., 1997; Kluck et al., 1997). However, in contradistinction to these studies, others have reported antiapoptosis activity of Bcl-2 in anaerobic systems (Jacobson and Raff, 1995) and have even ascribed a pro-oxidant function to Bcl-2 (Steinman, 1995).

Glutathione (GSH) is one of several antioxidant species the metabolism of which is thought to be altered by Bcl-2 overproduction. We examine herein the metabolism of GSH in native and bcl-2-transfected cell lines. Our results indicate that the implications of overproduction of Bcl-2 for GSH metabolism depend on the particular cell line involved. We illustrate this cell line-dependence and suggest its potential clinical application in our previously characterized (Cortazzo and Schor, 1996) chemotherapeutic model system.
MATERIALS AND METHODS

Chemicals and transfected cell lines: All chemicals used in enzyme assays were obtained from Sigma (St. Louis, MO). Media for tissue culture were obtained from GIBCO (Grand Island, NY). The enediyne antineoplastic agent, neocarzinostatin (NCS), was purchased as a powder from Kayaku Pharmaceuticals, Inc. (Tokyo, Japan). NCS is a reduction-dependent prodrug for a compound that induces apoptosis in cultured tumor cell lines (Cortazzo and Schor, 1996; Hartsell et al., 1995; Hartsell et al., 1996). Its intracellular activity is GSH-dependent (Cortazzo and Schor, 1996; DeGraff and Mitchell, 1985; DeGraff et al., 1985).

Mock- and bcl-2-transfected PC12 pheochromocytoma cells were prepared and isolated as a polyclonal population as previously published (Kane et al., 1993), and were the kind gift of Dr. Dale E. Bredesen (Burnham Institute, LaJolla, CA). Mock- and bcl-2-transfected MCF-7 human breast cancer cells were generated by electroporation (300 mV, 960 mFD) with pSFFV-neo and pSFFV-bcl-2 (plasmids described in Boise et al., 1993), respectively. Stably transfected MCF-7 clones were screened for Bcl-2 production by Western blot analysis using the N-19 anti-Bcl-2 antibody (Santa Cruz Biotechnology).

Enzyme assays: Mock- and bcl-2-transfected PC12 cells (10⁷ of each) were harvested with trypsin from the tissue culture surface, washed free of medium, and suspended in 1 ml of phosphate-buffered saline. The suspension was homogenized and assayed for γ-glutamylcysteine synthetase (GCS) and glutathione reductase (GR) activities by modifications of the methods of Seelig and Meister (1985) and Carlberg and Mannervik (1985).

In brief, GCS activity was measured by coupling the generation of ADP by the enzyme to the oxidation of NADH by lactate dehydrogenase. Enzyme activity was determined at 37°C in a reaction mixture that contained 150 mM KCl, 5 mM ATP, 2 mM
phosphoenolpyruvate, 10 mM α-aminobutyrate, 10 mM L-glutamate, 20 mM MgCl₂, 2 mm Na₂EDTA, 0.2 mM NADH, 17 µg pyruvate kinase, and 17 µg lactate dehydrogenase in Tris-HCl buffer (0.1 M, pH 8.0). GR activity was measured by following the oxidation of NADPH by GSSG as the optical density of the assay mixture at 340 nm, as described by Carlberg and Mannervik (Carlberg and Mannervik, 1985). Each determination was performed in triplicate in each of two separate experiments. Values obtained for each experiment were compared between transfectants using Student’s t test, with p ≤ 0.05 considered statistically significant.

**Glutathione efflux:** The rate of efflux of glutathione from mock- and bcl-2-transfected cells was measured by determining the rate of decrease in intracellular glutathione concentration after complete inhibition of new glutathione synthesis. Cells were incubated with 1 mM BSO for the duration of the experiment, and total glutathione content of the cells was determined by the method of Tietze (1969) at 0.25, 0.5, 1, 4, 8, 12, and 24 hr after initiation of BSO treatment. Samples were analyzed in triplicate, and the mean ± SEM of each determination was plotted on a time course. The initial slope of the best fit curve for these points was taken as the efflux rate for glutathione from the cell. These slopes were compared between transfectants using Student’s t test.

**Effects of N-acetylcysteine on sensitivity of transfectants to NCS:** N-acetylcysteine (10 mM) was added to the medium bathing the cells and the cells were then incubated at 37°C for 4 hr. Cells were washed free of extracellular N-acetylcysteine (to ensure that NCS did not get activated by this compound extracellularly), and an adherent cell number concentration-response curve to NCS was constructed for each transfectant as we have done in our previously published studies (Cortazzo and Schor, 1996). The ED₅₀ for NCS was determined for each transfectant as we have previously done for apoptosis induction in
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SH-SY5Y cells (Hartsell et al., 1995; Hartsell et al., 1996). The ED_{50} values obtained with and without N-acetylcysteine were compared for each transfectant using Student's t test, as we have previously described (Hartsell et al., 1996).

