JCI The Journal of Clinical Investigation

Bcl-2 and Bcl-X_L serve an anti-inflammatory function in endothelial cells through inhibition of NF-κB

A.Z. Badrichani, … , F.H. Bach, C. Ferran

J Clin Invest. 1999;103(4):543-553. https://doi.org/10.1172/JCI2517.

Article

To maintain the integrity of the vascular barrier, endothelial cells (EC) are resistant to cell death. The molecular basis of this resistance may be explained by the function of antiapoptotic genes such as *bcl* family members. Overexpression of Bcl-2 or Bcl-X_L protects EC from tumor necrosis factor (TNF)–mediated apoptosis. In addition, Bcl-2 or Bcl-X_L inhibits activation of NF-κB and thus upregulation of proinflammatory genes. Bcl-2–mediated inhibition of NF-κB in EC occurs upstream of IκBα degradation without affecting p65-mediated transactivation. Overexpression of *bcl* genes in EC does not affect other transcription factors. Using deletion mutants of Bcl-2, the NF-κB inhibitory function of Bcl-2 was mapped to *bcl* homology domains BH2 and BH4, whereas all BH domains were required for the antiapoptotic function. These data suggest that Bcl-2 and Bcl-X_L belong to a cytoprotective response that counteracts proapoptotic and proinflammatory insults and restores the physiological anti-inflammatory phenotype to the EC. By inhibiting NF-κB without sensitizing the cells (as with IκBα) to TNF-mediated apoptosis, Bcl-2 and Bcl-X_L are prime candidates for genetic engineering of EC in pathological conditions where EC loss and unfettered activation are undesirable.

Bcl-2 and Bcl-X_L serve an anti-inflammatory function in endothelial cells through inhibition of NF-κB

A.Z. Badrichani,[1] D.M. Stroka,[1] G. Bilbao,[2] D.T. Curiel,[2] F.H. Bach,[1] and C. Ferran[1]

[1]Immunobiology Research Center, Department of Surgery, Beth Israel-Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts 02215, USA
[2]Gene Therapy Program, University of Alabama at Birmingham, Birmingham, Alabama 35294, USA

Address correspondence to: Christiane Ferran, Immunobiology Research Center, Beth Israel-Deaconess Medical Center, 99 Brooklyn Avenue, Room 370, Boston Massachusetts 02215, USA. Phone: (617) 632-0840; Fax: (617) 632-0880; E-mail: cferran@bidmc.harvard.edu

Received for publication December 15, 1997, and accepted in revised form January 4, 1999.

To maintain the integrity of the vascular barrier, endothelial cells (EC) are resistant to cell death. The molecular basis of this resistance may be explained by the function of antiapoptotic genes such as *bcl* family members. Overexpression of Bcl-2 or Bcl-X_L protects EC from tumor necrosis factor (TNF)–mediated apoptosis. In addition, Bcl-2 or Bcl-X_L inhibits activation of NF-κB and thus upregulation of proinflammatory genes. Bcl-2–mediated inhibition of NF-κB in EC occurs upstream of IκBα degradation without affecting p65-mediated transactivation. Overexpression of *bcl* genes in EC does not affect other transcription factors. Using deletion mutants of Bcl-2, the NF-κB inhibitory function of Bcl-2 was mapped to *bcl* homology domains BH2 and BH4, whereas all BH domains were required for the antiapoptotic function. These data suggest that Bcl-2 and Bcl-X_L belong to a cytoprotective response that counteracts proapoptotic and proinflammatory insults and restores the physiological anti-inflammatory phenotype to the EC. By inhibiting NF-κB without sensitizing the cells (as with IκBα) to TNF-mediated apoptosis, Bcl-2 and Bcl-X_L are prime candidates for genetic engineering of EC in pathological conditions where EC loss and unfettered activation are undesirable.

J. Clin. Invest. **103**:543-553 (1999).

Introduction

Under physiological conditions, vascular endothelium is a multifunctional anticoagulant and anti-inflammatory barrier (1, 2). The endothelial interface can dynamically modify its phenotype (EC activation), to evoke an inflammatory environment in response to a variety of pathophysiological stimuli, including endotoxin, proinflammatory cytokines, and immunological insults such as those associated with graft rejection and autoimmunity (2–4). Acquisition by EC of this activated phenotype has been thoroughly analyzed in the literature and implicates the *de novo* expression of genes such as those encoding for adhesion molecules (E-selectin), chemokines (interleukin-8 [IL-8]), and procoagulant factors (tissue factor [TF]). The induction of most of these genes is regulated by a key transcription factor: NF-κB (5, 6). In most circumstances, EC resist the damaging potential associated with this proinflammatory environment and revert to their original quiescent phenotype. More recently, analysis of gene expression in EC of long-term surviving hamster to rat heart xenograft revealed the potential of EC to acquire a novel phenotype by expressing high levels of antiapoptotic proteins, namely the Zn finger protein A20 and the antiapoptotic *bcl* members Bcl-2 and Bcl-X_L (7). Expression of these genes correlated with the absence of inflammation, thrombosis, and endothelial cell death, features seen in rejecting xenografts that lack the expression of A20, Bcl-2, and Bcl-X_L in their EC (7).

The Zn finger protein A20 was originally identified as a tumor necrosis factor (TNF)–inducible gene in human umbilical vein endothelial cells (HUVEC) (8, 9). We recently demonstrated that A20 has a dual function in EC: protection from apoptosis and downregulation of EC activation through inhibition of the transcription factor NF-κB (10, 11). However, both expression and function of Bcl-2 and Bcl-X_L in EC remain poorly defined (12, 13). Bcl-2 and Bcl-X_L are prototypic cell-death regulators whose function is modulated by complex homo- and heterodimerizations with their proapoptotic homologues such as Bax and/or with other nonrelated molecules such as Raf-1 kinase (14–18). Both these proteins confer to cells resistance to a variety of proapoptotic stimuli, including hypoxia, radiation, growth factor withdrawal, and others (19), but their effect upon TNF-mediated apoptosis is still controversial (20, 21). TNF is associated with most proinflammatory conditions and is a potent activator of EC both *in vivo* and *in vitro* (22, 23). In this work, we studied the function of Bcl-2 and Bcl-X_L in EC. Our results demonstrate that expression of Bcl-2 or Bcl-X_L in EC significantly protects the cells from TNF-mediated apoptosis after sensitization with cycloheximide (CHX). In addition, we show that Bcl-2 and Bcl-X_L are able to downregulate EC activation independently of the agonist tested through specific inhibition of the transcription factor NF-κB. Inhibition occurs at a level upstream of IκBα degradation and involves stabilization of a slower migrating band that might represent a hyperphosphorylated form of IκBα. Bcl-2 and Bcl-X_L share the same function(s) in EC with the nonrelated antiapoptotic molecule A20. Their coexpression in EC of long-term surviving xenografts defines a novel protected pheno-

type of EC, whereby these molecules sum their potentials to protect the cell from death and from the untoward effect of EC activation (7). We suggest that the dual function in EC of Bcl-2 and Bcl-X_L (*i.e.*, antiapoptotic and anti-inflammatory, through inhibition of NF-κB) qualifies their cytoprotective role.

Methods

Cell culture and treatment. Bovine aortic endothelial cells (BAEC) were isolated and cultured in DMEM supplemented with L-glutamine (2 mM), penicillin G (100 U/ml), and FCS (10%). Primary cultures of BAEC were used between the fourth and the fifth passage. HUVEC were isolated and cultured as described (24).The 293 human embryonic kidney cell line was obtained from American Type Culture Collection (Rockville, Maryland, USA) and cultured in 10% FCS-supplemented DMEM. All cells were grown in culture at 37°C in a 5% humid CO_2 atmosphere. EC were stimulated with either 100 ng/ml of LPS from *Escherichia coli* 0B55 (Sigma Chemical Co., St. Louis, Missouri, USA), 100 U/ml of recombinant human TNF (kind gift of Sandoz Pharmaceuticals, East Hanover, New Jersey, USA), or 5×10^{-8} M of PMA (Sigma Pharmaceuticals, St. Louis, Missouri, USA).

Lipofection protocol. BAEC (3×10^5 per well) were plated in a 6-well plate and transfected when they reached 70% confluence. A total of 1.6 μg of DNA per well (test plasmids and reporter constructs) was added to 8 μg of Lipofectamine (GIBCO BRL, Grand Island, New York, USA), incubated at room temperature for 30 min, and then added to the cells in triplicate. In all experiments, 0.3 μg of the β-galactosidase (β-gal) reporter was used with 0.7 μg of the expression plasmids or the pAC control, and 0.6 μg of the E-selectin, IL-8, IκBα (ECI-6), or NF-κB-luciferase (luc) reporters. For the apoptosis experiments, the newly developed Lipofectamine Plus Reagent (GIBCO BRL) was used according to the manufacturer's instructions to achieve a higher percentage of transfection. In experiments involving the induction of the IκBα reporter by the p65 (RelA) expression vector, 40 ng of p65 was used. For the HIV-wt-chloramphenicol acetyltransferase (HIV-CAT) and HIV*Δκ*B-CAT reporters experiments, 0.5 μg of the expression plasmids (mBcl-2, mBcl-X_L, or pAC) were transfected with 0.3 μg of the c-Tat expression plasmid and 0.6 μg of the HIV-CAT, or the HIV*Δ–κB*-CAT reporter along with 0.2 μg of the β-gal reporter.

In all cotransfection experiments, FCS was added to the medium 5 h after transfection to achieve a final concentration of 10%. Forty-eight hours after transfection, the cells were stimulated with either human recombinant TNF (100U/ml), LPS (100 ng/ml), or PMA (5×10^{-8} M), harvested 7 h later, and assayed for β-gal, luciferase, and CAT.

Human embryonic kidney 293 cells were transfected using the Calcium-Phosphate method. Then 1 μg of the expression plasmids (mBcl-2, mBcl-X_L, or pAC), 0.7 μg of IκBα reporter, and 40 ng of p65 (RelA) were added per well.

β-gal, luciferase, and CAT assays. Cellular extracts were assayed for β-gal activity per the Galacto-Light protocol (Tropix Inc., Bedford, Massachusetts, USA). Luciferase activity was assayed by adding 10 μl of cellular extract to 90 μl of a solution containing 24 mM glyclglycine (pH 7.8), 2 mM ATP (pH 7.5), and 10 mM $MgSO_4$. Samples were read on the Microlumat LB 96P luminometer (EG&G Berthold, Wildbad, Germany) using an injection mix consisting of 24 mM glyclglycine and 0.1 mM luciferin (Sigma Chemical Co.).

