Endogenously generated or exogenously supplied nitric oxide (NO)-induced apoptotic cell death in the mouse macrophage cell line RAW 264.7. Apoptotic signaling caused an early accumulation of the tumor suppressor p53 prior to DNA fragmentation. Contrary to the notion of specific activating signals, inhibitory transcription mechanisms largely remain unknown. Therefore, RAW 264.7 macrophages were stably transfected with human Bcl-2, an anti-apoptotic protein. Bcl-2 transfectants showed substantial protection from cell death induced following the exposure to NO donors such as S-nitroso glutathione (GSNO) and spermine-NO. In contrast, in RAW 264.7 parent or in neomycin control-transformed cells, these NO donors induced internucleosomal DNA cleavage in a dose-dependent manner. Similarly, expression of the inducible NO synthase in response to lipopolysaccharide and interferon-γ also caused apoptosis in RAW macrophages and neo controls within 24 h. In contrast, Bcl-2 transfectants appeared highly resistant, although inducible NO synthase levels increased along with concomitant nitrite production similar to control cells. The expression of p53 and Bax was also explored in controls and Bcl-2 transfectants after GSNO addition. GSNO induced p53 expression in Bcl-2 transfectants at levels comparable with nontransfected RAW macrophages. Moreover, GSNO induced increases in the steady-state levels of Bax protein in parental and Bcl-2-transfected cells. We conclude therefore, that Bcl-2 acts downstream of p53, presumably nullifying the NO-mediated increase in Bax protein in RAW 264.7 cells.

Nitric oxide (NO) is generated from L-arginine by a family of constitutive or cytokine inducible NO synthase (NOS) isoenzymes (1). Signaling of NO via rich redox and additive chemistry can be broadly discriminated as cGMP-dependent versus independent. Activation of soluble guanylyl cyclase is considered a main physiological event of NO production. A pathological role for NO has been suggested for several diseases, including immune-mediated cytotoxicity, septic shock, neurotoxicity, β-cell destruction, autoimmune diseases, and chronic inflammation (see Ref. 2 for references). Besides activation of NOS, a chemically heterogeneous group of NO-releasing compounds preserve NO in their molecular structure and exhibit biological activity after NO liberation (3). These drugs are valuable biochemical tools because they permit the investigation of NO’s role in signaling pathways without interfering with second messenger systems involved in NOS activation or NOS induction.

In several systems, the progressive intra- or extracellular generation of NO results in cytostasis and/or cytotoxicity. Multiple target interactions, with protein thiol groups, iron-sulfur proteins, or a direct DNA damage, with either NO, ONOO−, or various NO oxidation products serving as the destructive species, have been implicated in NO-mediated cytotoxicity (4). In peritoneal macrophages (5, 6), β-cells (7), thymocytes (8), chondrocytes (9), and some other types of cells, NO-mediated cell death occurs through mechanisms consistent with apoptosis. This comprises morphological criteria such as chromatin condensation, nuclear fragmentation, and biochemical changes, most particularly as a result of internucleosomal genomic digestion (4). Our own Western blot data suggest accumulation of the tumor suppressor p53 in response to endogenously generated or exogenously supplied NO as an early accompanying event (10). Confirmingly, exposure of some human cells to NO, generated from an NO donor or from overexpression of inducible-type NOS, results in p53 protein accumulation (11). Expression of wild-type p53 seems to be closely linked to apoptosis caused by most DNA-damaging agents (12). This nuclear phosphoprotein, originally characterized as a tumor suppressor protein, acts as a checkpoint control in the cell cycle, permitting the repair of damaged DNA. However, in case of severe DNA damage, p53 also signals apoptosis in at least some types of cells. Intriguingly, not all cells expressing inducible NO synthase activity, such as rat mesangial cells (13), are toxicologically affected. Cellular susceptibility toward NO varies between different types of cells and tissues. This is best exemplified in the brain, where NO-producing cells are selectively spared from toxicity (14).

