Platelet-activating factor-induced apoptosis is blocked by Bcl-2 in rat intestinal epithelial cells

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Platelet-activating factor (PAF) is involved in the pathogenesis of epithelial cell death. The role of PAF in epithelial apoptosis is not clear. In this study, we examined the effect of PAF on epithelial cell death using an adenovirus-based model of enterocyte apoptosis in vitro. Adenovirus-mediated bcl-2 expression was shown to block PAF-induced cell death, whereas adenovirus-mediated bax expression failed to induce apoptosis in cultured intestinal epithelial cells. The results suggest that PAF-induced cell death is mediated by the proapoptotic Bax protein, and that Bcl-2 plays a protective role in PAF-induced apoptosis.

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antia apoptotic effect on lymphocytes and a proapoptotic effect on enterocytes may be a plausible explanation for the potent injurious nature of PAF in the intestine.

Apoptosis can be induced in cells by a variety of mechanisms that may or may not involve mitochondria. When mitochondria are involved in the initiation of apoptosis, a balance of pro- and antia apoptotic members of the Bcl-2-related proteins plays a critical role in the decision between cell survival and apoptosis (1). Translocation of Bax, a proapoptotic member of the Bcl-2 family to mitochondria results in a collapse of mitochondrial membrane potential, release of cytochrome c or other mitochondrial proapoptotic molecules, and cell death. Bcl-2 can heterodimerize with Bax, and thus the interaction of these two molecules establishes a balance of pro- and antia apoptotic signals (38). Consequently, heterologous overexpression of bax results in increased cell death in tissue culture (29), and the bax/bcl-2 ratio in tissues is diagnostic of sensitivity to proapoptotic stimuli (27, 30). Enterocyte-targeted overexpression of bcl-2, the prototypical antia apoptotic member of this family, has been shown to be a potent defense against ischemia-reperfusion-induced intestinal epithelial apoptosis (16).

The purpose of the present study was to determine whether PAF induces apoptosis in enterocytes and to delineate the mechanism of action. We have found that PAF induces apoptosis in enterocytes and that bcl-2 overexpression provides potent protection to IEC-6 from PAF-induced apoptosis by blocking a PAF-induced translocation of Bax to mitochondria and loss of mitochondrial membrane potential. This study is the first to demonstrate a proapoptotic effect of PAF on enterocytes and to establish a link between two major regulators of mucosal injury, PAF and the Bcl-2 family of apoptosis regulatory gene products.

MATERIALS AND METHODS

Cell culture. IEC-6 cells were maintained in DMEM (GIBCO-BRL, Rockville, MD) supplemented with 5% FBS, 100 μg/ml penicillin and streptomycin, 4 mM glutamine, and 0.1 mg/ml insulin. Later-derived stable cell lines were maintained in the same medium supplemented with 0.6 mg/ml G418 and 75 μg/ml hygromycin.

Plasmid construction. The 937 bp bcl-2 cDNA was obtained from American Type Culture Collection (Manassas, VA) and was subcloned into the pOPRSV-MCS vector of the LACSwitch II expression system (Stratagene, La Jolla, CA) resulting in the pOPRSV/bcl-2 vector. IEC-6 cells were transfected with the plasmid pcMVlacI, encoding the lac repressor under the control of the CMV promoter. Stable lac repressor-expressing clones were generated by using hygromycin selection and named IEC-6/acl–10. These clones were tested for their abilities to regulate the expression of chloramphenicol acetyltransferase (CAT; a reporter molecule), and the clone IEC-6/acl2 was chosen for further use on the basis of the very low background and high-level-induced expression of CAT by isopropyl- β-d-thiogalactopyranoside (IPTG). IEC-6/acl2 cells were transfected with the plasmid pOPRSV/bcl-2, and 10 clones were selected in 0.6 mg/ml G418. In this construct, bcl-2 gene expression is under control of the lac repressor/operator system. Resulting cell lines were named IEC/bcl-2/1 to IEC/bcl-2/10.