Luciferase activity was normalized for β-gal by using the formula: luciferase activity / β-gal activity × 1,000. Normalized luciferase activity is given in relative light units (RLU). Significance was determined by the Student's *t* test. The CAT assay was performed for the HIV-wt reporter by means of a standard method using a Promega Kit (Promega Corp., Madison, Wisconsin, USA) according to the manufacturer's recommendation. A portion of the xylene phase was mixed with scintillation liquid and counted in a scintillation counter (1900 TR; Packard Instrument Co., Downers Grove, Illinois, USA).

Reporter constructs. E-selectin reporter. The reporter construct used was described previously (25). Briefly, it represents bp –1286 to +484 of the porcine E-selectin promoter. This region includes the first complete intron and exon, as well as the beginning of the second exon up to the ATG site. The promoter was cloned into the pMAMneo-luc plasmid vector by replacing the mmTV promoter (CLONTECH Laboratories Inc., Palo Alto, California, USA).

IL-8 reporter. A gift from E. Hofer (VIRCC, Vienna, Austria) represents the human IL-8 promoter linked to the luciferase gene (p-UBT luc).

IκBα (ECI-6) reporter. The construction of this reporter has been described previously (26). It represents a 600-bp fragment of the porcine ECI-6/IκBα promoter ligated into the luciferase expression vector p-UBT (p-UBT-luc), with the creation of an additional *Hin*dIII site.

NF-κB reporter. This reporter is a kind gift from A. Palmetshofer (Beth Israel Deaconess Medical Center, Boston, Massachusetts, USA). It consists of four copies of NF-κB elements taken from the porcine E-selectin promoter inserted upstream of a TK minimal promoter driving a luciferase gene. The vector backbone is a Bluescript KS+ plasmid (Stratagene, La Jolla, California, USA).

RSV β-gal reporter. The full-length *E. coli* β-gal gene (CLONTECH Laboratories Inc.) was inserted into the pRc/RSV vector (Invitrogen Corp., San Diego, California, USA) at the *Not*I site. This reporter is not inducible upon stimulation by the agonists used in this paper. Thus, its activity was used to correct for transfection efficiency.

Expression plasmids. The murine Bcl-2 and Bcl-X_L cDNA are a kind gift of T. Behrens (University of Minnesota, Minneapolis, Minnesota, USA). The full-length human Bcl-2 is a kind gift of G. Nünez (University of Michigan, Ann Arbor, Michigan, USA), and the deleted forms (Δ1, Δ2, Δ3, Δ4, Δ6, Δ8, Δ12) are a kind gift of T.G. Parslow (University of California, San Francisco, San Francisco, California, USA). These cDNA were subcloned in the pAC expression vector. The pAC 8.8-kb plasmid vector contains a CMV promoter, a pUC19 polylinker site, and a SV40 splice/polyA site (a kind gift of R. Gerard, University of Texas Southwestern, Dallas, Texas, USA).

p65. The p65 expression plasmid is a kind gift of J. Anrather (Beth Israel Deaconess Medical Center, Boston, Massachusetts, USA) and represents the human RelA (from amino acid 2 to 551) fused to a NH_2-terminal *c-myc* Tag and cloned into the pcDNA3 expression plasmid (Invitrogen Corp.) at the *Hin*dIII/*Xba*I polycloning sites.

Recombinant Bcl-2 adenovirus. The human recombinant Bcl-2 adenovirus (Ad.hBcl-2) was generated by Bilbao *et al*(27) according to the two-plasmid recombination method developed by Graham and Prevec (28) and McGrory *et al.* (29). In brief, a 0.7-kb fragment corresponding to the human Bcl-2 cDNA lacking the transmembrane domain was excised from the pGEX-4T-1-hBcl-2 plasmid (kind gift of J. Reed, La Jolla Cancer Research Foundation, La Jolla, California, USA) and subcloned in the shuttle plasmid PCA13 (Microbix Inc., Toronto, Ontario, Canada). The resultant plasmid, pCAhBcl-2, was then cotransfected in 293 cells together with the adenoviral rescue plasmid PJM17 (Microbix Inc.). To reduce the possibility of wild-type virus being produced from a plasmid that contains adenovirus genomic DNA, the pJM17 vector has a plasmid vector sequence inserted in the E1 region that makes the DNA molecule too large to package in an adenovirus particle (30). Individual plaques of rAd.hBcl-2 were picked approximately 10 days after cotransfection and carried through three additional steps of plaque purification. Identity of the rAd.hBcl-2 was proved by restriction enzyme analysis of genomic DNA and by Western blot analysis of cell extracts from infected cells using the N19 rabbit

anti–mouse and human Bcl-2 polyclonal antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, California, USA). Furthermore, the presence of the expression cassette in the viral genome was confirmed by DNA sequencing. The rAd.β-gal used as a control adenovirus is a kind gift of R. Gerard (University of Texas Southwestern). Production of rAd was done in the embryonic kidney 293 cell line. Recombinant adenoviruses were subsequently purified by two consecutive cesium chloride centrifugation and titered by limiting dilution on 293 cells.

Cells extracts. Transfected BAEC or HUVEC infected with rAd were harvested in PBS 48 h after transfection or infection and lysed in Ripa buffer (10 mM Tris [pH 7.5], 150 mM NaCl, 1% [vol/vol] Triton X-100, 0.5 μg/ml each of aprotinin, leupeptin, and antipain, 1 μg/ml of pepstatin, and 0.5 mM PMSF) for 20 min at 4°C. Cellular debris were pelleted by microcentrifugation for 20 min at 4°C, and supernatants were recovered and kept at –80°C until assayed. The protein concentration of these cell extracts was evaluated by the Lowry assay (Bio-Rad DC Protein Assay; Bio-Rad Laboratories Inc., Hercules, California, USA)

Immunoblots. Proteins (15–20 μg) were resolved on a reducing 12% SDS-polyacrylamide gel and transferred onto Immobilon-P transfer membranes (Millipore Corp., Bedford, Massachusetts, USA) at 0.8 mA/cm². Membranes were preblocked at room temperature for 1 h in 5% (wt/vol) BLOTTO nonfat dry milk in 0.1% (vol/vol) Tween-20 PBS. Membranes were then labeled for 1 h with a first specific antibody. After four 10-min washings in PBS containing 0.1% Tween-20, membranes were incubated for 1 h at room temperature with secondary donkey anti–rabbit IgG antibody conjugated to horseradish peroxidase (1:3,000 dilution) (Pierce Chemical Co., Rockford, Illinois, USA). Detection was then performed by enhanced chemiluminescence (ECL) using a commercially available kit (Amersham Corp., Arlington Heights, Illinois, USA) according to the manufacturer's instructions.

Murine Bcl-2 (mBcl-2) expression was detected using a 1:500 dilution of a polyclonal rabbit antibody (N-19) directed against the amino acids 4–21 of the murine or human Bcl-2. Human wild-type Bcl-2 and Bcl-2 deletion proteins were detected using a 1:200 mouse monoclonal IgG antibody against Bcl-2 (DAKO Corp., Carpinteria, California, USA) or using the N-19 antibody. Bcl-X_L expression was detected with a 1:500 dilution of a polyclonal rabbit antibody raised against the human and mouse Bcl-$X_{S/L}$. IκBα expression was detected using a rabbit polyclonal antibody (C-21; dilution 1:2,000). All these antibodies were purchased from Santa Cruz Biotechnology.

Flow cytometric analysis of E-selectin and vascular cell adhesion molecule-1 expression. HUVEC were cultured to 90% confluence in 6-well plates and infected at a moiety of infection (MOI) of 100 with either the rAd.hBcl-2, the rAd.β-gal, or noninfected. Thirty-six to 48 h after infection, HUVEC were treated with 200 U/ml of TNF for 4 h (E-selectin expression) and 8 h (vascular cell adhesion molecule-1 [VCAM-1] expression), after which cells were harvested and labeled for surface expression of both adhesion molecules. Approximately 10^5 cells were incubated with 40 μl (1 μg/ml) of mouse anti–human monoclonal antibodies to either E-selectin (CD62E) or VCAM-1 (CD106) (R&D Systems Inc., Minneapolis, Minnesota, USA) or an isotype-matched control monoclonal antibody (IgG_1) for 20 min on ice. Cells were then washed and incubated with 40 μl of FITC-conjugated goat anti–mouse IgG (1:300) for 20 min on ice. Surface antigen expression was analyzed with a FACScan bench-top model (Becton Dickinson Immunocytometry Systems, San Jose, California, USA) using Cellquest acquisition and analysis software (Becton Dickinson Immunocytometry Systems). Data were collected from viable cells only as determined by forward- and side-scattered light properties and propidium iodide staining.

Evaluation of apoptosis in a transient-transfection assay. BAEC grown in 6-well plates were cotransfected using Lipofectamine Plus (GIBCO BRL) with 0.5 μg of the reporter plasmid CMV-βgal together with 1 μg of the expression plasmids (mBcl-2, mBcl-X_L, hBcl-2, and the different hBcl-2 mutants, or the pAC control plasmids). Twenty-four hours after transfection, cells were treated with 2 μg/ml of CHX (Sigma Chemical Co.), or with CHX followed 30 min later by 200 U/ml of TNF. After 12 h incubation with the respective treatments, the cells were fixed with 0.05% glutaraldehyde and stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside for 4 h. BAEC were then visualized by phase-contrast microscopy, and the number of living β-gal–positive cells were evaluated in random 10 high-power fields per well (minimal number of 100 blue cells). Viable or apoptotic cells were distinguished based on morphological alterations of adherent cells undergoing apoptosis, including becoming rounded, condensed, and detached from the dish (31). The number of blue cells in CHX-treated BAEC and for each given expression plasmid was considered as corresponding to 100% of survival; the percentage of survival in the CHX-TNF–treated wells was calculated relative to that number as described elsewhere (32). CHX alone does not induce apoptosis in BAEC but sensitizes these cells to TNF-mediated apoptosis.

Results

Expression of Bcl-2 or Bcl-X_L in BAEC protects CHX-sensitized cells from TNF-mediated apoptosis. Expression of murine Bcl-2 and Bcl-X_L after Lipofectamine transfection in BAEC was confirmed by Western blot analysis (Fig. 1*a*). BAEC transfected with the empty expression plasmid show little expression of Bcl-2 or Bcl-X_L (lanes *1* and *3*). In contrast, BAEC transfected with the Bcl-2 or Bcl-X_L expression plasmids show high levels of expression (lanes *2* and *4*). We then tested whether expression of Bcl-2 or Bcl-X_L protected CHX-sensitized EC from undergoing TNF-mediated apoptosis.

