Expression of BCL-2 Protein Enhances the Survival of Mouse Fibrosarcoal Cells in Tumor Necrosis Factor-mediated Cytotoxicity

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ABSTRACT

Tumor necrosis factor (TNF) kills some types of tumor cells in vitro and participates in tumor elimination in vivo. TNF has been shown to kill cells by altering their mitochondria structurally and functionally. The oncogene BCL-2 codes for a protein located in the inner membrane of mitochondria which is able to inhibit the commitment to cell death in various cell types. We have therefore investigated whether TNF-mediated killing of the cell line L929 could be modulated by expression of the protein BCL-2. We report here that L929 cells transfected with a BCL-2 expression vector have an increased survival compared to wild type cells after TNF challenge. The protective effect is greatest at moderate TNF concentrations and is still significant at concentrations that killed 100% of wild type cells. The action of BCL-2 is selective inasmuch as cells are not protected against other cytotoxic agents blocking various mitochondrial functions. We show that cells expressing BCL-2 have a higher mitochondrial membrane potential (ΔΨ) than wild type cells. The increase in ΔΨ could be linked with the enhanced survival of cells after TNF challenge. Indeed, we found that treatment of wild type L929 cells with the ionophore nigericin, which increases ΔΨ, protects them even at high TNF concentrations.

INTRODUCTION

In human follicular B-cell lymphoma the BCL-2 gene is translocated from chromosome 18 to chromosome 14 and its expression participates in overexpression of the oncogene (1, 2). The BCL-2 gene codes for a M, 26,000 membrane protein with no similarity to any other known protein (3). Its localization in the inner membrane of mitochondria suggests that it could influence some functions of this organelle (4). Beside its mitochondrial location, BCL-2 has the unique property for an oncogene to enhance cell survival rather than to promote cell proliferation (5) as demonstrated by its protective effect against apoptotic cell death (6). However, the precise function of BCL-2 within mitochondria has not been elucidated. BCL-2 is of physiological importance in the establishment of B-cell memory (7), and its pattern of expression in different tissues suggests that it could be involved in the life span control of various cell types (8, 9).

We were interested whether the expression of certain rescue proteins can protect cells against immune effector mechanisms. This could be a way used by some tumor cells to escape immunosurveillance. In a first attempt, we focused our attention on the role of BCL-2 expression in tumor cells challenged by the cytotoxic cytokine TNF in vitro.

The mechanism of TNF-mediated cell death varies according to the target cells, some dying by necrosis and others by apoptosis (10). Oxidative stress (11, 12), induction of endogenous nucleases (13), and depletion of ATP/NADH stores (14) have been proposed to contribute to cell killing. Further, some groups have observed that cytotoxicity is related to structural and functional alterations of mitochondria in cells treated with TNF (15-17). We have shown that mitochondria of TNF-treated L929 cells generate increased amounts of O2− (18). Mitochondria are the first cellular target to show damage, and the loss of mitochondrial integrity leads to cell death apparently because of a depletion of energy stores.

Since the BCL-2 protein is located within mitochondria, we investigated whether it can protect TNF-treated cells by preventing mitochondrial damage. To this end, we concentrated on the mouse fibrosarcoal L929 cell, one of the most thoroughly studied targets of TNF cytotoxicity. We report that expression of BCL-2 in L929 cells enhances the resistance of these cells to TNF-mediated cytotoxicity. Protection is specific inasmuch as cells are still sensitive to an array of other cytotoxic agents. In addition, we show that the effect of BCL-2 is not due to a change in the expression of TNF receptors or to a decreased ability to produce O2− after TNF addition. Since BCL-2 is located in mitochondria, we also investigated the mitochondrial electron transport system in BCL-2-transfected cells. While the activity of the mitochondrial respiratory chain remains unchanged, we observed a higher ΔΨ in cells expressing BCL-2. This parameter could be linked to the TNF resistance inasmuch as we found that the ionophore nigericin, known to increase ΔΨ, protects the cells against killing by TNF.

MATERIALS AND METHODS

Materials. Recombinant mouse TNFα was purchased from Genzyme (Boston, MA). The activity was 4 x 10⁷ units/mg protein. The mouse BCL-2 cDNA was a generous gift of S. J. Korsmeyer (Howard Hughes Medical Institute, St. Louis, MO). W. Lesslauer (Hoffmann LaRoche Ltd., Basel, Switzerland) kindly provided the cDNA of mouse TNF receptors. The expression vector BCMGSNeo was obtained from F. Melchers (Basel Institute for Immunology, Basel, Switzerland) (19). Unless indicated the chemicals were from Sigma Chemical Co. (St. Louis, MO).