Cell death is controlled, in part, by a complex interplay between regulatory proteins. The prototypic regulator of mammalian cell death is the proto-oncogene bcl-2 (15). The bcl-2 gene was first discovered because of its involvement in t(14:18) chromosomal translocations found in follicular B-cell lymphomas, where it contributes to neoplastic expansion of germinal center B-cells by prolonging cell survival rather than by accelerating the rate of cell proliferation (16, 17). Constitutive expression of high levels of Bcl-2 protein by transfection experiments has proven that Bcl-2 can protect many cell types from apoptosis induced by exposure to a wide variety of adverse conditions and stimuli. This suggests that the protein controls a distal step in a signaling pathway leading to apoptotic cell death. Despite reports that Bcl-2 functions as an antioxidant (18), modulates some aspects of nuclear transport (19), inter-

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† To whom correspondence should be addressed: University of Erlangen-Nürnberg, Faculty of Medicine, Dept. of Medicine IV, Loschgestraße 8, 91054 Erlangen, Germany. Tel.: 49-9131-856311; Fax: 49-9131-859202.

The abbreviations used are: NO, nitric oxide; NOS, nitric oxide synthase; iNOS, inducible nitric oxide synthase; GSNO, S-nitroso glutathione; IFN-γ, murine interferon-γ; LPS, lipopolysaccharide.
venes in calcium signaling (20), and associates with several other proteins (21), many questions regarding its biochemical mechanism of action remain unanswered.

With the notion that Bcl-2 promotes cell survival in cases of cell death mediated by reactive oxygen species, we were interested in exploring the effect of Bcl-2 on apoptosis induced by reactive nitrogen species. Our data demonstrate that Bcl-2 protects RAW 264.7 macrophages against apoptosis induced in response to nitric oxide. Notably, Bcl-2 blocks NO-mediated apoptosis, while accumulation of the tumor suppressor p53, an early indicator of NO-initiated apoptotic signaling, remains unperturbed.

EXPERIMENTAL PROCEDURES

Materials—Spermine-NO was provided by Biotrend, Cologne, Germany. Hoechst dye 33258, protein A-Sepharose, diphenylamine, bovine serum albumin, LPS (Escherichia coli serotype 0127:B8), and sodium nitroprusside were purchased from Sigma, Deisenhofen, Germany. [153-153] I-Protein A (10 mCi/mg) was bought from DuPont NEN, Dreieich, Germany. Recombinant murine interferon-γ was provided by Boehringer Mannheim, Mannheim, Germany. Genetin was from Life Technologies, Berlin, Germany. The mouse monoclonal anti-human Bcl-2 antibody (clone 124) was obtained from DAKO, Hamburg, Germany. The rabbit polyclonal anti-mouse Bax antibody was purchased from Santa Cruz, Ismaning, Germany. RPMI 1640, cell culture supplements, and fetal calf serum were ordered from Biochrom, Berlin, Germany. All other chemicals were of the highest grade of purity commercially available.

Cell Culture—The mouse monocyte/macrophage cell line RAW 264.7 was maintained in RPMI 1640 supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated fetal calf serum (complete RPMI). All experiments were performed using complete RPMI.

Transfection—The plasmid pRc/CMVbcl2 was constructed by subcloning the human bcl-2 gene into the HindII and XbaI sites of pRc/CMV (Invitrogen). For transfection, 2 × 10⁶ RAW 264.7 macrophages were grown in a 10-cm Petri dish. After overnight adhesion, cells were transfected either with 20 μg of the plasmid pRc/CMVbcl2 or a control plasmid lacking the bcl-2 gene (neo control) by using the calcium phosphate precipitation method (22). Stable transfected single clones were picked randomly after selection with 400 μg/ml G418. RAW 264.7 cells were grown in RPMI 1640 supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated fetal calf serum (complete RPMI). All experiments were performed using complete RPMI.

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cally following the method as described for p53. Filters were incubated with the mouse anti-human Bcl-2 antibody (clone 124, 1:100) or the rabbit anti-mouse Bax antibody (clone P-19, 0.5 μg/ml) overnight at 4°C. Detection was by horseradish peroxidase-conjugated goat anti-mouse monoclonal antibody (1:5000) or horseradish peroxidase-conjugated goat anti-rabbit monoclonal antibodies (1:5000) for 1 h at room temperature using the ECL method (Amersham Corp.). Video densitometry quantification was performed with a Vilber Lourmat BIO1DV 6.02c.

Statistical Analyses—Each experiment was performed at least three times and statistical analysis was performed using the two-tailed Student's t test. Otherwise representative data are shown.