Preparation of cell extracts and cellular fractions. For detection of inducible overexpression of bcl-2, cells were harvested by centrifugation at 700 g after 24 h treatment with or without IPTG. Pellets were washed twice with PBS and applied to ice-cold cell lysis buffer containing 0.02 M HEPES, pH 7.4, 0.15 M NaCl, 0.001 μM EDTA, 1% Nonidet P-40 (IGEPAL-CA-630), 0.1 mg/ml aprotonin, 0.1 mg/ml leupeptin, and 0.0625 mg/ml 4-aminophenylacetamide fluoride on ice for 20 min. The suspension was spun down at 7,000 g for 10 min, and the supernatants were collected. For translocation analysis, cytosol and mitochondrial fractions of IEC-6 and bcl-2/6 cells were obtained by using ApoAlert cell fractionation kit (Chontech, Palo Alto, CA). Briefly, cells were harvested by centrifugation at 700 g for 10 min at 4°C. Pellets were suspended in 1 ml of ice-cold wash buffer and resuspended at 700 g for 5 min at 4°C. Pellets were resuspended in 0.8 ml of ice-cold fractionation buffer mix and incubated on ice for 10 min. Cells were then homogenized in an FDA-Depth tissue grinder with a pestle and a pestle. Homogenates were centrifuged at 700 g for 4°C for 10 min. Supernatants were transferred to a fresh 1.5 ml tube and centrifuged at 10,000 g for 25 min at 4°C. Supernatants were collected as cytosolic fraction. Pellets were resuspended in 0.1 ml fractionation buffer mix and stored as mitochondrial fraction.

Western blot analysis. Each lane of a 12% SDS-PAGE was loaded with extracts representing equal amounts of protein and after separation was transferred to polyvinylidene difluoride membranes. After blocking with 5% nonfat milk in 0.1% Tween 20, the membrane was incubated with mouse monoclonal antibodies against human Bcl-2, or Bax (1:2,500 dilution; BioVision, Mountain View, CA) followed by horseradish peroxidase-conjugated goat anti-mouse IgG (1:10,000 dilution). Blots were visualized by enhanced chemiluminescence (ECL Plus) detection solution (Amersham Pharmacia Biotech, Piscataway, NJ) and scanned with a phosphorImager (Molecular Dynamics; Piscataway, NJ).

Immunocytochemistry. For immunocytochemistry analysis, bcl-2 cells were seeded onto glass coverslips and incubated with or without 3 mM IPTG for 24 h. MitoTracker [MitoFluor Red 594 (1:2,000 in medium); Molecular Probes, Eugene, OR] was applied to cells for 30 min before fixation. Cells on coverslips were fixed in 100% methanol at −20°C and permeabilized with 0.1% Triton X-100 in PBS for 5 min. Cells were then blocked with 10% goat serum in PBS for 30 min. Each step was followed by several washes with PBS and performed at room temperature. Cells were probed with mouse anti-Bcl-2 (diluted 1:50 in PBS + 10% goat serum; BioVision) overnight at 4°C in a humid chamber, washed once with 0.1% Triton X-100 in PBS, and then washed with PBS for 20 min three times. Cells were incubated with fluorescein-labeled goat anti-mouse secondary antibody (diluted 1:50 in PBS + 10% goat serum; IGN Pharmaceuticals, Aurora, OH) for 1 h at 37°C in a humid chamber, followed by a 5-min washing with 0.1% Triton X-100 in PBS. Coverslips were placed on a glass slide in antiadhesive mounting medium (Molecular Probes) and observed by confocal fluorescence microscopy.