As described in Methods, BAEC were cotransfected with mBcl-2, mBcl-X_L, or pAC expression plasmids together with the CMV-βgal plasmid. After 12 hours of CHX and TNF treatment, the percentage of cell survival was decreased to 3 ± 1% in pAC-transfected cells (Fig. 1*b*, lane *2* vs. lane *1*). Overexpression of mBcl-2 or mBcl-X_L leads to significant protection: the percentage of cell survival reaches 52 ± 2% and 50 ± 4% in mBcl-2– or mBcl-X_L–expressing cells (Fig. 1*b*, lane *4* vs. lanes *3* and *6* vs. lane *5*). These results demonstrate that the antiapoptotic genes *bcl-2* and *bcl-X_L* can also interrupt TNF-mediated apoptotic signaling in EC, consistent with those obtained in EC using another *bcl* family member, A1 (33).

Expression of Bcl-2 or Bcl-X_L inhibits EC activation. The effect of overexpression of murine Bcl-2 or Bcl-X_L on the upregulation of proinflammatory genes, which constitute a major part of EC activation, was first tested on the inducibility of an E-selectin reporter. E-selectin is a cell-specific marker of EC activation (34, 35). BAEC were cotransfected with the porcine E-selectin reporter construct, along with the expression plasmids encoding for mBcl-2, mBcl-X_L, or with the control empty plasmid. Bcl-2 or Bcl-X_L expression inhibits the induction of the E-selectin reporter in a dose-dependent manner (Fig. 2, *a* and *b*, lanes *6–10*). Stimulation of the E-selectin reporter with 100 U/ml of TNF leads to a threefold induction of the corrected luciferase activity. Transfection of BAEC with mBcl-2 or mBcl-X_L expression plasmids in amounts ranging from 0.25 μg to 0.7 μg/5×10^5 BAEC leads to a significant inhibition of this reporter activity. The inhi-

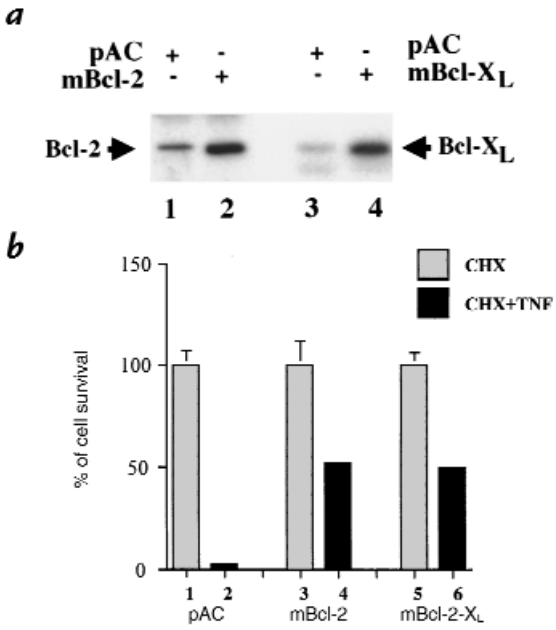

Figure 1
Expression of Bcl-2 or Bcl-X_L in BAEC after Lipofectamine-mediated transfection inhibits TNF-induced apoptosis in CHX-sensitized EC. (***a***) Immunoblot detection of Bcl-2 (lanes *1* and *2*) and Bcl-X_L (lanes *3* and *4*) in BAEC-transfected cells using polyclonal anti–Bcl-2 and anti–Bcl-X_L antibodies. Arrows indicate that transfected BAEC express high levels of Bcl-2 or Bcl-X_L as opposed to control nontransfected cells. (***b***) BAEC were cotransfected with a CMVβ-gal reporter (0.5 μg) and 1 μg of pAC (lanes *1* and *2*), mBcl-2 (lanes *3* and *4*), or mBcl-X_L (lanes *5* and *6*). CHX was added to transfected cells (*all lanes*) that were subsequently stimulated (lanes *2*, *4*, and *6*) or not (lanes *1*, *3*, and *5*) with TNF for 12 h . The percent cell survival was calculated as described in Methods. Expression of Bcl-2 or Bcl-X_L rescues CHX-sensitized EC from TNF-mediated apoptosis. Error bars are ± SE. Graph shown is representative of three experiments.*BAEC*, bovine aortic endothelial cells; *β-gal*, β-galactosidase; *CHX*, cycloheximide; *EC*, endothelial cells; *TNF*, tumor necrosis factor.

bition is significant at doses of 0.5 μg and higher for mBcl-2 (Fig. 2*a*, lanes *8* and *9* vs. lane *6*; $P < 0.03$, $P = 0.02$, respectively) and at 0.6 μg for Bcl-X_L (Fig. 2*b*, lane *9* vs. lane *6*; $P = 0.008$). Inhibition is complete at 0.7 μg for either Bcl-2 or Bcl-X_L compared with the basal levels detected in the nonstimulated cells (Fig. 2, *a* and *b*, lane *10* vs. lane *1*). These results demonstrate that expression of Bcl-2 and Bcl-X_L in EC not only protects from TNF-mediated apoptosis but also downregulates TNF-mediated EC activation as evaluated by analysis of the E-selectin reporter activity.

The inhibitory effect of the *bcl* genes was associated not only with activation of EC by TNF but also by LPS and PMA. Stimulation by LPS (100ng/ml) or PMA (5.10^{-8}M) of pAC-transfected BAEC leads to seven- and twofold induction of the E-selectin reporter activity, respectively (Fig. 2, *a* and *b*, lanes *11* and *16* vs. lane *1*). Similar to the observation with TNF, expression of either Bcl-2 or Bcl-X_L inhibits E-selectin reporter activity in a dose-dependent manner (Fig. 2, *a* and *b,* lanes *12–15*, *17–20*). This inhibition is complete when 0.7 μg of the given expression plasmid was used.

The inhibitory effect of Bcl-2 and Bcl-X_L is also seen with other proinflammatory genes that are upregulated with EC activation. Reporters comprising the promoters of porcine IL-8 and IκBα (ECI-6) linked to the luciferin gene were tested in cotransfection experiments along with 0.7 μg of mBcl-2 or mBcl-X_L expression plasmids (previously established as the optimal inhibitory amount). Expression of Bcl-2 or Bcl-X_L inhibited the activity of the two reporters after stimulation with either TNF, LPS, or PMA (Fig. 2, *c* and *d*). The luciferase activity of the IL-8 reporter, when cotransfected with pAC alone, increases 1.7-, 2.8-, and 1.8-fold after stimulation with TNF, LPS, or PMA, respectively (Fig. 2*c,* lane *1* vs. lanes *4*, *7*, and *10*). Expression of Bcl-2 or Bcl-X_L completely inhibited the inducibility of the IL-8 reporter by TNF, LPS, and PMA (lanes *5*, *6*, *8*, *9*, *11*, and *12* vs. lane *1*). The same results were obtained when the porcine IκBα reporter is cotransfected along with Bcl-2 or Bcl-X_L (Fig. 2*d*). Induction of the IκBα reporter activity after stimulation with TNF, LPS, and PMA reached 1.7-, 2.8- and 1.8-fold, respectively. Overexpression of Bcl-2 or Bcl-X_L led to significant inhibition of reporter activity after TNF (lanes *5* and *6*; $P < 0.03$), LPS (lanes *8* and *9; P* < 0.04), or PMA stimulation (lanes *11* and *12; P* = 0.0002). Taken together, these results indicate that both Bcl-2 and Bcl-X_L serve a new function in the EC (*i.e.*, inhibition of EC activation) and that this function is independent of the agents tested.

Bcl-2 or Bcl-X_L inhibits the activation of NF-κB without affecting p65-mediated transactivation. We and others have shown that the induction of many proinflammatory genes that are upregulated upon EC activation is transcriptionally regulated and depends on NF-κB (3, 5, 26, 36). Our results demonstrating that the induction of 3 NF-κB-dependent genes (E-selectin, IL-8, and IκBα) is inhibited by the expression of Bcl-2 or Bcl-X_L prompted us to investigate whether inhibition relates to blockade of activation of NF-κB. BAEC were cotransfected with a reporter construct dependent only on NF-κB for its activation and mBcl-2, mBcl-X_L expression plasmids, or the empty vector, pAC (0.7 μg). In pAC-transfected BAEC, the induction of the NF-κB reporter reached 24- and 52-fold after TNF and LPS stimulation, respectively (Fig. 3*a*, lanes *4* and *7* vs. lane *1*). In Bcl-2–transfected BAEC, the induction of the NF-κB reporter activity decreased by 71.3% after TNF (Fig. 3*a,* lane *5* vs. lane *4*; $P = 0.006$) and by 78.3% after LPS stimulation (Fig. 3*a*, lane *8* vs. lane *7*; $P < 0.001$). Similarly, Bcl-X_L expression in BAEC inhibits the inducibility of the NF-κB reporter by 79.5% after TNF (Fig. 3*a*, lane *6*; $P = 0.01$) and by 61% after LPS stimulation (Fig. 3*a,* lane *9*; $P < 0.001$). In addition, expression of Bcl-2 or Bcl-X_L abrogates the 5.6-fold induction of the NF-κB reporter after stimulation with PMA (Fig. 3*b*, lanes *5* and *6* vs. lane *4*; $P = 0.001$).

A recent report by Grimm *et al.* (37) demonstrated that expression of Bcl-2 in 293 cells inhibits NF-κB activation.

These authors showed that overexpression of Bcl-2 in 293 cells did not prevent IκBα degradation but rather downmodulated the transactivating potential of nuclear p65. To test whether Bcl-2 and Bcl-X_L would also affect p65-mediated transactivation in EC, we cotransfected BAEC with the Bcl-2 or Bcl-X_L expression plasmids, or the empty vector pAC together with the p65-dependent IκBα reporter. Expression of this reporter was then induced by cotransfection with a p65 expression plasmid. The IκBα reporter activity in pAC-transfected cells was increased 53-fold (Fig. 3*c*, lane *2*). This induction was not inhibited by expression of Bcl-2 or Bcl-X_L, which showed 68- and 74-fold induction, respectively (Fig. 3*c*, lanes *4* and *6*). Thus, the inhibitory effect of Bcl-2 or Bcl-X_L expression upon p65-mediated transactivation does not occur in EC as opposed to 293 cells, a result that we reproduced in our system (Fig. 3*d*). The difference could be explained by our use of primary cell cultures as opposed to the 293 cell line or could indicate cell-type specific function of the *bcl* genes.