Effect of NCS on formation of glutathionyl radical: Mock- and bcl-2-transfected PC12 cells treated with varying concentrations of NCS were assayed for formation of the glutathionyl radical as a measure of glutathione turnover using the free radical trapping agent DMPO as we have previously described (Stoyanovsky et al., 1996). Briefly, cells were incubated with different amounts of NCS in the presence of DMPO (100 mM) for 1 hr at 37°C in the dark in medium (115 mM NaCl, 5 mM KCl, 1 mM MgCl_{2}, 5 mM NaH_{2}PO_{4}, 10 mM glucose and 25 mM HEPES, pH 7.4). After incubation, cells were washed twice with medium, and GS-DMPO nitron was determined by HPLC.

Effect of NCS on GSH and protein thiol content: Total protein sulfhydryl and GSH concentration in cells were determined using ThioGlo™ 1, a maleimide reagent that produces a highly fluorescent product upon its reaction with sulfhydryl groups (Langmuir et al., 1996). A standard curve was established by addition of GSH (0.04 - 4.0 μM) to 0.1 M phosphate buffer, pH 7.4, containing 200 μM ThioGlo™ 1 (from a stock solution in DMSO). GSH content was determined by an immediate fluorescence response registered upon addition of ThioGlo™ 1 to the cell homogenate. Total protein sulfhydryls were measured as an additional fluorescence response after addition of SDS (1.0 mM) to the same cell homogenate. A Shimadzu spectrofluorophotometer RF-5301PC was employed for these determinations using an excitation slit of 1.5 nm and an emission slit of 5 nm. The wavelengths employed in the assay were 388 nm (excitation) and 500 nm (emission). The data acquired were exported from the spectrofluorophotometer using RF-5301PC Personal Fluorescence Software (Shimadzu).
Effects of bcl-2 transfection on accumulation of glutathione in N-acetylcysteine-treated PC12 cells: N-acetylcysteine (10 mM) was added to the medium bathing the cells and the cells were then incubated at 37°C for 0.5 - 4 hr. Cells were washed free of extracellular N-acetylcysteine and assayed at various timepoints for total glutathione content by the spectrophotometric method of Tietze (1969).
RESULTS

Effects of bcl-2 transfection on glutathione accumulation in N-acetylcysteine-treated PC12 and MCF-7 cells: Incubation of mock-transfected PC12 cells with N-acetylcysteine (10 mM) for up to 8 hr results in a 3-fold increase in intracellular GSH that is apparent by 2 hr and plateaus until at least 4 hr of incubation (Fig. 1A). In contrast, similar incubation of bcl-2-transfected PC12 cells results in a GSH level 5-times baseline by 4 hr of treatment. Note that in these cultures that had not been fed for four days at t = 0, there was no significant difference in GSH content between mock- and bcl-2-transfected PC12 cells. Without additional N-acetylcysteine supplementation, GSH content of the bcl-2-transfected cells declines and the difference in GSH content between the two transfectants loses statistical significance by 12 hr after addition of N-acetylcysteine to the medium (data not shown).

Analogous treatment of mock- and bcl-2-transfected MCF-7 cells with N-acetylcysteine resulted in GSH content kinetics that resembled those seen with mock-transfected PC12 cells (Fig. 1B). There was no significant difference between the MCF-7 bcl-2- and mock-transfectants in this regard.

Effect of bcl-2 transfection on GSH turnover in response to NCS treatment: Transfection of PC12 cells with bcl-2 results in GSH accumulation during incubation with N-acetylcysteine. Furthermore, the GSH content of bcl-2-transfected PC12 cells is augmented relative to native cells even in the absence of a supplemental cysteine source (Kane et al., 1993). This elevation of native GSH content and N-acetylcysteine-induced GSH accumulation could result from augmented GSH cycling in response to oxidative consumption. As such, we have examined the effects of bcl-2 transfection on the ability of PC12 cells to exchange and recycle GSH reducing equivalents (so-called "functional thiol reserves"; Hubel et al., 1997) in response to treatment with NCS, a known "consumer" of
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GSH. We have done this by measuring the generation of glutathionyl radical (a mirror of oxidative GSH consumption) over a 1 hr incubation with NCS, and the cellular GSH content after such an incubation.