Measurement of mitochondrial membrane potential and cell death by fluorescence microscopy. 5,5′,6′,6′-Tetra-O-methyloxyluciferinylbenzoylcarbocyanine iodide (JC-1; Molecular Probes) accumulates and aggregates in normal mitochondria and emits a red-orange fluorescence detected at 590 nm, whereas it forms monomers in the cytosol and emits a green fluorescence detected at 525 nm when mitochondria loses membrane potential (ΔΨm). Therefore, loss of ΔΨm can be measured by uptake of JC-1 and indicated by a decrease in the red/green fluorescence intensity ratio. JC-1 was dissolved in DMSO to produce a 1 mg/ml stock solution in our study. To measure the changes of ΔΨm, cells were plated in black 96-well, optically clear assay plates (Corning, Corning, NY) and incubated with 50 μl incubation buffer (in mM: 116 NaCl, 5.4 KCl, 0.4 MgCl2, 1.8 CaCl2, 5.5 glucose, 26 NaHCO3, 0.9 Na2HPO4, and 10 HEPES, pH 7.4) containing JC-1 (1:500 dilution from stock solution) for 30 min analysis.

Caspase activity. Caspase-3 activity was measured by quantifying cleavage of a fluorogenic peptide substrate Ac-DEVD-AFC (BioVision). Cell lysates were collected as the following categories: control, IPTG, and PAF + IPTG. Cells were pretreated with or without 3 mM IPTG for 24 h and then treated with or without 20 μM PAF for 1, 3, and 6 h. Cells were harvested and spun down at 700 g and then the resulting pellets were washed with PBS twice and lysed in cell
lysis buffer containing 10 mM Tris·HCl (pH 7.5), 10 mM NaH₂PO₄/NaHPO₄ (pH 7.5), 130 mM NaCl, 1% Triton X-100, and 100 mM NaPPi on ice for 20 min. After centrifugation at 10,000 g for 10 min, supernatants were collected and mixed with either substrate (Ac-DEVD-AFC) or substrate plus inhibitor (Ac-DEVD-CHO), and fluorescence was measured at 400 nm excitation and 505 nm emission after 1 h of incubation (the development of fluorescence signal was linear between 10 min and 4 h). Specific caspase activity was calculated by subtracting the fluorescence intensity measured in the presence of substrate plus inhibitor from the fluorescence observed by incubating with substrate alone. Sample fluorescence intensities were normalized to the intensity of untreated cells and were expressed as percentage of control.

**DNA fragmentation with ELISA.** DNA fragmentation in all cell lines was analyzed by using an ELISA kit (Roche, Indianapolis, IN) according to the manufacturer’s instructions. Absorbance at 405 nm was measured intermittently in 20-min intervals until absorbance values obtained from the sample representing cells were at least 0.2 optical density (OD) unit above the substrate blanks. Absorbance of blanks was subtracted, and OD values were normalized to the mean of OD values representing the samples of untreated cells and expressed as percentage of controls.

**RESULTS**

**Establishment of stably transduced bcl-2 cell lines.** To investigate the role of bcl-2 in PAF-induced apoptosis in small intestinal epithelial cells, we expressed bcl-2 in IEC-6 cells by using the LacSwitch inducible expression system. As described in detail in MATERIALS AND METHODS, we obtained 10 clones of IEC-6 cells stably transduced with IPTG-inducible bcl-2. Baseline expression and induction of bcl-2 expression by 3 mM IPTG was tested in these clones by Western blotting and immunocytochemistry. On the basis of the low baseline level and high level of induction, we chose two of these cell lines for further characterization and to determine whether the level of bcl-2 expression modifies PAF-induced apoptosis in enterocytes. We show data from one of these cell lines (bcl-2/6), but similar data were obtained from the bcl-2/1 cell line, also. Figure 1 illustrates the effect of treatment with IPTG on bcl-2 expression.
expression in bcl-2/6 cells. Figure 1, A–F, depicts indirect immunofluorescence labeling and fluorescence microscopy on bcl-2/6 cells with or without IPTG treatment. Without IPTG treatment, a small proportion of bcl-2/6 cells exhibit detectable Bcl-2 protein (A and E), whereas following treatment with IPTG, nearly all cells show positive staining (C and F). Mitochondria were visualized by using MitoTracker to confirm the presence of viable cells in all fields (B and D). In the parental IEC-6 or in the control CAT-expressing cells, Bcl-2 protein levels were below our detection limit using immunohistochemistry either with or without IPTG treatment (data not shown). Figure 1G illustrates the results of Bcl-2 Western blotting in bcl-2/6 cells, in the parental IEC-6 cells, and in a control cell line transduced with IPTG-inducible CAT reporter molecule. On treatment with 3 mM IPTG for 24 h, an intense 26-kDa band corresponding to the predicted size of Bcl-2, was readily detectable in cell lysates from bcl-2/6 cells, but remain undetectable in parental IEC-6 cells, the control CAT cell line, or in bcl-2/6 cells in the absence of IPTG.