Bcl-2 inhibits NF-κB activation at a level upstream of IκBα degradation. To determine the level at which Bcl-2 expression acts upon activation of NF-κB, a recombinant replication deficient human Bcl-2 adenovirus was generated as described. HUVEC were either noninfected or infected with rAd.hBcl-2 or rAd.β-gal at a MOI of 100, which usually leads to >90% of expression of the transgene in HUVEC (38). Expression of the transgene (human Bcl-2) was evaluated by Western blot analysis of total cell extracts recovered 48 hours after infection (Fig. 4*a*, lane *3*). In a first set of experiments, we confirmed that Bcl-2 retained its ability to inhibit EC activation in human cells. HUVEC were either noninfected or infected with rAd.hBcl-2 or rAd.β-gal. Forty-eight hours after infection, cells were treated with 200 U/ml of TNF, and both E-selectin and VCAM-1 surface expression was evaluated at four and eight hours after treatment, respectively. FACS analysis shows that overexpression of Bcl-2 significantly decreased TNF-mediated upregulation of E-selectin (Fig. 4*b*) and almost totally abrogated VCAM-1 upregulation (Fig. 4*c*). In addition, the impact of Bcl-2 upon IκBα degradation after TNF was also evaluated. Again, noninfected, rAd.Bcl-2, or rAd.β-gal–infected HUVEC were treated with 200 U/ml of TNF, and cell extracts were recovered 15 minutes and two hours after TNF stimulation. These extracts were evaluated by Western blot analysis for IκBα expression. We demonstrate that overexpression of Bcl-2 in EC inhibits IκBα degradation by mainly stabilizing the slower migrating form of IκBα (Fig. 4*d*, lane *5* vs. lanes *2* and *8*). The modest decrease of IκBα that follows TNF treatment in Bcl-2–expressing HUVEC might relate to the small percentage of cells not expressing the transgene. This data establishes that Bcl-2 inhibits NF-κB activation upstream of IκBα degradation.

Expression of Bcl-2 or Bcl-X_L in EC does not affect the transcription factor Sp1. To rule out nonspecific or toxic effects of Bcl-2 or Bcl-X_L expression upon the whole transcriptional machinery, NF-κB–independent induction of genes was examined. BAEC are cotransfected with the Bcl-2, Bcl-X_L, or pAC expression plasmids together with HIV-CAT or HIV ΔκB–CAT reporters. Induction of the HIV-CAT reporter by the viral protein c-Tat is dependent upon the Sp1 binding sites of the HIV-LTR promoter and

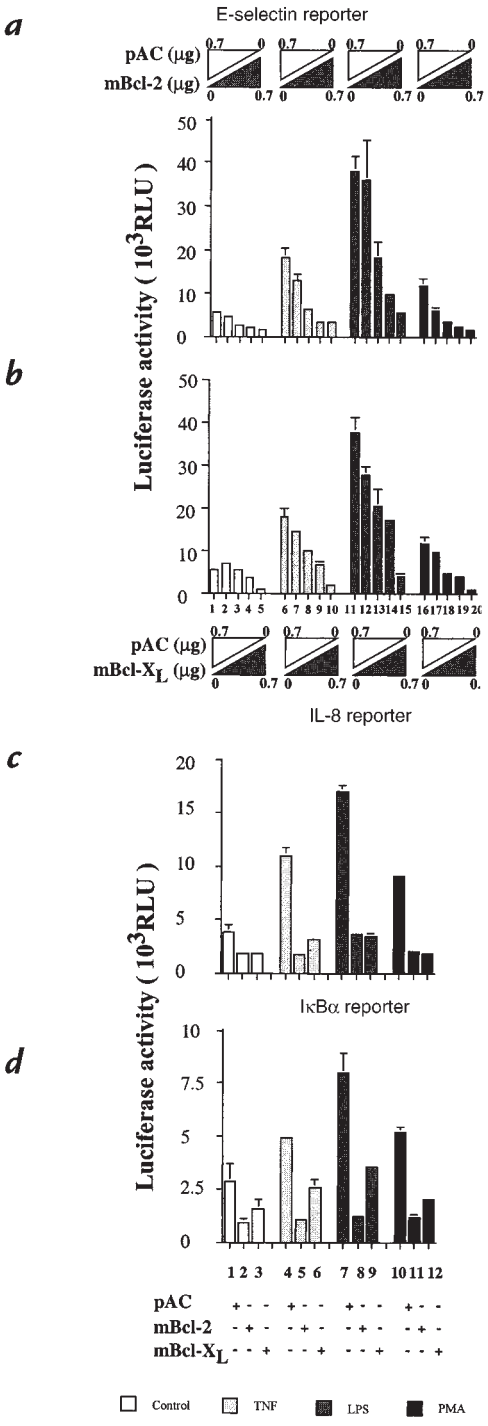

Figure 2

Expression of Bcl-2 or Bcl-X_L inhibits the induction by TNF, LPS, and PMA of (***a*** and ***b***) E-selectin, (***c***) IL-8, and (***d***) IκBα reporters. E-selectin reporter induction by TNF, LPS, and PMA is inhibited by (*a*) Bcl-2 and (*b*) Bcl-X_L in a dose-dependent manner. Both mBcl-2 and mBcl-X_L were titrated (0, 0.25, 0.5, 0.6, 0.7 μg) with pAC to equal 0.7 μg. The graphs shown are representative of four experiments. Significant inhibition is achieved at doses of 0.5 μg and higher. For (*c*) IL-8 and (*d*) IκBα reporters, 0.7 μg of either Bcl-2, Bcl-X_L, or pAC expression plasmids was used for BAEC transfection. The graphs shown are representative of four and three experiments, respectively. For all the reporters studies, BAEC were treated with TNF at a concentration of 100 U/ml, LPS at a concentration of 100 ng/ml, or PMA at a concentration of 5×10^{-8} M. Results are given in RLU. Error bars represent ± SE. *RLU*, relative light units.

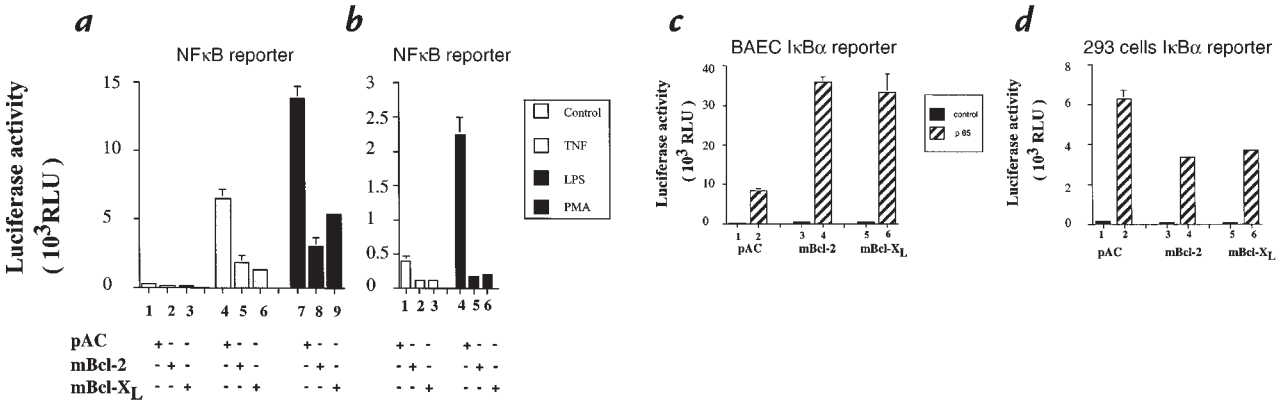

Figure 3

(***a*** and ***b***) Bcl-2 or Bcl-X_L expression in BAEC prevents the induction of an NF-κB reporter after TNF, LPS, and PMA stimulation without interfering with (***c***) p65-mediated transactivation, in contrast with (***d***) 293 cells. (*a* and *b*) BAEC were cotransfected with 0.7 μg of pAC (*a*; lanes *1*, *4*, and *7*), *Bcl*-2 (*a*; lanes *2*, *5*, and *8*), or mBcl-X_L (*a*; lanes *3*, *6*, and *9*) expression plasmids along with 0.6 μg of the NF-κB reporter and 0.3 μg of β-gal reporter. Cells were stimulated with either 100 U/ml TNF (*a*; lanes *4–6*), 100 ng/ml LPS (*a*; lanes *7–9*), or 5×10^{-8} PMA (*b*; lanes *4–6*). The graphs are representative of at least three experiments performed. Results are given in RLU. Error bars are ± SE. (*c*) BAEC were cotransfected with 0.3 μg β-gal, 0.6 μg IκBα reporter, 40 ng p65 (RelA), and 0.7 μg of either pAC (lanes *1* and *2*), mBcl-2 (lanes *3* and *4*), or mBcl-X_L (lanes *5* and *6*) expression plasmids. Overexpression of Bcl-2 or Bcl-X_L does not inhibit the induction of IκBα by p65 (lanes *4* and *6* vs. lane *2*). (*d*) 293 cells were transfected with the same plasmids with the exception of higher concentrations of the expression plasmids (1 μg). Results show that expression of Bcl-2 or Bcl-X_L inhibits the ability of p65 (RelA) to induce an IκBα reporter. Data shown are representative of three experiments performed. Results are expressed in RLU. Error bars represent ± SE.

represents a means of gene induction independent of NF-κB (39). Bcl-2 or Bcl-X_L expression does not affect the threefold induction of this reporter stimulated by 0.3 μg of a c-Tat expression plasmid (Fig. 5). To further confirm that the presence in the wild-type HIV reporter of two κB binding sites did not interfere with its induction by c-Tat, a similar experiment was performed with the HIV ΔκB-CAT reporter that is deleted from its κB binding sites. The results obtained were similar to those achieved using the wild-type reporter (data not shown), establishing that the transactivation property of the Sp1 transcription factor is not altered by Bcl-2 or Bcl-X_L expression.