Treatment of bcl-2-transfected PC12 cells with NCS (0.2-0.6 nmol/10⁶ cells) results in the trapping of 4 nmol/mg protein of glutathionyl radical over 1 hr (Fig. 2A). In a parallel study, analogous treatment of sister cultures results in a much smaller (< 2 nmol/mg protein) drop in the level of cellular GSH. In sharp contrast, treatment of mock-transfected PC12 cells with NCS results in no significant change in the cumulative generation of glutathionyl radical or the cellular content of GSH (Fig. 2B).

Parallel studies of the effect of NCS treatment on cellular protein thiol content revealed no significant change in either PC12 transfectant (data not shown).

GSH-related enzyme activity and resting GSH efflux in mock- and bcl-2-transfected PC12 cells: In an effort to determine the mechanism of GSH accumulation and increased functional GSH reserves in bcl-2-transfected PC12 cells, we have measured the activities of GR and GCS in mock- and bcl-2-transfected PC12 cells both before and after 4 hr treatment with 10 mM N-acetylcysteine. As is shown in Fig. 3, there was no difference between the transfectants with respect to GCS activity either in the native state or after incubation with N-acetylcysteine. Furthermore, the observed lower GR activity in bcl-2-transfected PC12 cells than in mock-transfected cells is counter to the predicted increase in GR activity hypothesized to result in increased intracellular GSH levels after bcl-2 transfection.

The rate of efflux of GSH from native mock- and bcl-2-transfected PC12 cells was approximated by treatment of the cells with BSO (1 mM; to block new GSH synthesis) followed by timed measurement of total GSH per cell. There was no significant difference in the rate of decrease in the total cellular GSH content of the two transfectants after BSO treatment (Fig. 4).
Effects of bcl-2 transfection on the sensitivity of PC12 and MCF-7 cells to NCS: Our previous studies have demonstrated the paradoxical increase in sensitivity of PC12 rat pheochromocytoma cells to NCS afforded by bcl-2 transfection (Cortazzo and Schor, 1996; Fig. 5A). This potentiation of NCS toxicity was abrogated by prior incubation of PC12 cells with BSO. The difference in effect of bcl-2 transfection on GSH handling in PC12 and MCF-7 cells led us to predict that, in MCF-7 cells, the effects of bcl-2 transfection on apoptosis induced by reduction-dependent drugs like NCS would not differ from its effects on apoptosis induced by non-reduction-dependent drugs. That is, we predicted that bcl-2 transfection would prevent apoptosis in MCF-7 cells treated with NCS.

As predicted, unlike PC12 cells, bcl-2 overexpression protected MCF-7 cells from death induced by NCS (Fig. 5B). At each concentration, the survival of the bcl-2 transfectants exceeds that of the mock transfectants. Note also that mock-transfected MCF-7 cells are 10-fold more sensitive than mock-transfected PC12 cells to the effects of NCS.

Effect of bcl-2-transfection on potentiation of NCS-induced apoptosis by incubation with N-acetylcysteine: In light of the accumulation of GSH seen only in bcl-2-transfected PC12 cells after incubation with N-acetylcysteine, and the concentration-dependent role of GSH in activation of NCS (DeGraff and Mitchell, 1985; DeGraff et al., 1985; Beerman et al., 1977; Schor, 1992), we hypothesized that pretreatment of bcl-2-transfected PC12 cells with N-acetylcysteine would result in further potentiation of NCS-induced apoptosis, while the response of mock-transfected PC12 cells to NCS would remain unaltered. Fig. 6A demonstrates the shift in the concentration-response curve of bcl-2-transfected PC12 cells to NCS after a 4 hr incubation with N-acetylcysteine. In contrast, the concentration-response curve of bcl-2-transfected MCF-7 cells to NCS is unaffected by N-acetylcysteine treatment (Fig. 6B). The sensitivity to NCS of mock-transfected cells of both types is unaffected by N-acetylcysteine exposure (data not shown).
DISCUSSION

The induction of apoptosis in neural cells has been hypothesized to play a role in the morphological and physiological changes associated with a variety of developmental and pathological states (Narayanan, 1997; Gelbard et al., 1997). For many of these, reactive oxygen species (ROS) have been implicated in the disease pathogenesis itself and/or as final common mediators of apoptosis (Wood and Youle, 1994; Wood and Youle, 1995). Similarly, species known to influence the induction and enactment of apoptosis have been proposed as modulators of development and disease and cellular handling of ROS.

