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

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Submitted 21 April 2003; accepted in final form 16 September 2003

Platelet-activating factor-induced apoptosis is blocked by Bcl-2 in rat intestinal epithelial cells. Am J Physiol Gastrointest Liver Physiol 286: G340–G350, 2004. First published September 25, 2003; 10.1152/ajpgi.00182.2003.—Platelet-activating factor (PAF) is a key mediator in pathogenesis of inflammatory bowel diseases (IBDs) but mechanisms of PAF-induced mucosal injury are poorly understood. To determine whether apoptosis and the Bcl-2-family of apoptosis regulatory gene products play a role in PAF-induced mucosal injury, we stably and conditionally overexpressed bcl-2 in rat small intestinal epithelial cell lines under the control of a lactose-inducible promoter. Western blot analysis and immunohistochemistry were used to verify inducible Bcl-2 and to analyze Bcl-2 and a proapoptotic member of the Bcl-2 family, Bax, subcellular distribution. DNA fragmentation was quantified by ELISA, caspase activity was measured by using fluorogenic peptide substrates, and mitochondrial membrane potential was assayed by 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) and fluorescence digital imaging. Bcl-2 expression was highly inducible by lactose analog isopropyl-β-D-thiogalactoside (IPTG) and was localized predominantly to mitochondria. In the absence of bcl-2 overexpression and after treatment with PAF, Bax translocated to mitochondria, and mitochondrial membrane potential collapsed within 1 h, followed by caspase-3 activation, which peaked at 6 h with an ensuing DNA fragmentation maximizing at 18 h. After IPTG-induction of bcl-2 expression, PAF failed to induce DNA fragmentation, caspase-3 activation, Bax translocation, or a collapse of mitochondrial membrane potential. These data are the first to show that PAF can activate apoptotic machinery in enterocytes via a mechanism involving Bax translocation and collapse of mitochondrial membrane potential and that both of these events are under control by bcl-2 expression levels. A better understanding of the role of PAF and Bcl-2 family of apoptosis regulators in epithelial cell death might aid design of better therapeutic or preventive strategies for IBDs.

inflammatory bowel diseases; necrotizing enterocolitis; DNA fragmentation; caspase; mitochondrial membrane potential; Bax

Platelet-activating factor (PAF), identified as 1-alkyl-2-acetyl-sn-glycero-3-phosphatidylcholine, is rapidly released during the inflammatory response and plays a central role in the pathogenesis of several inflammatory bowel diseases (IBDs). High concentrations of PAF have been found in colonic biopsies from patients with ulcerative colitis (UC) (18, 40), in cultured inflamed mucosa from patients with UC (50), from stool of patients with UC and Crohn’s disease (22), and in plasma from premature infants with necrotizing enterocolitis (NEC) (9), whereas the level of PAF-degrading enzyme PAF acetylhydrolase (PAF-AH) was significantly lower in mucosal samples from the distal ileum in Crohn’s disease patients than in controls (28). These findings suggest that increased PAF production and/or decreased PAF degradation may result in increased levels of PAF in these disease states. Studies in experimental models of intestinal injury suggest that the increase of PAF levels is not coincidental but might be a significant underlying cause of tissue injury. In animal models, exogenous PAF results in ischemic bowel necrosis (21) and gastric ulceration (41). Endotoxin, hypoxia, or TNF-induced intestinal injury can be prevented by PAF receptor antagonists (10, 11). PAF receptor antagonists or PAF-AH given with enteral feeding interferes with the initiation of NEC in a neonatal rat model (10, 12). These data underscore the importance of PAF in mucosal injury occurring in IBD but leave the mechanism of injury unanswered.

In addition to an important role for PAF, a faulty regulation of apoptosis appears to play a role in intestinal mucosal injury. Apoptosis is a tightly regulated cellular suicide program that is required for many essential processes in multicellular organisms, including organogenesis, the proper functioning of the immune system, and for the physiological turnover of cells in constantly regenerating organs. The intestinal epithelium has a high rate of turnover (23), which necessitates the apoptotic removal of cells at the end of their useful life (4). In addition to epithelial cells, T cells in the lamina propria have to undergo apoptosis to control mucosal inflammation (32). Accelerated epithelial apoptosis has been thought to have pathogenic significance in UC (44), celiac disease (14), NEC (19), and ischemia-reperfusion-induced intestinal injury (16). To the contrary, defective epithelial apoptosis may lead to tumor formation (8, 35), and lamina propria T cells that fail to undergo apoptosis might contribute to an exaggerated inflammatory state and mucosal pathology such as Crohn’s disease (27). Interestingly, PAF protects lymphocytes from apoptosis (46), whereas it is a potent proapoptotic molecule in a variety of cell types such as hippocampus-derived cells (43), human epidermal cells (3), astrocytes, and oligodendrocytes (24). Intestinal epithelial cells (IECs) express PAF receptor (34), and we have shown that PAF receptor is present exclusively in the apical plasma membrane and its activation leads to regulation of transepithelial ion transport in colocytes (15). Curiously, PAF activates epithelial functions at a much higher concentration than in other cell types (15, 45). We hypothesized that PAF induces apoptosis in enterocytes and that a combined...
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 prognostic 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 pCMVlal, 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/lac1–10. These clones were tested for their abilities to regulate the expression of chloramphenicol acetyltransferase (CAT; a reporter molecule), and the clone IEC-6/lac1–10 was used for transfection. Resulting cell lines were named IEC-6/lac1–10/lac2–10.