Colocalization of Bcl-2 and mitochondria. Mitochondrial localization is thought to be necessary for the antiapoptotic activity of Bcl-2. Therefore, to test whether heterogeneous Bcl-2 is localized to mitochondria in bcl-2/6 cells, we utilized Bcl-2 immunohistochemistry in conjunction with MitoTracker labeling to colocalize Bcl-2 with mitochondria in situ, and we performed Western blot analysis of Bcl-2 from isolated mitochondrial and cytosolic fractions from IEC-6 and bcl-2/6 cells. Figure 2, A and B, illustrates colocalization of Bcl-2 with mitochondria in situ. After IPTG treatment, cells were intensely labeled with the Bcl-2 antibody (Figure 2B). Mitochondria were visualized with MitoTracker (Fig. 2A). Arrowheads point to mitochondria that are clearly decorated with Bcl-2. Figure 2C depicts the results of Western blotting. An intense protein band of 26 kDa was detected in the lane corresponding to the mitochondria fraction from bcl-2/6 cells, and only a faint band was observed in the lane corresponding to cytosolic fraction from bcl-2/6 cells. We could not detect Bcl-2 from either mitochondrial, or cytosolic fractions from IEC-6 cells.

Bcl-2 overexpression protects cells from PAF-induced loss of mitochondrial $\Delta \Psi_m$. Mitochondrial potential loss has been reported to be an early event in apoptosis in various experimental systems. To study whether a collapse of $\Delta \Psi_m$ was involved in the PAF-induced apoptosis cascade, we treated IEC-6 and bcl-2/6 cells with PAF (10 $\mu$M) for various lengths of time and evaluated $\Delta \Psi_m$ by measuring the accumulation of JC-1 in mitochondria using fluorescence ratio digital imaging microscopy. JC-1 accumulates in the mitochondria as a function of $\Delta \Psi_m$ and shifts its fluorescence from a green (525) to a red (590) emission maximum proportionately to its concentration. Measurement of the red/green (590:525) emission ratio at 480-nm excitation in JC-1-treated cells gives an estimate of the change of $\Delta \Psi_m$. In IEC-6 cells, treatment with 10 $\mu$M PAF resulted in a time-dependent loss of $\Delta \Psi_m$ (Fig. 3, top row). Similarly, in bcl-2/6 cells in the absence of IPTG, PAF treatment resulted in a loss of membrane potential (Fig. 3, middle row). However, in bcl-2/6 cells pretreated with IPTG for 24 h, $\Delta \Psi_m$ was well preserved even in the presence of PAF (Fig. 3, bottom row).

To evaluate the change of $\Delta \Psi_m$ after treatment with PAF, we quantified red/green fluorescence ratios in cells from five consecutive, randomly selected microscopy fields and plotted the histogram distribution of ratios from untreated cells and from cells treated with 10 $\mu$M PAF for 1 h with or without pretreatment with IPTG for 24 h (Fig. 4). In the parental IEC-6 cells, treatment with PAF (Fig. 4A, thick line) resulted in a shift of ratios toward lower values compared with untreated cells (Fig. 4A, thin line). A threshold ratio of 0.75 (vertical line) clearly divides the cells into two populations, one representing the untreated cells with a ratio $>0.75$ and the other representing cells with injured mitochondria with a ratio $<0.75$. On the basis of this clear distinction, we designated the 0.75 threshold
ratio to distinguish between cells with intact (high ratio) and injured mitochondria (low ratio). Using this threshold, we evaluated the distribution of ratios for each treatment group and provided the values in a table insert for each histogram. As shown by this analysis, in IEC-6 cells, either with or without IPTG or in bcl-2/6 cells in the absence of IPTG, treatment with PAF for 1 h resulted in a significant shift of ΔΨm toward low ratio values. In contrast, in bcl-2/6 cells after the induction of bcl-2 expression with IPTG, the majority of cells (Fig. 4D) remained in the high ratio group. These data indicate that bcl-2 overexpression protects mitochondria in IEC-6 cells from PAF-induced loss of ΔΨm.