Some studies of the effects of bcl-2 overexpression on ROS handling in neural cells have suggested a role for Bcl-2 in increasing the tolerance of such cells to oxidant stress (Kane et al., 1993; Albrecht et al., 1994; Tyurina et al., 1997). Conversely, some studies have proposed a pro-oxidant role for Bcl-2, and view the enrichment of the intracellular reducing potential as a compensatory, cell-generated phenomenon (Steinman, 1995). Still others have pointed out the Bcl-2 overproduction is protective from apoptosis under near-anaerobic conditions (Jacobson and Raff, 1995), and that GSH depletion does not abolish the protective effects of Bcl-2 (Kane et al., 1993). The present results demonstrate the cell-dependence of the effects of Bcl-2 on GSH metabolism. The impact of Bcl-2 on the reducing potential of the cell is milieu-dependent. This may contribute to the variability of the involvement of ROS and their scavenging in the enactment and prevention of apoptosis.

The determination by the intracellular environment of the redox activity of Bcl-2 and/or the species that are produced or altered as a result of its expression is not unique. The potential for other redox-active agents, including vitamins C and E, to either potentiate or inhibit oxidation is well documented (Halliwell and Gutteridge, 1985).

Our studies of NCS treatment of PC12 pheochromocytoma and MCF-7 breast cancer cells demonstrate the consequences of this biochemical variability and exemplify the potential therapeutic importance of our findings. We have previously reported that, unlike
the case for other chemotherapeutic agents from which cells are protected by Bcl-2 (Dole et al., 1994; Dole et al., 1995; Teixeira et al., 1995; Bonetti et al., 1996; Beham et al., 1996; Campos et al., 1993; Reber et al., 1998), Bcl-2 potentiates the induction of apoptosis by the enediyne NCS (Cortazzo and Schor, 1996). From the mechanistic standpoint, the present results suggest increased intracellular activation of NCS in bcl-2-overexpressing cells, as evidenced by increased production of the glutathionyl radical. The relatively small concentration ratio of added NCS in the bathing medium to GSH intracellularly may be deceptive, since NCS is actively taken up into cells by endocytosis and may therefore be considerably more concentrated intracellularly than extracellularly. Additional increments in NCS concentration may arise from specific compartmentalization of this compound within cellular organelles (Maeda, 1984). In addition, our finding that the rate of turnover of GSH is higher in Bcl-2-overproducing PC12 cells than in native producers exposed to NCS implies that not only the endogenous rate of production of ROS, but also the role of GSH in and ability to compensate for consumption of reducing equivalents is aberrant in some bcl-2-overexpressing cells. This increased GSH turnover, as evidenced by increased formation of the glutathionyl radical, is not related to changes in GSH peroxidase activity, as such changes are not associated with glutathionyl radical formation (Stoyanovsky et al., 1996). They are rather related to the non-enzymatic generation of glutathionyl radical via interaction with NCS (Stoyanovsky et al., 1996; Chin et al., 1988). This finding, along with our previous observation that the downstream block in the apoptosis final common pathway produced by Bcl-2 is lifted in NCS-treated cells (Schor et al., 1999), explains the enhanced apoptotic rate in bcl-2-transfected PC12 cells treated with the reduction-dependent prodrug, NCS. The exploitation by NCS of the effects of Bcl-2 on GSH handling in some cells make NCS a potential chemotherapeutic drug for these chemoresistant tumors. However, the cell-dependent effects of Bcl-2 on GSH handling imply that Bcl-2 content alone could not be used as a criterion for predicting the efficacy of NCS against tumor cells.
That incubation with N-acetylcysteine accentuates the difference in GSH handling between bcl-2- and mock-transfected PC12 cells, but not MCF-7 cells, suggests that such incubation would augment the potentiation of apoptosis in bcl-2-overexpressing PC12 cells, but not in the analogous MCF-7 cells. Indeed, our results bear this out, suggesting that an in vitro assay of GSH accumulation after N-acetylcysteine incubation might be developed to predict the likely responsiveness of a particular tumor to NCS.
ACKNOWLEDGEMENTS

The authors wish to acknowledge the expert assistance of Karen D. Nylander.
REFERENCES


Schor, NF et al.


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This work was funded by grants CA74289 from the National Institutes of Health and DAMD17-97-1-7247 from the Department of the Army. Y.Y.T. was partly supported by the International Neurological Science Fellowship Program # F05 NS 10669 administered by NIH/NINDS in collaboration with WHO, Unit of Neuroscience, Division of Mental Health and Prevention of Substance Abuse.