The *bcl* homology domains BH2 and BH4 are required for the inhibitory effect of Bcl-2 upon NF-κB activation in EC, whereas all BH domains are necessary for the antiapoptotic effect of Bcl-2. To test whether the inhibitory effect of Bcl-2 upon NF-κB activation is associated with any of the described *bcl* homology domains, several human Bcl-2 deletion mutants were coexpressed together with the NF-κB reporter. Deletion of BH1, BH2, BH3, BH4, and the recently described negative regulatory domain (NRD) were included (Table 1). Expression of each of the mutants after transfection of BAEC was confirmed by Western blot analysis (Fig. 6*a*). The ability of these mutants to inhibit NF-κB activation was evaluated after stimulation with TNF and LPS. Bcl-2 mutants lacking BH4 (Δ1) or BH2 (Δ12) were no longer able to inhibit NF-κB activation (Fig. 6*b*, lanes *C2–3* and *G2–3* vs. lane *B2–3*). Additionally, expression of Δ1 (lane C1) or Δ12 (lane G1) leads to a significant increase in the basal luciferase activity of the NF-κB reporter compared with control (lane *A1*). Although still able to inhibit TNF- and LPS-mediated NF-κB activation, deletion of the BH3 domain (Δ4) led to significantly less inhibition than that achieved by wild-type Bcl-2 (lanes *E2–3* vs. lanes *B2–3*). In contrast, deletion of BH1 (Δ8) or the newly described NRD (Δ2) did not affect the ability of Bcl-2 to inhibit EC activation upon TNF and LPS stimulation (Fig. 6*b*, lanes *F2–3* and *D2–3* vs. lanes *B2–3*).The impact of the different Bcl mutants upon TNF-mediated apoptosis was also evaluated as described previously. Results demonstrate that all BH domains, as well as the NRD, are required to maintain the antiapoptotic function of Bcl-2 (Fig. 6c). The percentage of surviving EC after CHX and TNF treatment was comparable between the control pAC and all the Bcl-2 mutant–transfected cells (6%–13% of the transfected cells). In contrast, 55% cell survival was achieved in cells expressing the full-length hBcl-2 (Fig. 6c). Taken together, these results indicate the absence of a complete overlap between the Bcl-2 domains required for protection from apoptosis and those necessary for inhibition of NF-κB activation.

Table 1
Bcl-2 deletion mutants

Proteins	Amino acids deleted	Domains deleted
wt-Bcl-2[A]	0	0
Δ1	6–31	BH4
Δ2	30–79	NRD
Δ4	50–101	BH3
Δ8	138–151	BH1
Δ12	188–203	BH2

BH, *bcl* homology domain; *NRD*, negative regulatory domain. [A]Amino acids from 293 cell line.

Discussion

Programmed cell death or apoptosis of EC can occur *in vivo* and has recently been involved in the pathogenesis of certain pathological conditions, including vasculitis, atherosclerosis, and graft rejection (7, 40–42). Different

stimuli can trigger apoptosis in cultured EC, including growth factor deprivation, hemorrhagic snake venom, inhibition of anchorage-dependent cell spreading, and proinflammatory molecules, *i.e.*, LPS and TNF (43–47). TNF is a potent proinflammatory cytokine always detected at sites of inflammation. One of the functions of TNF is to trigger a pathway leading to programmed cell death. This function has been mapped to a specific cytoplasmic domain within the TNF-R type I protein (p55 TNF-R) that associates with a spectrum of newly defined molecules, including TRADD, FADD/MORT1, and MACH/Flice-1; the latter links TNF stimulus to death effectors (48–50). Although TNF-RI is expressed on EC and plays a crucial role in the acquisition by EC of a proinflammatory phenotype by activating NF-κB, these cells are highly resistant to TNF-mediated cell death (51). This resistance can be modulated by the addition of RNA or protein synthesis inhibitors such as CHX (52). We demonstrate that two antiapoptotic genes, *bcl-2* and *bcl-X_L*, extensively studied in other cell types, also interrupt the cell-death pathway initiated by TNF in CHX-sensitized EC. Although Bcl-2 and Bcl-X_L were described for their ability to counteract a number of proapoptotic programs, including protection of murine aortic endothelial cells from growth factor withdrawal (53), their effect upon TNF-mediated apoptosis is still questioned (14) and has not yet been evaluated in EC. Our data concur with recent results showing that A1, a *bcl-2* homologue, protects EC from TNF-mediated apoptosis (33).

In addition, we demonstrate that the function of Bcl-2 and Bcl-X_L in EC is not limited to their antiapoptotic potential. Our data establishes that both Bcl-2 and Bcl-X_L

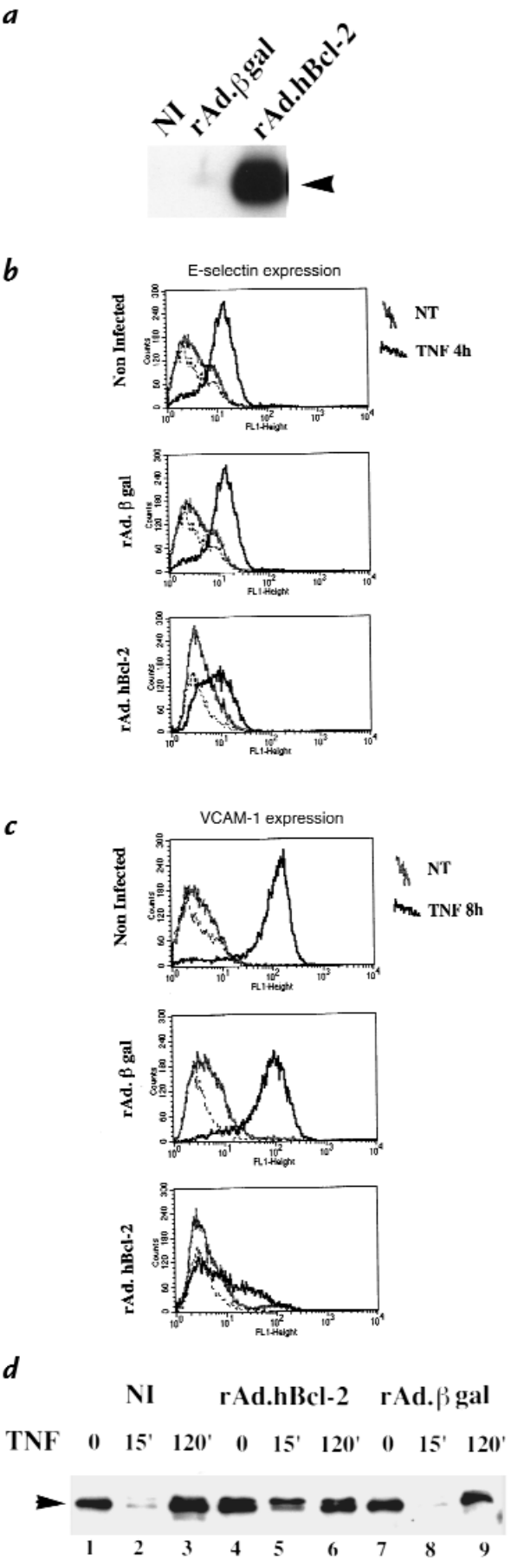

Figure 4

(***a***) Adenoviral-mediated gene transfer of Bcl-2 to HUVEC achieves high levels of expression of the transgene. (***b***) Bcl-2 expression inhibits E-selectin, and (***c***) VCAM-1 inhibits upregulation by inhibiting NF-κB activation at (***d***) a level upstream of IκBα degradation. (*a*) 90%-confluent HUVEC monolayers were either noninfected or infected with the control rAd.β-gal or the rAd.hBcl-2 at a MOI of 100. Forty-eight hours after infection, cell extracts were recovered, and 1.5 μg of protein was run on a SDS-PAGE and checked by Western blot analysis for the expression of the transgene using a rabbit polyclonal anti–human Bcl-2 antiserum. Results show high levels of expression of Bcl-2 in HUVEC infected with the rAd.hBcl-2 (*arrow*). (*b* and *c*) Expression of Bcl-2 in HUVEC significantly decreases TNF-mediated upregulation of E-selectin and VCAM-1 as assessed by flow cytometry. In all cases, E-selectin and VCAM-1 expression levels on nonstimulated HUVEC is represented by a light line, expression after TNF treatment is shown by a dark line, and labeling of an isotype-matched control monoclonal antibody is illustrated by a broken line. (*d*) Western blot analysis of IκBα expression after TNF treatment. Extracts from noninfected (lanes *1–3*), rAd.hBcl-2 (lanes *4–6*), and rAd.β-gal (lanes *7–9*) were recovered before and 15 min and 2 h after TNF treatment, and assayed for IκBα expression. Results show that Bcl-2 expression in HUVEC inhibits the usual IκBα degradation that occurs 15 min after TNF stimulation (lane *4* vs. lanes *2* and *8*). A second, slower migrating form of IκBα is strongly stabilized in the Bcl-2–expressing EC. *HUVEC*, human umbilical vein cells; *MOI*, moiety of infection; *VCAM-1*, vascular cell adhesion molecule-1.

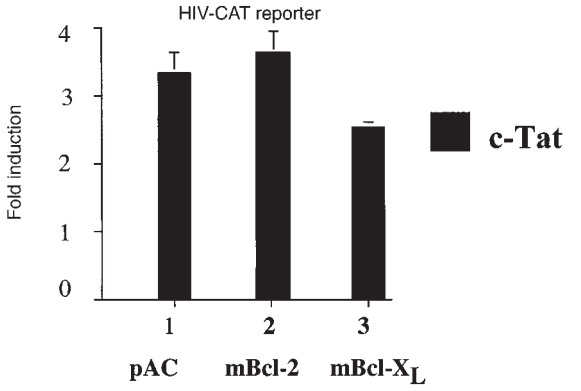

Figure 5

Overexpression of Bcl-2 and Bcl-X_L does not affect the transactivation properties of Sp1. BAEC were cotransfected with mBcl-2, mBcl-X_L, or pAC together with an HIV-CAT reporter. Induction of this reporter was achieved by cotransfecting the viral protein c-Tat, which requires the Sp1 binding sites of the HIV-CAT reporter. Results show no difference in the c-Tat–mediated induction of the HIV-CAT whether the cells were transfected with pAC (lane *1*), Bcl-2 (lane *2*), or Bcl-X_L (lane *3*). Data shown is representative of two experiments performed. Results are expressed in fold induction. *CAT*, chloramphenicol acetyltransferase.

are involved in a complex regulatory network that serves to downregulate EC activation and its associated gene upregulation. We show that expression of Bcl-2 or Bcl-X_L in BAEC inhibits the activation of reporter constructs, including promoters of E-selectin, IL-8, and IκBα used as readouts for EC activation. Although inducibility of these three markers is highly dependent upon activation of NF-κB, additional transcription factors can interfere with their upregulation; *e.g.*, *c-jun* together with activating transcription factor (ATF-2) and cyclic AMP–related element binding (CREB) can synergize with NF-κB for the upregulation of E-selectin and IL-8, respectively (54–56). By showing that the expression of Bcl-2 or Bcl-X_L inhibits the upregulation of the activity of a reporter solely dependent upon NF-κB for its induction, we demonstrate that blockade of EC activation by Bcl-2 and Bcl-X_L relates to the inhibition of the transcription factor NF-κB. The downregulatory effect of Bcl-2 and Bcl-X_L upon EC activation is not limited to stimulation with TNF but applied to all stimuli tested. This agonist-independent inhibitory effect upon activation of NF-κB establishes the broad inhibitory potential of the *bcl* genes in EC. The novel dual role that we describe for *bcl-2* and *bcl-X$_L$* in EC (*i.e.*, protection from apoptosis and blockade of activation through inhibition of NF-κB) parallels our recent findings with the non–*bcl*-related antiapoptotic A20 protein (10, 11), suggesting that in EC, cell-death regulators interfere with NF-κB activation.

