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Bcl-2 protects from oxidative damage and apoptotic cell death without interfering with activation of NF-κB by TNF

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Abstract

The recent demonstration of the anti-oxidant properties of the Bcl-2 gene product suggested that expression of Bcl-2 may interfere with the nuclear migration of the NF-κB transcription factor, which is thought to depend on the presence of reactive oxygen intermediates. In mouse L cells, overexpression of Bcl-2 interfered with the activation of NF-κB by H_2O_2. However, Bcl-2 had no effect on the activation of NF-κB by TNF, even though it protected cells from TNF-induced apoptosis. The effects of exogenous pyrrolidine dithiocarbamate were very similar to those of Bcl-2 overexpression. We conclude that the protective effects of anti-oxidants against induced apoptotic cell death are unrelated to their ability to interfere with NF-κB activation.

Key words: Apoptosis; Bcl-2; Reactive oxygen species; NF-κB; Tumor necrosis factor

1. Introduction

The *bcl-2* proto-oncogene was initially defined as lying near one of the breakpoints of the t(14;18)(q32;q21) chromosomal translocations characteristic of follicular lymphomas. It was subsequently shown to be overexpressed in these cells, having come under the control of regulatory elements in the IGH locus at 14q32. The Bcl-2 gene product has recently received much attention because it interferes with endogenously programmed or externally induced cell death which proceeds along the apoptotic pathway [1,2]. The oncogenic potential of the Bcl-2 protein lies in its ability to promote the survival of cell populations that would otherwise be programmed to die off, and thus *bcl-2* has become the prototype of a newly defined class of oncogenes which act in a direction opposite to tumor suppressers such as p53 [2–4].

The mode of action of Bcl-2 has remained unclear until quite recently. The demonstration of its association with the outer mitochondrial membrane [5–7], at least in some cell types, has fueled speculations about its potential implication in regulating respiratory processes. However, cells lacking mitochondria are still protected by Bcl-2 expression [8], and C-terminal truncations of the protein which prevent its association with intracellular membranes do not necessarily reduce its protective effects [5,9]. Important clues as to the physiological role of Bcl-2 have come from experiments demonstrating that it functions as an anti-oxidant, and interferes with cell damage induced by reactive oxygen intermediates (ROI), by reducing the production of ROI, by protecting cells from their toxic effects, or perhaps by both mechanisms [10,11]. The association of Bcl-2 with mitochondrial, nuclear or endoplasmic reticular membranes [6,7] may then be explained by the fact that these are preferential sites of intracellular ROI-induced damage.

The cytotoxic effects of the pleiotropic pro-inflammatory cytokine, tumor necrosis factor (TNF), are due in part to its ability to induce the generation of ROI [12]. Overexpression of Mn-SOD has been shown to be a major pathway by which cells can protect themselves against the cytotoxic effects of TNF [13,14]. Consistent with this observation, overexpression of Bcl-2 has also been shown to protect cells against TNF-mediated cytotoxicity [15–17]. One of the most prominent early events induced by TNF in its target cells is an induction of the nuclear migration of the transcription factor NF-κB [18–20], which in turn induces the transcription of a large number of genes which participate in the inflammatory response [21]. Several studies have indicated that the generation of ROI may be an important step in the activation of NF-κB, presumably by inducing the proteolytic degradation of IκB family proteins [22]. Specifically, induction of nuclear NF-κB is inhibited by anti-oxidants and iron chelators [23,24], and by drugs that block ROI generation by the respiratory chain [12]. However, there is also evidence indicating that there are pathways of NF-κB activation which may be independent of ROI generation; in particular, TNF has been shown to induce NF-κB in a T lymphocyte line through a cascade of phospholipase activations in which ROIs do not seem to play a role [19]. The aim of this study was

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to test whether Bcl-2 protects cells from the cytolytic activity of TNF by interfering with the ROI-dependent activation of NF-κB.

2. Materials and methods

2.1. Chemicals

Pyrrolidine dithiocarbamate (PDTC), and protease inhibitors (see below) were purchased from Sigma Chemical Co. (Buchs, Switzerland). Recombinant human TNF was from Boehringer-Ingelheim (Ingelheim, Germany). Radioactively labeled compounds were from NEN DuPont (Regensdorf, Switzerland). Enzymes were purchased from Boehringer-Mannheim (Mannheim, Germany).