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FIGURE LEGENDS

Fig. 1: Effects of bcl-2 transfection on accumulation of GSH in tumor cells continuously incubated with N-acetylcysteine (NACys; 10 mM). GSH was measured for the results depicted using the method of Tietze (1969). Independently-obtained samples assayed using the Thio-Glo™ method (Langmuir et al., 1996) gave analogous results. (A) PC12 pheochromocytoma cells. Four independent experiments were performed and gave comparable results. The results of duplicate determinations from one of these experiments are shown. (B) MCF-7 breast cancer cells. The results of single determinations from each of two mock- (Neo.1-mock and Neo.2-mock) and two bcl-2-transfected (Bcl-2.1 and Bcl-2.2) clones of MCF-7 cells are shown. A second independent experiment gave comparable results.

Fig. 2: Effect of NCS on the formation of GS-DMPO nitrone in mock- (B) and bcl-2- (A) transfected PC12 pheochromocytoma cells. Cells were incubated with different amounts of NCS in the presence of DMPO (100 mM) for 1 hr at 37°C in the dark in medium. After incubation, cells were washed twice with medium, and GS-DMPO nitrone and GSH content were determined as described in Experimental Procedures. Results shown are means ± SEM of three independent experiments.

Fig. 3: Activity of GR and GCS in native and N-acetylcysteine-treated (10 mM; 4 hr; 37°C) mock- and bcl-2-transfected PC12 pheochromocytoma cells. Results shown are means ± SEM from triplicate determinations of the change in OD measured in each of the assays (Seelig and Meister, 1985; Carlburg and Mannervik, 1985) on homogenates from 10^7 cells.
Fig. 4: Effect of bcl-2 transfection on the native efflux rate of GSH from PC12 pheochromocytoma cells. Mock- and bcl-2-transfected PC12 cells were continuously treated with BSO (1 mM) and GSH was measured at various times after BSO addition. Data shown represent the means ± SEM from triplicate determinations.

Fig. 5: Effects of bcl-2 transfection on sensitivity of cultured tumor cells to NCS. In all cases, cells were treated for 1 hr with NCS (37°C) on day 0 and counted daily as described previously (Cortazzo and Schor, 1996). Points represent the mean of counts from three separate high power fields expressed as a percent of the count on day 0. Error bars signify the SEM. (A) PC12 pheochromocytoma cells; (B) MCF-7 breast cancer cells. Transfection and selection of these lines is described in Experimental Procedures.

Fig. 6: Effect of N-acetylcysteine (10 mM; 37°C; 4 hr) pretreatment on NCS sensitivity of PC12 pheochromocytoma cells (A) and MCF-7 cells (B). NCS treatment (1 hr; 37°C) was performed on sister cultures immediately after washout of N-acetylcysteine (NAcCys) or vehicle (no NAcCys) from the medium. Results shown represent the mean cell counts of three determinations from one of two independent and comparable experiments. SEMs of the triplicate determinations are plotted but in some cases are too small to be resolved on the plot. Adherent cell counts are shown during mid-log-phase growth (day 5 for PC12 cells and day 3 for MCF-7 cells).
Figure 1A

![Graph showing GSH+GSSG (nmoles x 10^-6/cell) vs Hours Incubation with NAcCys. The graph compares Mock-Transfected and bcl-2-Transfected conditions.](image-url)
Figure 1B

GSH+GSSG (nmol x 10^-6/cell)

Hours Incubation with NAcCys
Figure 2A

*bcl-2-TRANSFECTED PC12 CELLS*

![Graph showing the relationship between [NCS] (nmol/10^6 cells) and nmol/mg protein for GS-DMPO Nitrone and Glutathione.](image-url)
Figure 2B
MOCK-TRANSFECTED PC12 CELLS

[nmol/mg protein]

[NCS] (nmol/10^6 cells)

GS-DMPO Nitrone
Glutathione
Figure 3

[Bar graph showing the relative activity of Glutathione Reductase and γ-Glutamylcysteine Synthetase in mock-transfected and bcl-2-transfected cells. The x-axis represents pre- and post-treatment samples, and the y-axis represents relative activity per cell.]

- Mock-transfected
- bcl-2-transfected
Figure 4

Relative Glutathione/Cell

Mock-transfected
bcl-2-transfected

\[ y = 99.468 \times 10^{-0.080x} \quad r^2 = 0.983 \]

\[ y = 93.409 \times 10^{-0.073x} \quad r^2 = 0.911 \]

Hours Incubation with BSO (1 mM)
Figure 5B

Day 7 Cell Count ( % Day 0 Count)
REFERENCES


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INDEX TERMS

apoptosis
Bcl-2
glutathione
neocarzinostatin
thiols