Preparation of cell extracts and cellular fractions. For detection of inductive overexpression of bcl-2, cells were harvested by centrifugation at 700 g for 10 min at 4°C. Supernatants were collected. For translocation analysis, cytosol and mitochondrial fractions of IEC-6 and bcl-2 cells were obtained by using ApoAlert cell fractionation kit (Chontec, Palo Alto, CA). Briefly, cells were harvested by centrifugation at 700 g for 10 min at 4°C. Pellets were resuspended in 0.1 ml of ice-cold wash buffer and resuspended at 500 g for 5 min at 4°C. Cell pellets were washed twice in wash buffer (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.05% Triton X-100) and suspended in wash 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 aprotinin, 0.1 mg/ml leupeptin, and 0.0625 mg/ml 4-aminophenyl-benzenesulfonyl 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 cells were obtained by using ApoAlert cell fractionation kit (Chontec, Palo Alto, CA). Briefly, cells were harvested by centrifugation at 700 g for 10 min at 4°C. Pellets were resuspended in 0.8 ml of ice-cold wash buffer and resuspended at 600 g for 5 min at 4°C. 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 polyvinyldene 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 using enhanced chemiluminescence (ECL Plus) detection solution (American 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 induced 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. Coverslips 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 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 antifade 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′-Tetramethyl-1,1′,3′,3′-tetrathylbenzimidazolylcarboxyanine 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 cytoplasm 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 96-well, optically clear, black-bottom 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 before analysis.

Caspase activity. Caspase-3 activity was measured by quenching the cleavage of a fluorogenic peptide substrate Ac-DEVD-AFC (BioVision). Cell lysates were collected as the following categories: control, stimulated with 3 mM 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
lisation 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 NaPi 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 control.

**RESULTS**

Establishment of stably transfected 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 in intestinal epithelial cells (IEC)-6/bcl-2 cells. Cells stably transduced with inducible bcl-2, cells transduced with a control construct encoding inducible chloramphenicol acetyltransferase (CAT), and the parental IEC-6 cells were grown in culture dishes and treated with or without 3 mM isopropyl-β-D-thiogalactoside (IPTG) for 24 h and were then lysed for Western blot analysis or were fixed for indirect immunofluorescence staining as described in MATERIALS AND METHODS. A–F: results of indirect immunofluorescence staining of Bcl-2 in bcl-2/6 cells. A, C, and E depict cells that were not treated with IPTG, whereas B, D, and F represent IPTG-treated cells. A and B are gray scale images of Bcl-2 immunohistochemistry, C and D are gray scale images of MitoTracker staining, and E and F are pseudocolored micrographs to illustrate the proportion of cells that exhibit Bcl-2 staining relative to the total number of cells. Without IPTG, a small proportion of cells exhibited Bcl-2 staining (E), whereas after induction with IPTG, almost all cells were stained very intensely (F).

G: Western blot. Cell lysates were analyzed with denaturing PAGE, transferred onto polyvinylidene difluoride membranes, and Bcl-2 protein was visualized by Western blot analysis by using mouse monoclonal anti-Bcl-2 antibody and subsequent fluorescence imaging as described in MATERIALS AND METHODS. Each lane represents 20 µg of cellular protein with (+) or without (−) IPTG treatment as indicated. Arrow points to the characteristic 26-kDa band corresponding to the predicted size for Bcl-2 protein. This experiment was repeated 2 more times with identical results.

**Statistical analysis.** All data were presented as means ± SE. Statistical analyses were performed by using ANOVA (GraphPad Prism, San Diego, CA). Differences were considered significant at P < 0.05.
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 $\Delta \Psi_m$ 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 $\Delta \Psi_m$.

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 $\Delta \Psi_m$, 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 $\Delta \Psi_m$ 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 $\mu$M PAF and loading with JC-1 (2 $\mu$M). 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 $\mu$M 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 $\mu$M PAF; C: bcl-2/6 cells without IPTG treatment and before (thin line) and 1 h after (thick line) the administration of 10 $\mu$M 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 $\Delta \Psi_m$ 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 $\Delta \Psi_m$.

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 $\mu$M 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 $P < 0.05$. 

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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.
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 $\Delta \Psi_m$ 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 Bcl2-L10 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.
GRANTS
This work was supported by March of Dimes Foundation Grant 6-F99–278, National Institute of Child Health and Human Development Grant RO1-HD-37581–01A, and by the Jessica Jacobi Goldner Endowment.

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