RESULTS

Stable Expression of Human Bcl-2 in RAW 264.7 Macrophages—NO-susceptible mouse RAW 264.7 macrophages were transfected with the plasmid pRcCMVbcl2 also carrying a neomycin resistance gene. Stable Bcl-2 protein expression was assessed by Western blot analysis with an anti-human Bcl-2 specific antibody. Four clones termed Rbcl2-2, Rbcl2-14, Rbcl2-18, and Rbcl2-25 showed substantial Bcl-2 expression (Fig. 1). Clone Rbcl2-14 expressed the highest levels of Bcl-2 protein among these four stable transfectants, whereas the amount of Bcl-2 in clone Rbcl2-2 was the lowest of these transfectants. As expected, parental RAW 264.7 cells as well as the neomycin-vector control-transfected clone (Rneo-2) lacked any human Bcl-2. Comparable cell morphology and proliferative behavior was evident for transfectants and parent RAW 264.7 macrophages (data not shown).

Bcl-2 Protects against NO Donor-induced Apoptosis—NO-releasing compounds permit the investigation of NO signaling irrespective of NOS involvement. Within 8 h after exposure to NO donors, RAW 264.7 parent cells, a neomycin-vector control transfected clone (Rneo-2) and one Bcl-2 transfected (Rbcl2-14) (8 × 10⁶ cells) were cultured as outlined under "Experimental Procedures" and incubated for 8 h with 1 mM GSNO or vehicle (control). DNA fragments were isolated, resolved on 1% agarose gels, stained with ethidium bromide, and visualized by UV transillumination.

FIG. 2. Bcl-2 blocks DNA fragmentation in response to NO donor exposure. Cells, cultured as outlined under "Experimental Procedures," were incubated for 8 h with GSNO (upper panel) or spermine-NO (lower panel). DNA fragmentation was measured with the diphenylamine reaction. Data are the means ± S.E., n ≥ 4.

Statistical Analyses—Each experiment was performed at least three times and statistical analysis was performed using the two-tailed Student's t test. Otherwise representative data are shown.

FIG. 3. Bcl-2 blocks NO-induced oligonucleosomal DNA fragmentation. RAW 264.7 parent cells, a neomycin-vector control transfectant clone (Rneo-2) and one Bcl-2 transfectant (Rbcl2-14) (8 × 10⁶ cells) were cultured as outlined under "Experimental Procedures" and incubated for 8 h with 1 mM GSNO or vehicle (control). DNA fragments were isolated, resolved on 1% agarose gels, stained with ethidium bromide, and visualized by UV transillumination.

FIG. 4. Delayed nitric oxide-induced apoptosis in Bcl-2-transfected RAW 264.7 macrophages. Bcl-2-transfected RAW 264.7 macrophages (clone Rbcl2-14) were exposed to increasing GSNO concentrations for 8 or 24 h. DNA fragmentation was quantitated using the diphenylamine assay. Values are means ± S.E. of four individual experiments.
titative fragmentation revealed about 35% DNA fragmentation in response to GSNO (1 mM), while spermine-NO (500 μM) produced 50% DNA degradation. Similar results were obtained with neomycin control-transformed cells (Rneo-2). In contrast, exposure of Bcl-2-transfected cells to GSNO or spermine-NO resulted in substantially less DNA cleavage. Clone Rbcl2-14, which contained the highest Bcl-2 levels, remained viable with no evidence of DNA fragmentation within the 8-h incubation period. Clone Rbcl2-2, which expressed the lowest amount of Bcl-2 among the stable transfectants, showed the least protection. In these cells 1 mM GSNO produced 10% DNA fragmentation. Intermediate responses were obtained for the clones Rbcl2-18 and Rbcl2-25, which contained moderately elevated levels of Bcl-2 protein.

Corroboratively, suppression of DNA fragmentation by Bcl-2 overexpression measured by the diphenylamine assay was verified by agarose gel electrophoresis. As demonstrated in Fig. 3, RAW 264.7 parent cells as well as a neomycin-vector control transfectant (Rneo-2) responded to GSNO treatment with the formation of a characteristic DNA ladder visualized after 8 h. In contrast, under the same conditions digonucleosomal DNA fragments were absent in the Bcl-2-transfected clone Rbcl2-14.