Cell Culture. The mouse fibrosarcoal L929 cell line was obtained from the American Type Culture Collection (Rockville, MD). The cells were subcloned by means of a cloning ring and the derived subline (WT) was used for all further experiments. The cells were cultured in Dulbecco's modified Eagle's medium (Gibco, Paisley, United Kingdom), supplemented with 2 mM glutamine, 10 μg 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4), penicillin (100 units/ml), streptomycin (100 μg/ml), and 7% heat-inactivated fetal calf serum.

Plasmid Construct. All enzymes and buffers were from Boehringer Mannheim (Mannheim, Germany). The BCL-2 gene was excised from the pN2-M-Bcl-2 vector with HindIII and EcoRI (5) and ligated into the polylinker of the Bluescript plasmid at the HindIII and EcoRI sites (Stratagene, La Jolla, CA). The resulting construct was then digested with Xhol and NotI and the 926-base pair BCL-2 fragment was inserted into the corresponding sites of the BCMG-SNeo vector.

Electroporation and Selection of G-418-resistant Cells. Cells were harvested after trypanosinization, washed twice with ice cold phosphate-buffered saline and resuspended at 1 x 10⁶ cells/ml. Plasmid DNA (25 μg) was added to the cells which were electroporated after 10 min incubation on ice using a Gene Pulser apparatus (Bio-Rad, Richmond, CA) set at 1000 V, 25 μF. Cells were then cultured in Dulbecco's modified Eagle's medium-7% fetal calf serum. The antibiotic G-418 (Gibco, Madison, WI) was added at 0.5 mg/ml to the culture medium after 48 h. Batches of 10,000 cells were transferred to
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culture dishes (Falcon; Becton Dickinson, Oxnard, CA) and cell clones were isolated with cloning rings after 15 days of culture.

RNA Analysis. Total cell RNA was purified according to the method of Chomczynski and Sacchi (20) using a kit (Promega, Madison, WI). RNA (5 μg) was dissolved in 50% formamide/6.6% formaldehyde, separated by electrophoresis in a 1.5% agarose/6.6% formaldehyde gel, and then transferred to Hybond-C extra membranes (Amersham, Amersham, United Kingdom) following standard procedures (21). Blots were hybridized using the following probes: HindIII/EcoRI BCL-2 fragment (5); EcoRI/EcoRI TNF-R55 fragment; BamHI/EcoRI TNF-R75 fragment (22). After removal of the primary probes by boiling, the membranes were rehybridized with a chicken β-actin probe.

TNF Cytotoxicity Assay. Cells were incubated with TNF and actinomycin D (1 μg/ml) at 37°C in Iscove's modified Dulbecco's medium (Gibco) supplemented with 0.2% Bacto-peptone (Difco, Detroit, MI), glutamine (2 mM), pyruvate (1 mM), penicillin (100 units/ml), streptomycin (100 μg/ml), nonessential amino acids (Gibco), minimal essential medium-vitamin solution (Gibco), and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4). Cell viability was measured by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide cleavage assay as described by Mosmann (23). The percentage of cytotoxicity was defined as the ratio

\[ \frac{A_i - A_b}{A_i} \times 100 \]

where \( A_i \) is the absorbance of 100% viable cell control minus background and \( A_b \) is the absorbance of the tested sample minus background (100% cytotoxicity). The clone WT expressed the same TNF sensitivity as the uncloned L929 cell population.

Membrane Potential. ΔΨ was analyzed by flow cytometry using a FACScan (Becton Dickinson) equipped with the software Lysys II. Fluorescence was recorded after 30 min staining of cells in suspension with rhodamine 123 at 0.2 μg/ml (24, 25). The amount of mitochondria within cells was evaluated by staining cells with 0.2 μM NAO (Molecular Probes, Eugene, OR) for 30 min as described (26).

Mitochondrial Enzyme Assays. Oxygen consumption was measured using a Clark-type electrode (YSI model 5331). Cells were set to a density of 6 × 10^6 in 25 ml Tris-HCl (pH 7.4)-0.25 mM sucrose-2 mM EDTA-5 mM MgCl_2-10 mM K_2HPO_4 and incubated under constant stirring at 37°C. Oxygen disappearance was monitored after consecutive addition of (final concentrations): digitonin (0.2 mg/10^7 cells); rotenone (0.2 μM); succinate (5 mM); antimycin A (0.2 μM) and N,N,N’,N’-tetramethyl-ρ-phenylenediamine/ascorbate (0.8 mM/4 mM). The activity of the mitochondrial respiratory chain downstream of NADH dehydrogenase (complex I) was defined as the rate of oxygen uptake after addition of the substrate succinate. The rate of oxygen uptake in the presence of the electron donors N,N,N’,N’-tetramethyl-ρ-phenylenediamine/ascorbate was used as a measure of cytochrome c oxidase activity (complex IV) (27). Respiratory activity was expressed in ng atoms oxygen/min/10^6 cells.