We further show in this paper that the inhibitory effect of Bcl-2 upon NF-κB activation relates to stabilization of IκBα, mainly of a slower migrating form. This slower migrating form of IκBα is already seen before the addition of TNF in Bcl-2–expressing EC and is no longer degraded upon TNF stimulation. The identity of this IκBα band, which might constitute a hyperphosphorylated form of the protein, will help to give insights into the precise effect of Bcl-2 expression on IκBα. Experiments are currently designed to check whether this form corresponds to a hyperphosphorylated form of IκBα and to map these phosphorylation sites. This effect of the *bcl* genes (*i.e.*, inhibition of NF-κB activation in EC at a level upstream of IκBα degradation) is demonstrated for the first time and confirms the data of Lin *et al.* (57), showing that Bcl-2 expression inhibits simian virus–induced NF-κB activation by inhibiting translocation of NF-κB to the nucleus. Our data, however, contrasts with other reported *bcl*-mediated inhibitory mechanisms upon NF-κB activation, *i.e.*, inhibition of p65-mediated transactivation (37) or decrease of nuclear levels of the transactivator RelA (p65)/p50 heterodimers to the advantage of the transinhibitor p50/p50 homodimers (58). The situation is even more complex than in other cell types: Bcl-2 or Bcl-X_L expression either has no effect on NF-κB (L929, MCF-7 breast carcinoma cell line, and Jurkat T cells) (59–62) or restores the transactivating potential of NF-κB, as in HeLa cells activated by Fas (63). Our data are the first reported in primary cells. This novel NF-κB inhibitory function of the *bcl* genes could relate either to differences between primary cells and tumor cell lines or alternatively could qualify as cell type–specific function of the *bcl* genes.

The molecular basis of the inhibitory effect of Bcl-2 or Bcl-X_L upon NF-κB activation is still not defined. However, two of the already established functions of these *bcl* genes could explain the inhibitory effect: their protease inhibitor and antioxidant potentials (64, 65). Proteolysis of IκBα in the proteasome that follows its phosphorylation and ubiquitination is an obligatory step for activation of NF-κB (66–68). White and Gilmore (69) have recently identified an interleukin-1β converting enzyme (ICE)-like consensus site within the IκBα protein and suggested that an ICE-like protease activity could be one of the pathways involved in the proteolysis of IκBα. Because Bcl-2 and Bcl-X_L exert their antiapoptotic function by inhibiting proteolytic cleavage of the ICE/ced-3 cysteine proteases (65), it is tempting to speculate that they would also inhibit an ICE-like protease involved in the proteolysis of IκBα in EC, which would explain the accumulation of an eventually hyperphosphorylated form of IκBα. An alternative mechanism relies on the described antioxidant function of Bcl-2 (64). We and others have shown that antioxidants, mainly belonging to the thiol group, are potent inhibitor of NF-κB activation and that this inhibition, like the one achieved by Bcl-2, occurs at an early step upstream of IκBα degradation (36, 70).

The *bcl* genes could also indirectly interrupt the pathway leading to activation of NF-κB by interfering with key signal transducers. Bcl-2 has been shown to interact physically with molecules such as p21Ras, p23Rras, calcineurin, and Raf-1 kinase (71–74). Interaction between Bcl-2 and Raf-1 targets this kinase to the mitochondrial membrane and enhances the antiapoptotic effect of Bcl-2. This interaction requires the BH4 domain of Bcl-2 (75). Raf-1 kinase is an early mediator involved in multiple signaling pathways (TNF, LPS) acting at a level downstream of Ras and upstream of mitogen-activated kinase (MAPK) (76, 77). Cross-talk between MAPK and NF-κB signaling pathways is still debated; however, Raf-1 kinase interaction with Bcl-2 could be of some relevance in the pathway leading to IκBα phosphorylation

and degradation (78, 79). We demonstrate that deletion of the BH4 domain of Bcl-2 abrogates its inhibitory effect upon activation of NF-κB, which argues for a potential involvement of Raf-1 kinase in Bcl-2–mediated inhibition of NF-κB activation. In addition to these hypotheses, our findings showing that expression of Bcl-2 in HUVEC stabilizes a lower (potentially hyperphosphorylated) form of IκBα might yet relate to a novel function of Bcl-2 that can modify IκBα. For instance, Bcl-2–mediated modification of IκBα could prevent its phosphorylation at the specific serine residues, alter adequate ubiquitination, or enhance its resistance to degradation. These hypotheses are currently being tested.

Our data also show that Bcl-2 or Bcl-X_L specifically interrupts activation of NF-κB without affecting another tested transcription factor, *i.e.*, Sp1. We demonstrate that the c-Tat–driven Sp1 HIV reporter is not altered by Bcl-2 or Bcl-X_L expression in EC (39). Recent data from Linette *et al.* (61) showed that in T cells, Bcl-2 expression specifically impairs the transactivation properties of the nuclear factor of activated T cells (NFAT) without affecting other transcription factors, *i.e.*, AP-1, NF-κB, and OCT-1. These data suggest that Bcl-2 and Bcl-X_L have different transcriptional targets depending on the cell type studied.

Bcl-2 family members can either promote or protect from cell death. A feature of these proteins is their ability to homo- or heterodimerize through the conserved Bcl-2 homology domains BH1, BH2, BH3, and BH4 (14, 17, 80, 81). Heterodimerization of Bcl-2 or Bcl-X_L with their proapoptotic partners Bax, Bak, and Bcl-X_S seems to determine the life–death decision of a cell, although some controversies remain (82–84). Our experiments probe whether interaction with Bax is equally as important for the inhibitory effect of Bcl-2 upon NF-κB activation as for regulation of apoptosis. Mutagenesis studies showed that both BH1 and BH2 are required for Bcl-2 to heterodimerize with Bax (85). Our results indicate that in EC, a Bcl-2 mutant lacking the BH1 domain is still able to inhibit NF-κB activation, whereas deletion of the BH2 domain abrogates this function. This result indicates that interaction between Bcl-2 and Bax is either not required for Bcl-2 function in EC or that only BH2 is necessary in EC to interact with Bax. Having mapped the inhibitory function of Bcl-2 on NF-κB activation to the BH4 and BH2 domains of the molecule, we are currently investigating which proteins in EC interact with these domains to sustain this novel *bcl* function.

In contrast with the specific mapping of the Bcl-2 inhibitory effect upon NF-κB activation to BH2 and BH4, all BH domains, as well as the NRD of Bcl-2, were required for protection from apoptosis. This result indicates that the Bcl-2 motifs required for the anti-inflammatory function (BH2, BH4) are also necessary for its antiapoptotic function, but not vice versa; indeed, certain motifs are indispensable for the antiapoptotic function (BH1, BH3, NRD) but have no impact upon the anti-inflammatory function. Our data does not rule out that a unique cellular target interacting with BH2 and BH4 could account for both functions of Bcl-2; this query is being investigated.

From a basic point of view, we demonstrate that Bcl-2 and Bcl-X_L play a major protective role in EC by blocking

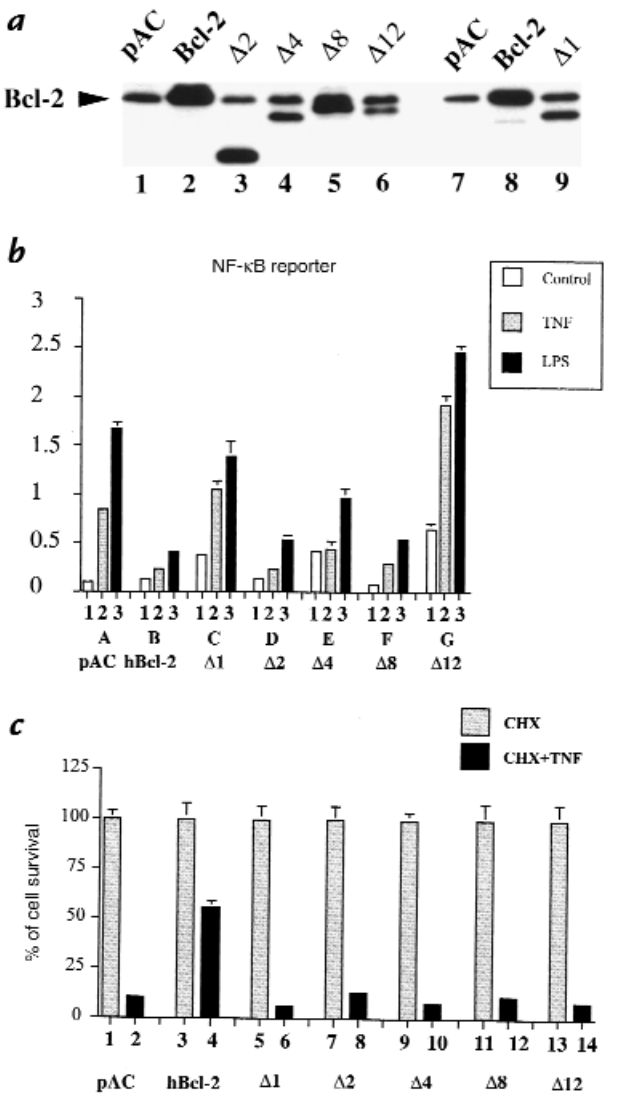

Figure 6

(***a***) Structure/function relationships of Bcl-2 deletion mutants. Expression of Bcl-2 deletion mutants in BAEC. (***b***) The Bcl homology domains BH4 and BH2 are required for the inhibitory effect of Bcl-2 upon NF-κB activation after TNF and LPS stimulation, whereas (***c***) all BH domains and the NRD are required for the antiapoptotic function of Bcl-2. (*a*) Immunoblot detection of human wild-type and deletion mutants of Bcl-2 (lanes *2–6*) in BAEC-transfected cells, using a polyclonal anti–Bcl-2 antibody. Mutant Δ1 is not recognized by this antibody and was detected using a monoclonal anti–Bcl-2 antibody (lane *9*). (*b*) BAEC were cotransfected with 0.3 μg of β-gal, 0.6 μg of NF-κB reporter along with 0.7 μg of pAC (lanes *A1–3*), human Bcl-2 (lanes *B1–3*), Δ1 (lanes *C1–3*), Δ2 (lanes *D1–3*), Δ4 (lanes *E1–3*), Δ8 (lanes *F1–3*), and Δ12 (lanes *G1–3*). Cells were stimulated with 100 U/ml TNF (lanes *2*) or 100 ng/ml LPS (lanes *3*) Overexpression of Δ2, Δ4, or Δ8 inhibits the induction by TNF or LPS of a NF-κB reporter, in contrast to Δ1 or Δ12. Graph shown is representative of four experiments. Results are expressed in RLU. Error bars represent ± SE. (*c*) BAEC were cotransfected with a CMVβ-gal reporter (0.5 μg) and 1 μg of pAC (lanes *1* and *2*), hBcl-2 (lanes *3* and *4*), Δ1 (lanes *5* and *6*), Δ2 (lanes *7* and *8*), Δ4 (lanes *9* and *10*), Δ8 (lanes *11* and *12*), Δ12 (lanes *13* and *14*). CHX was added to transfected cells (*all lanes*) that were subsequently stimulated (*even lanes*) or not (*odd lanes*) with TNF for 12 h . The percent cell survival was calculated as described in Methods. Results demonstrate that all the Bcl-2 deletion mutants lose their ability to protect EC from CHX-TNF mediated apoptosis. Error bars represent ± SE. Graph shown is representative of three experiments. *NRD*, negative regulatory domain.