Fig. 5. Bcl-2 expression blocks apoptosis after iNOS induction. RAW 264.7 macrophages, a neomycin-vector control clone (Rneo-2), and Bcl-2 transfectants were incubated for 24 h with vehicle or 10 μg/ml LPS and 100 units/ml IFN-γ, fixed, and stained with the DNA-specific fluorochrome Hoechst dye 33258. A, nitrite was determined in the culture supernatant with the Griess reaction (upper panel), and apoptotic cells exhibiting characteristic chromatin condensation were counted by fluorescence microscopy (lower panel). Values are means ± S.E. of four independent experiments. B, control RAW 264.7 macrophages, untreated Bcl-2-transfectants, as well as LPS/IFN-γ-stimulated Bcl-2-transfectants (clone Rbcl2-14) showed normal nuclear morphology characterized by diffuse chromatin structure. LPS/IFN-γ-stimulated RAW 264.7 macrophages exhibited apoptotic chromatin condensation (arrowheads). The figures are representative for four different experiments.

however, protection was less pronounced with a 24-h exposure. These data imply that overexpression of Bcl-2 delays but does not prevent the NO-mediated induction of apoptosis.

Bcl-2 Protects against NO Toxicity after iNOS Induction—The combination of LPS and IFN-γ results in NOS induction and apoptosis in RAW 264.7 cells. Both nitrite accumulation in the cell supernatant and apoptosis were blocked by the NOS inhibitor Nω-monomethyl-L-arginine (Fig. 5). To determine the effect of Bcl-2 on iNOS induction, endogenous nitrite formation, and apoptosis, RAW 264.7 cells and the Bcl-2 transfectants were stimulated with LPS/IFN-γ for 24 h. Cells responded with increased nitrite levels after LPS/IFN-γ challenge, with insignificant differences between RAW 264.7 cells, Rneo-2, and the Bcl-2-transfected clones (Fig. 5A, upper part). In parallel, chromatin structure was determined with the Hoechst fluorochrome 33258 to quantify apoptotic cells. As shown in Fig. 5 (Fig. 5A, lower part, and B), Bcl-2 overexpression protected against apoptotic cell death in response to LPS/IFN-γ, despite concomitant production of nitrite.

p53 and Bax Expression during NO Intoxication—NO has been shown to cause accumulation of the nuclear phosphoprotein p53 in RAW 264.7 cells. As shown in Fig. 6, p53 accumulation precedes subsequent apoptotic DNA laddering (10). To explore a possible interaction between p53 and Bcl-2, RAW cells, neomycin-control (Rneo-2), and Bcl-2 transfectants were tested for GSNO-induced p53 expression. GSNO evoked a dramatic increase in p53 protein levels within 4 h (Fig. 6). This p53 response was equivalent in the Bcl-2-transfectants (data
 FIG. 6. NO-induced p53 accumulation in RAW 264.7 and Bcl-2-transfected macrophages. RAW 264.7, Rneo-2, and Rbcl2-14 macrophages (2 x 10^5 cells/10 ml) were incubated for 4 h with 1 mM GSNO or remained untreated (control). p53 was immunoprecipitated using the monoclonal antibody PAb122, followed by Western blot analysis as described under “Experimental Procedures.” p53-associated radioactivity was quantitated using the PhosphorImager system. The blot is representative for three similar experiments.

The data presented in this paper demonstrate that NO-mediated apoptosis can be effectively antagonized by bcl-2 gene transfer. Previous investigations established that RAW 264.7 macrophages are highly susceptible to endogenously generated or exogenously supplied NO (4). The cellular response to NO induction, with concomitant massive and sustained NO formation, is compatible with apoptosis, as characterized by chromat condensation and DNA laddering. We also previously noticed a relatively early accumulation of the tumor suppressor p53, which clearly precedes DNA fragmentation in these cells in response to NO generation (10). In previous studies, we demonstrated that chromatin condensation, endonuclease activation, and p53 accumulation are linked to an active, LPS/cytokine-inducible iNOS system. In those experiments all apoptotic alterations were blocked by addition of the NO synthase inhibitor N(G)-monomethyl-L-arginine, thereby relaying endogenous NO generation to macrophage apoptosis (4, 10). Cytotoxic and cytostatic actions of NO are not only directed against invading pathogens but also can affect susceptible host cells. Therefore, the existence of cellular defense mechanisms that oppose the damaging potential of these radicals and that account for differential cellular susceptibilities to NO seem likely. Protective mechanisms may be attributable to an altered NO target interaction, scavenging of NO, or efficient repair mechanisms. Alternatively, anti-apoptotic proteins such as Bcl-2 may protect cells from NO-induced cell death without affecting these upstream processes.