Evaluation of the role of Bax in PAF-induced apoptosis. Because Bcl-2 and Bax are classic antagonistic regulators of apoptosis, we aimed to determine whether Bax plays a role in PAF-induced epithelial apoptosis and to analyze Bax-Bcl-2 interactions. For this purpose, we detected Bax from cytosolic and mitochondrial fractions by using Western blotting, quantified band intensities on resulting phosphor images, and calculated mitochondrial/cytosolic ratios both in the absence and presence of Bcl-2 overexpression. Figure 5 illustrates the results of these experiments. We have found that the mitochondrial/cytosol ratio of Bax increases as a function of time and reaches a significant difference compared with control ratios at 1 h after in the presence of PAF (Fig. 5A). Furthermore, induction of Bcl-2 with IPTG completely abrogates the increase of Bax mitochondrial/cytosol ratios (Fig. 5B). These results indicate that PAF induces a significant Bax translocation from cytosol to mitochondria, the timing of this translocation correlates with the collapse of ΔΨm, and Bcl-2 overexpression prevents PAF-induced Bax translocation.

Bcl-2 effects on PAF-induced activation of caspases. Caspase activation precedes DNA fragmentation in the cascade of events that lead to apoptosis after the activation of cell surface receptors. Caspase-3 functions as one of the principle effector caspases of apoptosis and cleaves the tetrapeptide sequence DEVD (Asp-Glu-Val-Asp). In IEC-6 cells, treatment with PAF results in a time-dependent increase of caspase activity that peaks at 6 h (Fig. 6A). Because we have shown that the inducible Bcl-2 in our cell system was dominantly
Fig. 4. Quantification of red/green fluorescence ratios indicates a significant protection from PAF-induced loss of ΔΨm in bcl-2-overexpressing cells. IEC-6, or bcl-2/6 cells were incubated for 24 h with or without 3 mM IPTG followed by treatment with 10 μM PAF and loading with JC-1 (2 μM). Red/green fluorescence ratios were visualized and quantified as described in MATERIALS AND METHODS. Histograms depict the distribution red/green fluorescence ratios in cells observed (obs.) over 5 different fields for each condition. A: IEC-6 cells without IPTG treatment and before (thin line) and 1 h after (thick line) the administration of 10 μM PAF; B: IEC-6 cells after 24 h of IPTG treatment and before (thin line) and 1 h after (thick line) the administration of 10 μM PAF; C: bcl-2/6 cells without IPTG treatment and before (thin line) and 1 h after (thick line) the administration of 10 μM PAF; D: bcl-2/6 cells after 24 h of IPTG treatment and before (thin line) and 1 h after (thick line) the administration of 10 μM PAF. A ratio of 0.75 was identified as a threshold that clearly distinguishes the intact and injured cell population, and it is indicated by a vertical line. Table insets, distribution of cells exhibiting <0.75 vs. >0.75 ratios before and after treatment with PAF. In IEC-6 cells, treatment with PAF resulted in a dramatic decrease of ΔΨm regardless of treatment with IPTG as indicated by a shift of red/green ratios toward lower values. In bcl-2/6 cells without IPTG treatment, PAF induced a similar shift of ratios toward the low values. However, after IPTG treatment in approximately two-thirds of cells, ratio values remained above the 0.75 threshold indicating well-preserved ΔΨm.