TNF-mediated apoptosis in addition to inhibiting EC activation through blockade of NF-κB activation. The mechanism by which inhibition of NF-κB is accomplished (*i.e.*, stabilization of IκBα) is novel.

From a therapeutic standpoint, blockade of NF-κB has been suggested as a means of preventing proinflammatory consequences of EC activation implicated in different pathologies, including allograft and xenograft rejection (4, 86). Bcl-2 and Bcl-X_L represent prime candidates for genetic engineering of EC to achieve this purpose. In support of this approach is our data showing that expression of Bcl-2 and Bcl-X_L in EC of long-term surviving xenografts is associated with the absence of apoptosis, inflammation, and atherosclerosis, whereas they are not expressed in EC of rejecting xenografts (7). Although indirect, these results further argue for their protective and broad anti-inflammatory potential *in vivo* as well as establish the safety of their use.

Acknowledgments

We are grateful to Timothy Behrens, Gabriel Nunez, Tristram G. Parslow, and J. Reed for providing us with the murine Bcl-2 and Bcl-X_L, the human Bcl-2, and the deletion mutants expression plasmids. We acknowledge Joseph Anrather for providing us with the p65 expression plasmid and for valuable advice. We also thank Maria-Beatriz Arvelo and E. Czismadia for help with the virus purification and skilled technical assistance in cell culture. We are also grateful to Shane T. Grey for criticism and advice.

1. Pober, J.S., *et al*. 1990. The potential roles of vascular endothelium in immune reactions [review]. *Hum. Immunol.* **28**:258–262.
2. Cotran, R.S., and Pober, J.S. 1990. Cytokine-endothelial interactions in inflammation, immunity, and vascular injury. *J. Am. Soc. Nephrol.* **1**:225–235.
3. Bach, F.H., *et al*. 1994. Endothelial cell activation and thromboregulation during xenograft rejection [review]. *Immunol. Rev.* **141**:5–30.
4. Gimbrone, M.A.J. 1995. Vascular endothelium: an integrator of pathophysiologic stimuli in atherosclerosis [review]. *Am. J. Cardiol.* **75**:67B–70B.
5. Read, M.A., Whitley, M.Z., Williams, A.J., and Collins, T. 1994. NF-κB and IκBα — an inducible regulatory system in endothelial activation. *J. Exp. Med.* **179**:503–512.
6. Collins, T., *et al*. 1995. Transcriptional regulation of endothelial cell adhesion molecules — NF-κB and cytokine-inducible enhancers. *FASEB J.* **9**:899–909.
7. Bach, F.H., *et al*. 1997. Accommodation of vascularized xenografts: expression of "protective genes" by donor endothelial cells in a host Th2 cytokine environment. *Nat. Med.* **3**:196–204.
8. Opipari, A.J., Boguski, M.S., and Dixit, V.M. 1990. The A20 cDNA induced by tumor necrosis factor alpha encodes a novel type of zinc finger protein. *J. Biol. Chem.* **265**:14705–14708.
9. Opipari, A.J., Hu, H.M., Yabkowitz, R., and Dixit, V.M. 1992. The A20 zinc finger protein protects cells from TNF cytotoxicity. *J. Biol. Chem.* **267**:12424–12427.
10. Ferran, C., and Stroka, D.M. 1997. Adenovirus-mediated gene transfer of A20 renders endothelial cells resistant to activation: a means of evaluating the role of EC activation in xenograft rejection. *Transplant Proc.* **29**:879–880.
11. Cooper, J.T., *et al*. 1996. A20 blocks endothelial cell activation through a NF-κB-dependent mechanism. *J. Biol. Chem.* **271**:18068–18073.
12. LeBrun, D.P., Warnke, R.A., and Cleary, M.L. 1993. Expression of bcl-2 in fetal tissues suggests a role in morphogenesis. *Am. J. Pathol.* **142**:743–753.
13. Lu, Q.L., Poulsom, R., Wong, L., and Hanby, A.M. 1993. Bcl-2 expression in adult and embryonic non-haematopoietic tissues. *J. Pathol.* **169**:431–437.
14. Reed, J.C. 1994. Bcl-2 and the regulation of programmed cell death. *J. Cell Biol.* **124**:1–6.
15. Boise, L.H., *et al*. 1993. bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. *Cell*. **74**:597–608.
16. Oltvai, Z.N., Milliman, C.L., and Korsmeyer, S.J. 1993. Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. *Cell*. **74**:609–619.
17. Sedlak, T.W., *et al*. 1995. Multiple Bcl-2 family members demonstrate selective dimerizations with Bax. *Proc. Natl. Acad. Sci. USA*. **92**:7834–7838.
18. Wang, H.G., Takayama, S., Rapp, U.R., and Reed, J.C. 1996. Bcl-2 interacting protein, BAG-1, binds to and activates the kinase Raf-1. *Proc. Natl. Acad. Sci. USA*. **93**:7063–7068.
19. White, E. 1996. Life, death, and the pursuit of apoptosis [review]. *Genes Dev*. **10**:1–15.
20. Vanhaesebroek, B., *et al*. 1993. Effect of bcl-2 proto-oncogene on cellular sensitivity to tumor necrosis factor-mediated cytotoxicity. *Oncogene*. **8**:1075–1081.
21. Hennet, T., Bertoni, G., Richter, C., and Peterhans, E. 1993. Expression of BCL-2 protein enhances the survival of mouse fibrosarcoid cells in tumor necrosis factor-mediated cytotoxicity. *Cancer Res.* **53**:1456–1460.
22. Pober, J.S., *et al*. 1986. Two distinct monokines, interleukin 1 and tumor necrosis factor, each independently induce biosynthesis and transient expression of the same antigen on the surface of cultured human vascular endothelial cells. *J. Immunol.* **136**:1680–1687.
23. Cotran, R.S., Gimbrone, M.A., Jr., Bevilacqua, M.P., Mendrick, D.L., and Pober, J.S. 1986. Induction and detection of a human endothelial activation antigen in vivo. *J. Exp. Med.* **164**:661–666.
24. Gimbrone, M.J., Cotran, R.S., and Folkman, J. 1974. Human vascular endothelial cells in culture. Growth and DNA synthesis. *J. Cell Biol.* **60**:673–684.
25. Brostjan, C., *et al*. 1996. Deletion analysis of the porcine E-selectin promoter. *Transplant Proc.* **28**:649–651.
26. de Martin, R., *et al*. 1993. Cytokine-inducible expression in endothelial cells of an IκBα-like gene is regulated by NF-κB. *EMBO J.* **12**:2773–2779.
27. Bilbao, G., *et al*. 1999. Genetic cytoprotection of human endothelial cells during the preservation time with an adenoviral vector encoding the anti-apoptotic human Bcl-2 gene. *Transplant Proc.* In press.
28. Graham, F.L., and Prevec, L. 1991. Manipulation of adenovirus vectors. In *Methods in molecular biology: gene transfer and expression protocols*. 7th ed. E.J. Murray, editor. Press Inc. Clifton, NJ. 109–128.
29. McGrory, W.J., Bautista, D.S., and Graham, F.L. 1988. A simple technique for the rescue of early region I mutations into infectious human adenovirus type 5. *Virology.* **163**:614–617.
30. Bett, A.J., Prevec, L., and Graham, F.L. 1993. Packaging capacity and stability of human adenovirus type 5 vectors. *J. Virol.* **67**:5911–5921.
31. Kerr, J.F., Wyllie, A.H., and Currie, A.R. 1972. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics [review]. *Br. J. Cancer*. **26**:239–257.
32. McCarthy, J.V., Ni, J., and Dixit, V.M. 1998. RIP2 is a novel NF-κB-activating and cell death-inducing kinase. *J. Biol. Chem.* **273**:16968–16975.
33. Karsan, A., Yee, E., and Harlan, J.M. 1996. Endothelial cell death induced by tumor necrosis factor a is inhibited by the Bcl-2 family member A1. *J. Biol. Chem.* **271**:27201–27204.
34. Pober, J.S., and Cotran, R.S. 1990. Cytokines and endothelial cell biology. *Physiol. Rev*. **70**:427–451.
35. Collins, T., Palmer, H.J., Whitley, M.Z., Neish, A.S., and Williams, A.J. 1993. A common theme in endothelial cell activation: insights from the structural analysis of the genes for E-selectin and VCAM-1. *Trends Card. Med.* **3**:92–97.
36. Ferran, C., *et al*. 1995. Inhibition of NF-κB by pyrrolidine dithiocarbamate blocks endothelial cell activation. *Biochem. Biophys. Res. Commun.* **214**:212–223.
37. Grimm, S., Bauer, M.K.A., Bauerle, P.A., and Schultze-Osthoff, K. 1996. Bcl-2 down-regulates the activity of transcription factor NF-κB induced upon apoptosis. *J. Cell. Biol.* **134**:13–23.
38. Wrighton, C.J., *et al*. 1996. Inhibition of endothelial cell activation by adenovirus-mediated expression of IκBα, an inhibitor of the transcription factor NF-κB. *J. Exp. Med.* **183**:1013–1022.
39. Zimmermann, K., *et al*. 1991. trans-activation of the HIV-1 LTR by the HIV-1 Tat and HTLV-I Tax proteins is mediated by different cis-acting sequences. *Virology*. **182**:874–878.
40. Collins, T. 1993. Biology of disease – endothelial nuclear factor-κB and the initiation of the atherosclerotic lesion. *Lab. Invest.* **68**:499–508.
41. Laine, J., Etelamaki, P., Holmberg, C., and Dunkel, L. 1997. Apoptotic cell death in human chronic allograft rejection. *Transplantation*. **63**:101–105.
42. Thompson, C. 1995. Apoptosis in the pathogenesis and treatment of disease. *Science*. **267**:1456–1462.
43. Abello, P.A., Fidler, S.A., Bulkley, G.B., and Buchman, T.G. 1994. Antioxidants modulate induction of programmed endothelial cell death (apoptosis) by endotoxin. *Arch. Surg.* **129**:134–140.
44. Buchman, T.G., Abello, P.A., Smith, E.H., and Bulkley, G.B. 1993. Induction of heat shock response leads to apoptosis in endothelial cells previously exposed to endotoxin. *Am. J. Physiol.* **265**:165–170.
45. Araki, S., Ishida, T., Yamamoto, T., Kaji, K., and Hayashi, H. 1993. Induction of apoptosis by hemorrhagic snake venom in vascular endothelial cells. *Biochem. Biophys. Res. Commun.* **190**:148–153.
46. Re, F., *et al*. 1994. Inhibition of anchorage-dependent cell spreading triggers apoptosis in cultured human endothelial cells. *J. Cell Biol.* **127**:537–546.
47. Robaye, B., Mosselmans, R., Fiers, W., Dumont, J.E., and Galand, P. 1991.