Our results provide evidence that Bcl-2 overexpression completely suppressed NO-mediated apoptosis and DNA laddering within the first few hours after NO generation or NO donor application (Figs. 2 and 3), while protection was less pronounced after prolonged NO donor exposure (Fig. 4). Bcl-2 neither blocked LPS/IFN-γ signaling pathways resulting in inducible NOS up-regulation or endogenous NO formation (Fig. 5), nor the decomposition of NO releasing compounds such as GSNO or spermine-NO. Although Bcl-2 blocks NO-induced apoptosis, p53 accumulation in response to NO remained unchanged (Fig. 6). Obviously, therefore, Bcl-2 blocks NO-mediated cell death events downstream of p53. Our results corroborate previous reports where Bcl-2 prevented p53-dependent apoptotic cell death (28). Recently the existence of alternative cell death pathways has been suggested by differential effects of Bcl-2 and CrmA, with Bcl-2 blocking staurosporine but not Fas-induced death and CrmA blocking Fas- but not staurosporine-induced death (29). Despite the differential effects of Bcl-2 and CrmA, both staurosporine and Fas induce the processing and activation of the ICE/Ced-3 family of cysteine proteases. Activation of an ICE-like protease during NO-initiated apoptosis which results in poly(ADP-ribose) polymerase cleavage has been demonstrated (30). Protease activation and poly(ADP-ribose) polymerase digestion were blocked in Bcl-2-transfected cells. Taken together, these observations suggest that Bcl-2 intervenes downstream of the p53 but upstream of protease activation in NO-exposed cells.

Among the potential explanations for Bcl-2 protection is re-
duced formation of oxygen free radicals and oxidative damage to lipids (31). In lymphoid cell lines, bcl-2 overexpression protected from lipid peroxidation and promoted survival although free radical generation was not affected (18). In bacteria, Bcl-2 was reported to function as a pro-oxidant that induced endogenous cellular antioxidant pathways (32). We found that Bcl-2 did not interfere with endogenous NO• generation and/or oxidation of the radical to nitrite. Although NO-initiated signaling pathways or the transduction mechanisms of NO-derived species leading to apoptosis are unknown, p53 accumulation remained intact in Bcl-2 transfectants. We conclude, therefore, that the DNA damaging activity of NO is not antagonized by Bcl-2. Similarly, previous studies have shown that the protection afforded by Bcl-2 does not involve reductions in drug-induced DNA damage with respect to chemotherapeutic anticancer drugs. By analogy to a wide variety of other apoptotic stimuli, NO-mediated apoptosis is blocked by Bcl-2 downstream of the initial toxic insult (15). This might explain why Bcl-2 overexpression delays the onset of apoptosis but does not abrogate the mechanism completely.

As Bcl-2 neither affects the formation of the cytotoxic NO species nor p53 accumulation, we searched for alternative explanations. The Bcl-2 protein is a suppressor of programmed cell death that homodimerizes with itself and forms heterodimers with a homologous protein Bax (33). The latter is considered a promoter of cell death, which is functionally neutralized by heterodimerization with Bcl-2. Western blot analysis revealed constitutively expressed Bax in both RAW 264.7 cells and Bcl-2 transfectants (Fig. 7). In response to exogenously supplied NO, Bax levels increased 2-fold, suggesting that Bax up-regulation is linked to NO-mediated apoptosis. Given that Bcl-2 is able to suppress Bax-mediated apoptosis, we presume a Bcl-2/Bax protein-protein interaction, which accounts at least in part for the protective mechanism of Bcl-2 in RAW 264.7 cells. Taking into account that p53 mediates Bax up-regulation (34, 35), our data are compatible with a pathway initiated by NO, transduced by p53, and ultimately leading to increased Bax protein expression. This hypothesis, however, requires additional testing before cause and effect relations can be attributed to the changes in p53 and Bax expression described here in NO-treated cells.

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