2.2. Cell lines

L929 cells were transformed by exposing them to supernatants from ψ2 cells transfected with pMV12 retroviral vector DNA, or with pMV12 containing either wild-type Bcl-2 or a C-terminal truncation (T3.1) which prevents Bcl-2 association with intracellular membranes. The Bcl-2 wt and Bcl-2 T3.1 L929 lines both strongly overexpress Bcl-2, in either a predominantly membrane-associated (Bcl-2 wt) or cytosolic form (Bcl-2 T3.1). These Bcl-2 expressing lines have been characterized in detail earlier [9].

2.3. Gel mobility shift assays

As a probe for NF-κB-like DNA binding activity, we used a double-stranded oligonucleotide derived from the κB enhancer of the mouse κ light chain locus (5'-GATCCGGAAAGTCCCCTGATC-3'), labeled at its 5' ends with ^{32}P using T4 polynucleotide kinase. Nuclear extracts were prepared by lysing cells on ice in 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA with 0.5% NP-40, collecting the nuclei by spinning at 800 × *g* for 5 min, and extracting proteins from the nuclear pellet with 20 mM HEPES (pH 7.9), 400 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, supplemented with protease inhibitors (1 mM PMSF, 200 ng/ml chymostatin, 5 μg/ml leupeptin, 10 μg/ml antipain, 10 μg/ml protinin, 5 μg/ml pepstatin, 0.4 μM benzamidine). Extracts were adjusted to 1 mg/ml protein with the same buffer, and 2 μg (2 μl) was mixed on ice with 15 fmol probe and incubated for 30 min before separation in an 8% acrylamide gel in 0.25 × TBE. The amount of probe trapped in a DNA/protein complex was quantitated by exposing the dried gel to a Phosphorimager plate (Molecular Dynamics).

3. Results and discussion

In a 16-h survival assay, both Bcl-2-overexpressing L929 lines were significantly more resistant to the cytotoxic action of TNF than the vector-transfected line (Fig. 1). The protective effect of Bcl-2 could be mimicked by the addition of pyrrolidine dithiocarbamate (PDTC), a metal chelator which inhibits ROI generation by interfering with the Fenton reaction which converts H_2O_2 to $OH^{\bullet}$ radicals (Fig. 1); exposing L929 cells overnight to 100 μM PDTC alone had no marked effect on their survival (data not shown). These data suggest that in this experimental system, TNF cytotoxicity is an ROI-dependent event, as has been shown in many other cases [25]. The fact that both Bcl-2 and PDTC protect from TNF cytotoxicity is consistent with the notion that the effect of Bcl-2 on cell survival is mediated at least in part by its ability to detoxify ROI or interfere with their generation.

We measured the nuclear localization of NF-κB by

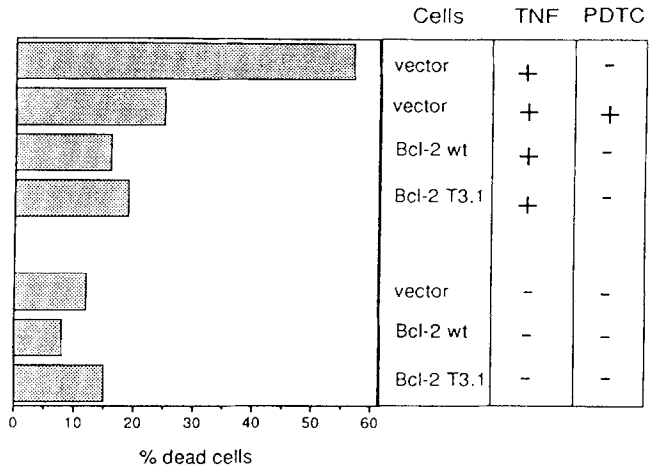

Fig. 1. Protection from TNF-mediated cell death by Bcl-2 and PDTC. L929 cells transfected with the pMV19 retroviral vector or with pMV19 containing wt Bcl-2 or the T3.1 C-Terminal truncated Bcl-2 were cultured for 16 h in medium alone, or medium containing 3 ng/ml TNF, or (for the vector-transfected cells) medium containing 3 ng/ml TNF and 100 μM PDTC. Cell death was assessed after 16 h by uptake of Trypan blue.

preparing nuclear extracts from these L929-derived lines after exposure to TNF and subjecting them to gel retardation assays. Surprisingly, early induction of NF-κB by TNF was equivalent in the three cell lines, independently of Bcl-2 expression and even at limiting TNF concentrations (Fig. 2A; and data not shown). This suggested that TNF-mediated NF-κB induction may be an ROI-independent event, at least under our experimental condi-