Fig. 5. PAF-induced Bax translocation to mitochondria is blocked by Bcl-2 overexpression. Bcl-2/6 cells were treated with or without 3 mM IPTG and were then exposed to 10 μM PAF before cells were harvested for subcellular fractionation and analysis of fractions for Bax protein by using Western blotting. Top insets: representative phosphor images of Western blots. Bottom, graphs depict the results of quantitative analysis of Bax band intensities from several experiments. A: time course of Bax translocation to mitochondria; B effect of Bcl-2 overexpression on PAF-induced Bax translocation. Each data point represents n = 12, except the 0.5-h time point (n = 9). *Statistical significance at P < 0.05.
localized on mitochondria and blocked the PAF-induced collapse of ΔΨm, we wanted to know whether the downstream event, caspase-3 activation, is blocked by overexpression of bcl-2 after PAF exposure. IEC-6 or bcl-2/6 cells were treated with 20 μM PAF for 6 h with or without pretreatment with 3 mM IPTG for 24 h, and caspase-3 activity was measured. In either the parental IEC-6 cells or in bcl-2/6 cells, without IPTG treatment with PAF resulted in a significant increase of caspase-3 activity (Fig. 6, B and C). In bcl-2/6 cells, pretreatment with IPTG for 24 h resulted in a significant decrease of baseline caspase-3 activity, and PAF failed to elicit a significant increase of this lowered activity (Fig. 6C). The inhibitory effect of IPTG treatment on PAF-induced caspase-3 activity must be mediated specifically through the induction of bcl-2 expression, because in the parental IEC-6 cells, IPTG treatment had no inhibitory effect on either baseline or PAF-induced caspase-3 activity (Fig. 6B). These data indicate that bcl-2 overexpression blocks the PAF-induced apoptotic signaling upstream of caspase-3 activation.

**Bcl-2 overexpression protects cells from PAF-induced DNA fragmentation.** To investigate whether bcl-2 overexpression has an effect on PAF-induced DNA fragmentation, IEC-6 and bcl-2/6 cells were treated with 10 μM PAF for 0, 8, 12, 18 h with or without pretreatment with IPTG (3 mM for 24 h). As shown in Fig. 7, DNA fragmentation was time dependent and peaked at 18 h for IEC-6 cells and at 12 h for bcl-2/6 cells. In the absence of IPTG, DNA fragmentation was not significantly different in IEC-6 and bcl-2/6 cells until the 12-h time point. Interestingly, by the 18-h time point bcl-2/6 cells exhibited significantly reduced DNA fragmentation even in the absence of IPTG induction of bcl-2 expression compared with the same time point in IEC-6 cells. After the induction of bcl-2 expression with IPTG in bcl-2/6 cells, PAF-induced DNA fragmentation was abolished and remained similar to the level of DNA fragmentation in untreated bcl-2/6 cells at all time points of PAF treatment. Levels of DNA fragmentation in IPTG- and PAF-treated bcl-2/6 cells were significantly lower at P < 0.001 from the corresponding time points of PAF-treated IEC-6 cells, or PAF-treated bcl-2/6 cells in the absence of IPTG. The lack of inhibitory effect by IPTG in IEC-6 cells on PAF-induced DNA fragmentation verifies that the inhibition of PAF-induced DNA fragmentation by IPTG treatment in bcl-2/6 cells is specifically mediated by the stimulation of bcl-2 expression. To assess whether bcl-2 overexpression uniformly affects DNA fragmentation induced by various stimuli, we treated parental IEC-6 cells and bcl-2/6 cells with TNF-α before or after treatment with IPTG. TNF-α significantly increased DNA fragmentation in both parental IEC-6 and bcl-2/6 cells (Fig. 7C and D), but overexpression of Bcl-2 failed to block DNA fragmentation induced by TNF-α. The fact that overexpression of Bcl-2 in IEC-6 interferes with the induction of DNA fragmentation by PAF but not TNF-α suggests that there are possibly multiple signaling pathways leading to epithelial apoptosis that are differentially affected by members of the Bcl-2 family in IEC-6 cells.