RESULTS

We cloned the mouse BCL-2 gene into the Xho/NotI site of the BCMGSNeo vector (Fig. 1). This plasmid replicates in transfected cells as an episome at elevated copy numbers (19). The panactive promoter cytomegalovirus that regulated BCL-2 expression ensured a high degree of transcription (28). We found elevated BCL-2 mRNA levels in most G-418-resistant L929 clones screened while no mRNA was detected in WT cells and in cells transfected with the BCMGSNeo vector alone (Fig. 2A). We selected for further investigations three clones, B11, B22, and B28, with high BCL-2 mRNA levels, and one clone, B38, with low BCL-2 mRNA level. These clones were compared to the original WT clone and to a clone (V3) transfected with the BCMGSNeo vector alone. As an essential control for TNF cytotoxicity assays, we compared the expression of TNF receptors in BCL-2- and mock-transfected cells. Fig. 2, B and C, shows that transfection of L929 cells had no effect on the expression of TNF-R55 and TNF-R75 as assayed in Northern blotting. We rehybridized the filters with a probe for β-actin to standardize our results (Fig. 2D).

TNF kills L929 cells in a dose-dependent manner. The cytotoxic effect can be further enhanced by inhibiting protein synthesis in target cells (29). We observed that BCL-2 expression protected L929 cells from TNF cytotoxicity (Fig. 3) in both the presence and absence of the transcriptional inhibitor actinomycin D. The protective effect was most pronounced at concentrations that killed about 50% of WT cells and was still significant at the highest concentrations tested (1000 pg/ml and 20 ng/ml). In the presence of actinomycin D, the survival of cell clones treated with TNF correlated with the extent of BCL-2 expression (Fig. 3A). In contrast, all clones transfected with BCL-2 had a similarly increased resistance when challenged with TNF in the absence of actinomycin D (Fig. 3B). Additional experiments showed that the enhanced survival of BCL-2 expressing cells treated with TNF...
change was observed in the rate of succinate-dependent oxygen consumption, which reflects the activity of complexes II, III, and IV. In all clones we found rates of oxygen uptake ranging from 2.5 to 3.3 ng atoms O/min/10^6 cells (results not shown). When measuring oxygen consumption through complex IV alone, we also failed to detect significant differences between clones expressing BCL-2 and WT cells. In all clones the rate of oxygen uptake through complex IV was between 4.6 and 5.0 ng atoms O/min/10^6 cells (results not shown).

In addition to determining the activity of mitochondrial electron transport, we compared the membrane potential \( \Delta \Psi \) in the BCL-2 clones with that in WT cells. The fluorescent dye rhodamine 123 permits the determination of \( \Delta \Psi \) without permeabilizing cells or isolating mitochondria. This lipophilic cation is taken up by mitochondria in proportion to the membrane potential (negative inside), allowing us to determine the latter parameter by measuring the fluorescence intensity issued from rhodamine 123. Compared to WT cells and clones transfected with the BCMGSNeo vector alone we detected an increased \( \Delta \Psi \) in clones B11, B22, B28, and, to a lesser extent, clone B38 (Fig. 5A). Because the difference in fluorescence intensity between the clones tested could originate from a difference in the amount of mitochondria within cells, we also measured this parameter in our cell clones. The fluorescent dye NAO specifically binds to mitochondria by interacting with lipids of the inner mitochondrial membrane (33) and binding was shown to be independent of \( \Delta \Psi \). NAO staining of cells revealed no difference in the amount of mitochondria between the different cell clones (Fig. 5B), indicating that the higher rhodamine 123 fluorescence measured in clones B11, B22, B28, and B38 was indeed due to an increase in \( \Delta \Psi \).

To determine the role of \( \Delta \Psi \) in TNF-mediated cytotoxicity, we treated WT L929 cells with the ionophore nigericin which increases \( \Delta \Psi \) by dissipation of the pH gradient across the mitochondrial membrane. Fig. 6 shows that nigericin protected cells from cytotoxicity in a dose-dependent fashion even at high concentrations of TNF, which otherwise resulted in killing of more than 95% of the cells. Nigericin alone was not cytotoxic at the concentrations used to modulate TNF-mediated cytotoxicity (results not shown). Treatment of cells with uncouplers like carbonyl cyanide p-trifluoromethoxyphenylhydrazone and the potassium-ionophore valinomycin failed to affect TNF-mediated cytotoxicity (results not shown), indicating that breakdown of \( \Delta \Psi \) did not further enhance cell death induced by TNF. In addition, we found that treatment of cells with monensin, which is structurally related to nigericin but lacks its capacity to enhance \( \Delta \Psi \), did not influence cytotoxicity after TNF challenge (results not shown). This latter finding further supports the interpretation that the effect of nigericin is due to its action on \( \Delta \Psi \).
DISCUSSION