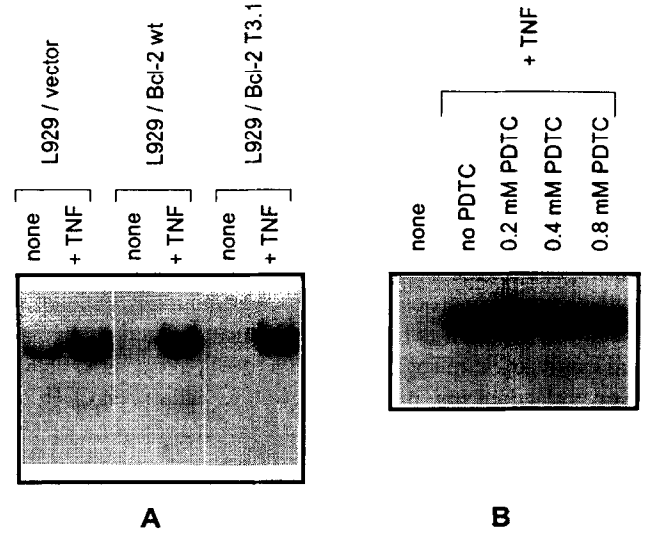

Fig. 2. Lack of effect of antioxidants on activation of NF-κB. L929 cells were exposed for 30 min to medium alone or medium containing 1.5 ng/ml TNF. Nuclear extracts were prepared and gel retardation assays were performed as described in section 2. (A) Comparison of the vector-transfected line with the lines containing the Bcl-2 wt and or the Bcl-2 T3.1 mutant. (B) PDTC was added to the vector-transfected line at the indicated concentrations 30 min before addition of TNF.

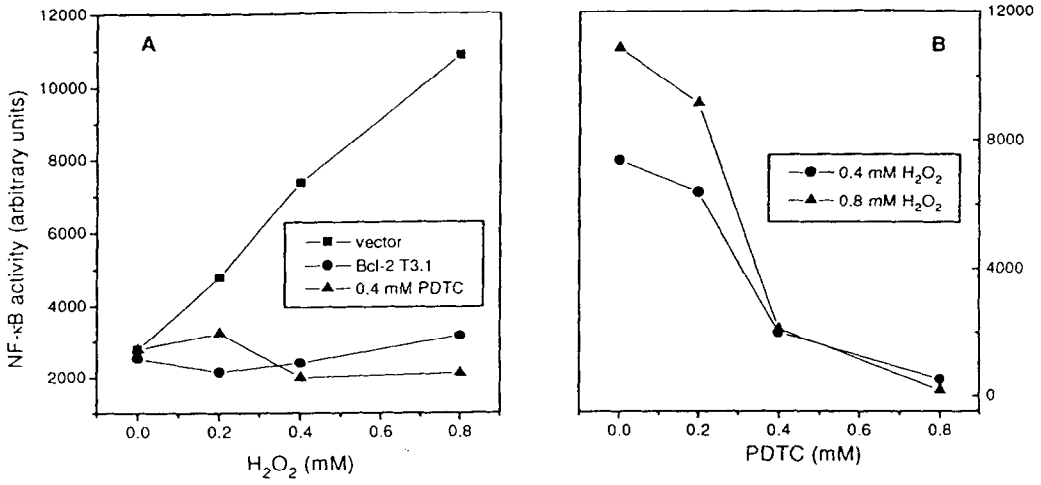

Fig. 3. Effects of Bcl-2 and PDTC on H_2O_2-mediated activation of NF-κB. Nuclear NF-κB activity 2 h after a 30 min exposure to H_2O_2 (in PBS) was measured by gel retardation assays as above, and the amount of protein-bound oligonucleotide was quantitated by exposing the dried gel to a phosphorimager plate. (A) Effect of increasing H_2O_2 concentrations on NF-κB activation in vector- or Bcl-2 T3.1-transfected L929 cells. (B) Effect of PDTC on vector-transfected cells activated by 0.4 or 0.8 mM H_2O_2.

tions. Consistent with this hypothesis, PDTC failed to inhibit rapid NF-κB activation by TNF (measured at 30 min), even at concentrations well above those required to interfere efficiently with ROI generation (Fig. 2B). It should be noted that these results are at variance with those published by Schreck et al. [23,24]; the difference may lie in the fact that these authors measured NF-κB activity 2 h after exposure to TNF, a time at which PDTC did also have an inhibitory effect in our experiments (see below).