**DISCUSSION**

Apoptosis of enterocytes is a physiological process that enables a high rate of turnover of these cells resulting in a renewal of the epithelial cell layer every 2–3 days (23). Epithelial cells germinate in the crypt, migrate toward the...
villous tip, and are removed from the monolayer in the tip area by apoptosis (4). A faulty regulation of this process may lead to pathological conditions such as cancerous growth in the case of a diminished rate of apoptosis (49) or compromised epithelial barrier in the case of accelerated apoptosis (2, 17). Neonatal NEC, celiac disease, and UC have all been linked to an increase in enterocyte apoptosis (14, 20, 44). Interestingly PAF has been implicated in each of these pathological conditions as well, but until now, there was no direct link established between the roles of PAF and epithelial apoptosis in IBD. Our findings establish a direct link between the role of apoptosis and PAF in intestinal injury.

Mechanisms by which mitochondria regulate apoptosis are closely linked to the expression of pro- and antiapoptotic members of the Bcl-2-related protein family (1). Pro- and antiapoptotic members of this family maintain a dynamic equilibrium in eukaryotes and, therefore, regulate cell survival. This dynamic equilibrium is maintained by multiple mechanisms involving the regulation of gene expression (33), phosphorylation and dephosphorylation of the expressed proteins (7), protein-protein interactions (38), and finally by regulation of degradation (7). In most model systems, overexpression of bcl-2 blocks apoptosis via an effect on mitochondrial membrane potential (47), but in some cases, bcl-2 overexpression has been shown to protect cells from apoptosis without having an effect on mitochondria (26). There could be several reasons for such a dichotomy of observations: 1) Bcl-2 can be localized to the cytoplasm, to the mitochondria, or to the endoplasmic reticulum, and the dominant localization between these compartments can be divergent among different kinds of cells depending on Bcl-2 phosphorylation, or protein-protein interactions; 2) in transgenic animals or in heterologous overexpression systems in which transgene expression is constantly at a high level, compensatory phenotypic changes may occur; and 3) in some model systems, the loss of mitochondrial membrane potential could be secondary to apoptosis elicited by means independent of mitochondria and thus could not be prevented by Bcl-2.