Cell death has emerged to be as important as cell proliferation in controlling biological functions. Cell death is crucial in processes like tissue modeling during development (34), immune tolerance (35), and immune killing mechanisms (36). Effectors responsible for the latter mechanisms, comprising cytotoxic T-cells and soluble factors like TNF, act by inducing cell death of target cells in vitro. It was therefore of interest, as suggested by Williams (37), to investigate whether genes that inhibit the commitment to cell death could protect tumor cells from killing by immune mediators such as TNF. To answer this question, we have transfected a TNF-sensitive clone from L929 cells with the BCL-2 gene and investigated the sensitivity of the transfected cells to the cytotoxic cytokine TNF. WT L929 cells were killed by pg amounts of TNF when their protein synthesis was blocked. When challenged with TNF, L929 cells expressing BCL-2 showed enhanced resistance compared to WT cells. In agreement with observations made by others in NIH3T3 fibroblasts transfected with BCL-2 (38), we are unable to detect endogenous BCL-2 transcripts in L929 cells. As expected, the transfection of L929 cells with the BCL-2 gene had no influence on the transcription of TNF receptor genes and on the proliferation of transfected cells (38). The increased survival of TNF-treated cells was observed in the presence and absence of protein synthesis inhibitors. However, when actinomycin D was added, clone B38, which had the lowest BCL-2 mRNA level, was more susceptible to TNF-mediated cytotoxicity. This is possibly linked to a decrease of the BCL-2 protein level under the threshold needed to protect cells against TNF once transcription is blocked by actinomycin D. We have not measured the half-life of the BCL-2 protein in transfected L929 cells, but data from others indicate that the level of this protein decreases within 12 h after arrest of synthesis in hemopoietic cells (5). Because we detected the same amount of O2− produced by cells expressing BCL-2 and WT cells, we conclude that BCL-2 confers protection rather than modulating the effector mechanisms of TNF-mediated cytotoxicity.

The enhanced susceptibility to the cytotoxic agents antimycin A and myxothiazol of BCL-2-transfected cells argues for a role of BCL-2 as a regulator of mitochondrial metabolism. These two drugs inhibit the mitochondrial complex III, which transfers electrons from CoQ to cytochrome c and participates in the build-up of the proton gradient across the mitochondrial membrane (39). Since antimycin A binds stoichiometrically to the cytochrome bc1 of complex III, the higher sensitivity to antimycin A and myxothiazol of cells expressing BCL-2 suggests that these cells had a decreased amount of complex III in mitochondria. However, proof for this is lacking because it was technically not possible to obtain sufficient mitochondria of L929 cells to study the composition of the respiratory chain enzymes. Along this line, we did not find any change in the activity of complexes II, III, and IV in L929 cells transfected with BCL-2 compared to WT cells. Nevertheless, it remains possible that BCL-2 is interacting with mitochondrial complex III without affecting its activity but making complex III more susceptible to inhibition by antimycin and myxothiazol.

A role for BCL-2 in mitochondrial function was further supported by the finding that L929 cells expressing BCL-2 have a higher ∆Ψ than WT cells. This increase is related to BCL-2 expression since all clones investigated are derived from a clone of L929 cells (WT) which displayed constant values for ∆Ψ over passages. According to the chemoosmotic theory of Mitchell (40) ∆Ψ is the driving force for ATP synthesis and is essential for viability of cells as its breakdown leads to cell death. In addition, ∆Ψ can vary according to the physiological status of cells. For example, it is increased in activated cells compared
to resting cells (41). $\Delta V$ is also increased in a variety of tumor cell types (42, 43). Although this increase is not sufficient to cause necrosis, it indicates that these cells have a higher rate of ATP synthesis, which could be related to their elevated proliferative activity. Further, it is tempting to link a higher $\Delta V$ with an increased resistance to stress caused by a decrease of ATP within cells. This could be especially true for TNF, which has been shown to act in that way (14, 18). To verify this hypothesis we used nigericin, a drug known to increase $\Delta V$ by eliminating the pH gradient across the mitochondrial membrane. As expected, nigericin-treated cells were protected against a TNF challenge, indicating that an enhancement of $\Delta V$ is an important factor in determining the sensitivity of cells to TNF.

The expression of BCL-2 may give a double advantage to tumors in vivo. It can enhance cell survival in low growth factor conditions (38) or even growth factor deprivation as observed for some myeloid cell types (5). Similar to our results in vitro, BCL-2 expression could render tumor cells more resistant to killing mechanisms mobilized by the host, like TNF. Along this line, it could be of interest to investigate whether BCL-2 expression can protect cells from lytic viral infections. This possibility seems attractive in view of the pattern of BCL-2 expression in cells like neurons (8, 9), which cannot be replaced when killed. In order to verify this hypothesis we are currently assaying the resistance of cells transfected with BCL-2 against various cytolytic viruses.

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