Since it has been reported that ROI can themselves induce the nuclear migration of NF-κB, we tested the effects of H_2O_2 in our system. Indeed, H_2O_2 efficiently induced NF-κB in the vector-transfected cell lines, but had very little effect in the Bcl-2 transfectants (Fig. 3A). Interestingly, the kinetics of NF-κB induction by H_2O_2 were slower than when TNF was used as the inducing agent, as nuclear NF-κB peaked at about 2 h instead of 30 min (data not shown). In the vector-transfected cells, PDTC interfered with the activation of NF-κB induced by H_2O_2, as expected (Fig. 3B). The data presented in Fig. 3 clearly show that ROI-induced NF-κB can be observed in our system, and that its induction is inhibited by both endogenous Bcl-2 and exogenous PDTC. They also establish the fact that Bcl-2 can interfere not only with ROI-induced damage, but also with physiological processes (i.e. NF-κB activation) induced by ROI.

Induction of NF-kB by TNF has been reported to occur in two waves, the first of which is independent of PKC activity and occurs within 30 min of exposure to the ligand, while the second reaches a peak at about 3 h and is dependent on PKC activity [26]. The kinetics of NF-κB induction by H_2O_2 suggested that ROI may only be effective in inducing the second wave. Accordingly, we tested whether expression of Bcl-2 or exogenous PDTC would influence the kinetics of appearance and disappearance of NF-κB in the nucleus. As shown in Fig. 4, PDTC caused TNF-induced nuclear NF-κB activity to decay much more rapidly than it did in control cells: at 4 h post-exposure to TNF, PDTC-treated cells had no residual nuclear NF-κB, while control cells still contained about 50% of the 30 min peak value. Surprisingly, Bcl-2 expression had no demonstrable effect on NF-κB activation kinetics, in spite of its effect as an anti-oxidant in other experimental situations.

Our results indicate that the hypothesis of a tight interconnection between ROI generation, NF-κB activation, and eventual cell death, is at the very least oversimplified. The evidence for a role of ROI in programmed or

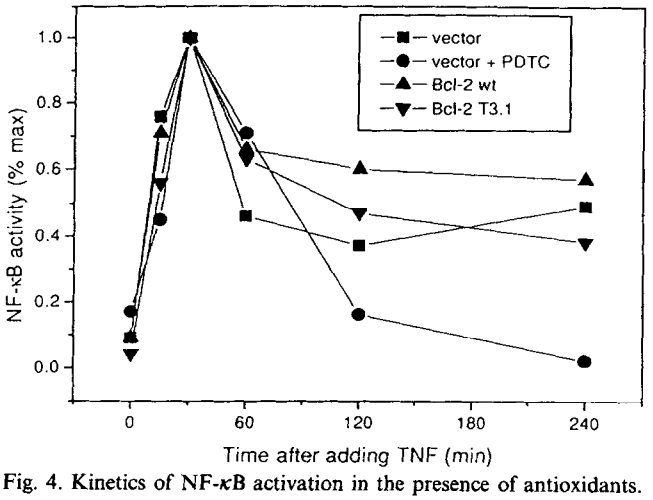

Fig. 4. Kinetics of NF-κB activation in the presence of antioxidants. Vector-transfected or Bcl-2 (wt or T3.1) transfected L929 cells were exposed to TNF (3 ng/ml) and nuclear NF-κB activity was measured at various times thereafter and quantitated as in Fig. 3. PDTC (400 μM) was added 30 min before induction by TNF.

TNF-induced apoptotic cell death is very strong indeed [25], and entirely consistent with the data presented here. However, the rapid induction of NF-κB which follows exposure to TNF does not seem to be linked to the generation of ROI, as it is not blocked by anti-oxidants or by Bcl-2. It is definitely not sufficient for the induction of a full apoptotic response. It is possible, however, that the NF-κB which appears early after exposure to TNF is necessary for the induction of genes whose expression will lead to the generation of ROI. Paradoxically, the prolonged NF-κB response induced by TNF was strongly inhibited by PDTC, but not by Bcl-2. This points to a difference between the modes of action of these two compounds, whose effects are otherwise rather similar. One possible explanation is that Bcl-2, while inhibiting the ROI-mediated reactions which eventually lead to cell death, cannot interfere with the induction of NF-κB by endogenously generated ROI, whose appearance is prevented by PDTC. Alternatively, PDTC could be interfering with a metal-requiring reaction unrelated to the generation of ROI. Anyway, the lack of an effect of Bcl-2 on the late phase of TNF-induced NF-κB activation excludes the possibility that this late phase can in itself trigger apoptotic cell death.