Tumor necrosis factor induces apoptosis (programmed cell death) in normal endothelial cells in vitro. *Am. J. Pathol.* **138**:447–453.
48. Chinnayian, A.M., O'Rourke, K., Tewari, M., and Dixit, V. 1995. FADD, a novel death domain-containing protein interacts with the death domain of Fas and initiates apoptosis. *Cell*. **81**:505–512.
49. Hsu, H., Xiong, J., and Goeddel, D.V. 1995. The TNF-receptor 1-associated protein TRADD signals cell death and NF-κB activation. *Cell*. **81**:495–504.
50. Hsu, H., Shu, H.B., Pan, M.G., and Goeddel, D.V. 1996. TRADD-TRAF2 and TRADD-FADD interactions define two distinct TNF receptor 1 signal transduction pathways. *Cell*. **84**:299–308.
51. Pohlman, T.H., and Harlan, J.M. 1989. Human endothelial cell response to lipopolysaccharide, interleukin-1, and tumor necrosis factor is regulated by protein synthesis. *Cell. Immunol.* **119**:41–52.
52. Polunovsky, V.A., Wendt, C.H., Ingbar, D.H., Peterson, M.S., and Bitterman, P.B. 1994. Induction of endothelial cell apoptosis by TNF-α: modulation by inhibitors of protein synthesis. *Exp. Cell Res.* **214**:584–594.
53. Kondo, S., *et al*. bcl-2 gene prevents apoptosis of basic fibroblast growth factor-deprived murine aortic endothelial cells. *Exp. Cell Res.* **213**:428–32.
54. Kaszubska, W., *et al*. 1993. Cyclic AMP-independent ATF family members interact with NF-κB and function in activation of the E-selectin promoter in response to cytokines. *Mol. Cell. Biol.* **13**:7180–7190.
55. DeLuca, L.G., Johnson, D., Whitley, M., Collins, T., and Pober, J. 1994. cAMP and tumor necrosis factor competitively regulate transcriptional activation through and nuclear factor binding to the cAMP-responsive element/activating transcription factor element of the endothelial leukocyte adhesion molecule-1 (E-selectin) promoter. *J. Biol. Chem.* **269**:19193–19196.
56. Stein, B., and Baldwin, A.S.J. 1993. Distinct mechanisms for regulation of the Interleukin-8 gene involve synergism and cooperativity between C/EBP and NF-κB. *Mol. Cell. Biol.* **13**:7191–7198.
57. Lin, K., *et al*. 1995. Thiol agents Bcl-2 identify an alphavirus-induced apoptotic pathway that requires activation of the transcription factor NF-kappa B.*J. Cell Biol.* **131**:1149–1161.
58. Ivanov, V.N., Deng, G., Podack, E.R., and Malek, T.R. 1995. Pleiotropic effects of Bcl-2 on transcription factors in T cells — potential role of NF-kappa-B P50-P50 for the anti-apoptotic function of Bcl-2. *Int. Immunol.* **7**:1709–1720.
59. Allbrecht, H., Tschopp, J., and Jongeneel, V. 1994. Bcl-2 protects from oxidative damage and apoptotic cell death without interfering with activation of NF-κB by TNF. *FEBS Lett.* **351**:45–48.
60. Jäättela, M., Benedict, M., Tewari, M., Shayman, J.A., and Dixit, V.M. 1995. Bcl-x and Bcl-2 inhibit TNF and Fas-induced apoptosis and activation of phospholipase A2 in breast carcinoma cells. *Oncogene*. **10**:2297–305.
61. Linette, G.P., Li, Y., Roth, K., and Korsmeyer, S.J. 1996. Cross talk between cell death and cell cycle progression: BCL-2 regulates NFAT-mediated activation. *Proc. Natl. Acad. Sci. USA*. **93**:9545–9552.
62. Dbaibo, G.S., *et al*. 1997. Cytokine response modifier A (CrmA) inhibits ceramide formation in response to tumor necrosis factor (TNF)-α: CrmA and Bcl-2 target distinct components in the apoptotic pathway. *J. Exp. Med.* **185**:481–490.
63. Mandal, M., *et al*. 1996. Bcl-2 prevents CD95 (Fas/APO-1)-induced degradation of lamin B and poly (ADP-ribose) polymerase and restores the NF-κB signaling pathway. *J. Biol. Chem.* **271**:30354–30359.
64. Hockenbery, D.M., Oltvai, Z.N., Yin, X.M., Milliman, C.L., and Korsmeyer, S.J. 1993. Bcl-2 functions in an antioxidant pathway to prevent apoptosis. *Cell*. **75**:241–251.
65. Chinnaiyan, A.M., *et al*. 1996. Molecular ordering of the cell death pathway. *J. Biol. Chem.* **271**:4573–4576.
66. Traenckner, E.B.M., and Baeuerle, P.A. 1995. Appearance of apparently ubiquitin-conjugated IκB-α during its phosphorylation-induced degradation in intact cells. *J. Cell Sci. Suppl.* **19**:79–84.
67. Traenckner, E.B.M., *et al*. 1995. Phosphorylation of human IκB-α on serines 32 and 36 controls IκB-α proteolysis and NF-κB activation in response to diverse stimuli. *EMBO J.* **14**:2876–2883.
68. Traenckner, E.B.M., Wilk, S., and Bauerle, P. 1994. A proteasome inhibitor prevents activation of NF-κB and stabilizes a newly phosphorylation form of IκBα that is still bound to NF-κB. *EMBO J.* **13**:5433–5441.
69. White, D.W., and Gilmore, T.D. 1996. Bcl-2 and CrmA have different effects on transformation, apoptosis and the stability of IκB-α in chicken spleen cells transformed by temperature-sensitive v-Rel oncoproteins. *Oncogene*. **13**:891–899.
70. Schreck, R., Meier, B., Mannel, D.N., Droge, W., and Baeuerle, P.A. 1992. Dithiocarbamates as potent inhibitors of nuclear factor κB activation in intact cells. *J. Exp. Med.* **175**:1181–1194.
71. Chen, C.Y., and Faller, D.V. 1996. Phosphorylation of Bcl-2 protein and association with p21Ras in Ras-induced apoptosis. *J. Biol. Chem.* **271**:2376–2379.
72. Fernandez-Sarabia, M., and Bischoff, J.R. 1993. Bcl-2 associates with the ras-related protein R-ras p23. *Nature*. **366**:274–275.
73. Wang, H.G., *et al*. 1994. Apoptosis regulation by interaction of Bcl-2 protein and Raf-1 kinase. *Oncogene*. **9**:2751–2756.
74. Shibasaki, F., Kondo, E., Akagi, T., and McKeon, F. 1997. Suppression of signalling through transcription factor NF-AT interactions between calcineurin and Bcl-2. *Nature*. **386**:728–731.
75. Wang, H.G., Rapp, U.R., and Reed, J.C. 1996. Bcl-2 targets the protein kinase Raf-1 to mitochondria. *Cell*. **87**:629–638.
76. Hambleton, J., McMahon, M., and DeFranco, A.L. 1995. Activation of Raf-1 and mitogen-activated protein kinase in murine macrophages partially mimics lipopolysaccharide-induced signaling events. *J. Exp. Med.* **182**:147–54.
77. Jelinek, T., Dent, P., Sturgill, T.W., and Weber, M.J. 1996. Ras-induced activation of RAF-1 is dependent on tyrosine phosphorylation. *Mol. Cell. Biol.* **16**:1027–1034.
78. Li, S.F., and Sedivy, J.M. 1993. Raf-1 protein kinase activates the NF-κB transcription factor by dissociating the cytoplasmic NF-κB-IκB complex. *Proc. Natl. Acad. Sci. USA.* **90**:9247–9251.
79. Chirillo, P., *et al*. 1996. Hepatitis B virus Px activates NF-κB-dependent transcription through a Raf-independent pathway. *J. Virol.* **70**:641–646.
80. Zha, J., Harada, H., Yang, E., Jockel, J., and Korsmeyer, S.J. 1996. Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-XL. *Cell*. **87**:619–628.
81. Sato, T., *et al*. 1994. Interactions among members of the Bcl-2 protein family analyzed with a yeast two-hybrid system. *Proc. Natl. Acad. Sci. USA.* **91**:9238–9242.
82. Cheng, E.H.Y., Levine, B., Boise, L.H., Thompson, C.B., and Hardwick, J.M. 1996. Bax-independent inhibition of apoptosis by Bcl-x_L. *Nature*. **379**:554–556.
83. Hunter, J.J., Bond, B.L., and Parslow, T.G. 1996. Functional dissection of the human Bcl-2 protein: sequence requirements for inhibition of apoptosis. *Mol. Cell. Biol.* **16**:877–883.
84. Yin, D.X., and Schimke, R.T. 1996. Inhibition of apoptosis by overexpressing Bcl-2 enhances gene amplification by a mechanism independent of aphidicolin pretreatment. *Proc. Natl. Acad. Sci. USA.* **93**:3394–3398.
85. Yin, X.M., Oltvai, Z.N, and Korsmeyer, S.J. 1994. BH1 and BH2 domains of Bcl-2 are required for inhibition of apoptosis and heterodimerization with Bax. *Nature*. **369**:321–323.
86. Bach, F.H., Winkler, H., Ferran, C., Hancock, W.W., and Robson, S.C. 1996. Delayed xenograft rejection. *Immunol. Today.* **17**:379–383.