**Fig. 7.** Bcl-2 overexpression protects bcl-2/6 cells from PAF-induced but not TNF-α-induced DNA fragmentation. Cells were plated in 96-well plates, treated with or without IPTG for 24 h, and then treated with 20 μM PAF for 0–18 h in the continuing presence or absence of IPTG. DNA fragmentation was quantified by using an ELISA kit as described in MATERIALS AND METHODS. The time course of DNA fragmentation in IEC-6 cells (A) and bcl-2/6 cells (B) is shown. For both cell lines in the absence of IPTG, there was time-dependent increased apoptosis induced by PAF treatment (*P < 0.01 vs. each group’s own untreated control). In IEC-6 cells, treatment with IPTG did not inhibit PAF-induced DNA fragmentation. Treatment with IPTG significantly reduced DNA fragmentation in bcl-2/6 cells (*P < 0.01 vs. bcl-2/6 without IPTG). DNA fragmentation was not significantly different from untreated controls at any time points after treatment with PAF in bcl-2/6 cells. C and D: effect of recombinant rat TNF-α (rTNF-α) and Bcl-2 on DNA fragmentation in IEC-6 and bcl-2/6 cells. Treatment with IPTG did not affect the ability of TNF-α to induce apoptosis in either of the two cell lines (***at least P < 0.001 vs. IEC-6 or bcl-2/6 without IPTG, respectively). All numbers were calculated as the percentage of each group’s untreated control. Statistical significances were calculated by using one-way ANOVA with Tukey’s postanalysis.
Our study was designed to circumvent the above outlined potential pitfalls, and we utilized a regulated expression system, verified the dominant mitochondrial localization of Bcl-2, and determined the relative timing of mitochondrial changes, caspase activation, and DNA fragmentation in our model. Our data show that the PAF-induced loss of ΔΨm occurs within a short time period (within 1 h) correlating with the timing of Bax translocation to mitochondria and preceding caspase activation and DNA fragmentation in enterocytes (Fig. 8). In bcl-2/6 cells before IPTG treatment, bcl-2 expression is at a low baseline comparable to the parental IEC-6, and it is induced to a high level 24 h after IPTG treatment, thus allowing the study of Bcl-2 function without major phenotypic changes of the cell line during routine culturing. We verified that the predominant localization of overexpressed bcl-2 in IEC-6 is on mitochondria, an observation that is in good correlation with our observation that Bcl-2 protects mitochondria from PAF-induced insult. Additionally, we used TNF-α in the same experimental setting to see whether the ability of Bcl-2 to block DNA fragmentation is relatively specific for the PAF-induced death pathway in IEC-6 cells. TNF-α is considered to be a key mediator in the pathogenesis of IBD (6, 42). TNF-α and its TNF superfamily initiate a so-called “death receptor pathway” of apoptosis that is different from “mitochondrial death pathway” because of the differential involvement of mitochondria (5, 31). The role of antiapoptotic proteins in the death receptor pathway remains controversial. Bcl-2 was shown to inhibit a Fas-induced conformational change in the Bax NH2 terminus and translocation to mitochondria (36). Yet, others (25) have shown that signaling of cell death by Fas is distinct from the apoptotic pathways regulated by Bcl-2 family, because neither Bcl-2 nor Bcl-xL blocked Fas-induced apoptosis. Another antiapoptotic member of the Bcl-2 family Bcl2L-10 was able to block apoptosis in the mitochondria pathway but not in the death receptor pathway (51). Therefore, the exact role of TNF-α in epithelial apoptosis needs to be defined better by future experiments. Nonetheless, our findings indicate that PAF-treated IEC-6 cells go through a mitochondrial-dependent apoptotic pathway that can be regulated by Bcl-2 protein and that the PAF-induced apoptotic pathway differs from TNF-α-induced apoptosis with regard to the significance of Bcl-2 in the regulatory pathway.

The role of Bcl-2 family of apoptosis regulators in intestinal mucosal homeostasis is well established. A differential expression of both bcl-2 and bax is established in the second part of fetal life along the crypt to villous axis with a presence of high bax expression and low bcl-2 expression in regions in which apoptotic rates are high and vice versa (30, 37, 48). Altered expression profiles of bcl-2-related genes can result in diminished apoptosis, causing neoplasias (8, 35) or, in increased apoptosis, resulting in mucosal injury (13). Experimental overexpression of a bcl-2 transgene in the murine intestinal epithelium provided protection from hypoxia/reoxygenation-induced mucosal injury (16), whereas bcl-2 knockout mice exhibited an increased susceptibility to damage-induced injury (39). In this respect it is notable that there is a crypt to villous decreasing gradient of bcl-2 expression in the ileum (48). On the basis of the findings described in this study, this particularly low level of bcl-2 expression makes the ileal villous tips susceptible to PAF-induced injury. Indeed, ileum is the most frequently affected region of the intestine in NEC, a disease in which the pathogenic role of PAF is well established.

In summary, our study provides evidence that PAF, a key molecule in intestinal mucosal injury elicits apoptosis in enterocytes via a mechanism that involves Bax translocation to mitochondria, collapse of mitochondrial membrane potential, and caspase activation. Conditional overexpression of bcl-2, the prototypical antiapoptotic member of the Bcl-2-related family of apoptosis regulators blocked all of these events by blocking Bax translocation to mitochondria and protecting mitochondria from PAF-induced collapse of membrane potential. This study is the first to demonstrate a connection among PAF, intestinal epithelial apoptosis, and the family of Bcl-2-related proteins, which have been identified as critical determinants of intestinal mucosal injury. An understanding of the process by which PAF induces epithelial apoptosis may provide insights into the cellular pathology of IBD and may lead to novel therapeutic interventions.