In summary, we have shown that although anti-oxidants have been reported to interfere with both NF-κB activation and apoptotic cell death, these two effects are not causally related. Moreover, we could not demonstrate any quantitative or qualitative effects of Bcl-2 expression on TNF-induced NF-κB activation, although Bcl-2 did interfere with H_2O_2-induced activation.

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References

[1] Reed, J.C. (1994) J. Cell Biol. 124, 1–6.
[2] Korsmeyer, S.J. (1992) Blood 80, 879–886.
[3] Wang, Y., Szekely, L., Okan, I., Klein, G. and Wiman, K.G. (1993) Oncogene 8, 3427–3431.
[4] Reed, J.C. and Tanaka, S. (1993) Leuk. Lymphoma 10, 157–163.
[5] Nguyen, M., Millar, D.G., Yong, V.W., Korsmeyer, S.J. and Shore, G.C. (1993) J. Biol. Chem. 268, 25265–25268.
[6] Nakai, M., Takeda, A., Cleary, M.L. and Endo, T. (1993) Biochem. Biophys. Res. Commun. 196, 233–239.
[7] Krajewski, S., Tanaka, S., Takayama, S., Schibler, M.J., Fenton, W. and Reed, J.C. (1993) Cancer Res. 53, 4701–4714.
[8] Jacobson, M.D., Burne, J.F., King, M.P., Miyashita, T., Reed, J.C. and Raff, M.C. (1993) Nature 361, 365–369.
[9] Borner, C., Martinou, I., Irmler, M., Schaerer, E., Mattman, C., Martinou, J.-C. and Tschopp, J. (1994) J. Cell Biol. (in press).
[10] Hockenbery, D.M., Oltvai, Z.N., Yin, X.M., Milliman, C.L. and Korsmeyer, S.J. (1993) Cell 75, 241–251.
[11] Kane, D.J., Sarafian, T.A., Anton, R., Hahn, H., Gralla, E.B., Valentine, J.S., Ord, T. and Bredesen, D.E. (1993) Science 262, 1274–1277.
[12] Schulze-Osthoff, K., Beyaert, R., Vandevoorde, V., Haegeman, G. and Fiers, W. (1993) EMBO J. 12, 3095–3104.
[13] Wong, G.H., Elwell, J.H., Oberley, L.W. and Goeddel, D.V. (1989) Cell 58, 923–931.
[14] Wong, G.H. and Goeddel, D.V. (1988) Science 242, 941–944.
[15] Vanhaesebroeck, B., Reed, J.C., De Valck, D., Grooten, J., Miyashita, T., Tanaka, S., Beyaert, R., Van Roy, F. and Fiers, W. (1993) Oncogene 8, 1075–1081.
[16] Itoh, N., Tsujimoto, Y. and Nagata, S. (1993) J. Immunol. 151, 621–627.
[17] Hennet, T., Bertoni, G., Richter, C. and Peterhans, E. (1993) Cancer Res. 53, 1456–1460.
[18] Hohmann, H.P., Remy, R., Poschl, B. and van Loon, A.P. (1990) J. Biol. Chem. 265, 15183–15188.
[19] Schütze, S., Potthoff, K., Machleidt, T., Berkovic, D., Wiegmann, K. and Krönke, M. (1992) Cell 71, 765–776.
[20] Hohmann, H.P., Remy, R., Scheidereit, C. and van Loon, A.P. (1991) Mol. Cell. Biol. 11, 259–266.
[21] Lenardo, M.J. and Baltimore, D. (1989) Cell 58, 227–229.
[22] Henkel, T., Machleidt, T., Alkalay, I., Krönke, M., Ben Neriah, Y. and Baeuerle, P. (1993) Nature 365, 182–185.
[23] Schreck, R., Rieber, P. and Baeuerle, P.A. (1991) EMBO J. 10, 2247–2258.
[24] Schreck, R., Meier, B., Männel, D.N., Dröge, W. and Baeuerle, P.A. (1992) J. Exp. Med. 175, 1181–1194.
[25] Buttke, T.M. and Sandstrom, P.A. (1994) Immunol. Today 15, 7–10.
[26] Hohmann, H.P., Remy, R., Aigner, L., Brockhaus, M. and van Loon, A.P. (1992) J. Biol. Chem. 267, 2065–2072.