Silencing of Bak Ameliorates Apoptosis of Human Proximal Tubular Epithelial Cells by *Escherichia coli*-Derived Shiga Toxin 2

C. Wilson, G. H. Foster, M. M. Bitzan

Abstract

Background: *Escherichia coli*-derived Shiga toxin (Stx), the cause of the enteropathic hemolytic uremic syndrome, is a potent inducer of apoptotic cell death. The present study was performed to examine the hypothesis that Stx initiates apoptosis by activating the mitochondrial pathway involving mitochondrial-associated, pro-apoptotic Bcl-2 family proteins Bax and Bak.

Materials and Methods: To determine if Stx2-mediated apoptosis is dependent on Bax or Bak, a gene-silencing approach was employed using sequence-specific small interfering (si)RNA duplexes. Silencing of Bax and Bak protein expression in human renal proximal tubular epithelial (HK-2) cells and its effect on Shiga toxicity was assessed by immunofluorescence microscopy and Western blotting.

Results: Transfection of HK-2 cells, shown to be exquisitely sensitive to Stx, with siRNA duplexes successfully diminished Bak, but not Bax protein expression. In order to determine if silencing of pro-apoptotic gene expression affects Stx-induced apoptosis, HK-2 cells were transfected with Bak-specific or control siRNA, exposed to lethal concentrations of Stx2 and assessed for cleavage of poly(ADP-ribose)polymerase-1 (PARP) as a marker of apoptosis, using Western blot technology. We observed that siRNA-induced reduction of Bak expression levels correlated with decreased PARP cleavage.

Conclusion: Results suggest that Stx-induced cell death involves pro-apoptotic Bak and that silencing of Bak gene expression affords partial protection against Stx-mediated apoptosis.

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Introduction

Enteric infection by Shiga toxin (Stx) producing *Escherichia coli* (STEC) O157:H7 and other STEC serotypes are the cause of hemorrhagic (ischemic) colitis and hemolytic uremic syndrome (HUS) [1–6]. STEC-mediated hemorrhagic colitis is important, because it mimics ulcerative colitis [4, 7]. Enteropathic HUS, which is defined by the triad of acute, nonimmune hemolytic anemia, thrombocytopenia and nephropathy [8] and which includes systemic complications of *Shigella dysenteriae* type I infections [9–11], is a leading cause of acute renal failure in young children with significant acute and long-term morbidity [12, 13]. It carries an estimated lethality of 3–5% in infants [12, 14], while higher mortality rates have been reported from outbreaks among elderly populations [15].

Despite recent advances, the precise mechanism leading to hemorrhagic colitis (HC) and HUS remains elusive and at present, there is no causal therapy [12, 5]. Stx is considered the primary culprit responsible for the gastrointestinal hemorrhage, and renal and extrarenal complications, in concert with an increasing number of bacterial proteins that regulate bacterial adhesion, protein export and host cell interaction [16]. *In vivo* produced Stx traverses the intestinal epithelium, enters the bloodstream and targets the colonic mucosa, the kidneys and the central nervous system. While Stx-mediated disease is characterized by small vessel injury [17–19], there is mounting evidence that Stx also affects glomerular epithelial cells and renal tubules [20–23] as well as hematopoietic cells [24–26].

Stx causes apoptosis of susceptible cells *in vitro* [27–30], and there is tentative evidence that Stx also induces apoptosis *in vivo*, of renal parenchymal cells, both in animal models of Shiga toxinemia [31, 32] and in humans with HUS [21, 22, 33].

Two major pathways of apoptosis have been identified in mammalian cells, the death receptor (tumor necrosis factor [TNF] receptor I/Fas receptor) dependent and the

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death receptor independent, mitochondrial pathway [34]. The latter is regulated by Bcl-2 family proteins with pro-apoptotic (Bax, Bak, Bad, Bid, Bcl-X_S) or anti-apoptotic (Bcl-2, Bcl-X_L) properties. Activation of the pro-apoptotic multidomain proteins Bax and Bak is now recognized as a key event leading to the alteration or *de novo* formation of mitochondrial membrane pores and the release of cytochrome c. Both pathways result in the activation of effector caspases and subsequent fragmentation of the cellular nucleus [34, 35]. Under physiological conditions, Bax is present in the cytosol in monomeric form (reviewed in [36, 37]), whereas Bak is attached to the mitochondrium and kept in check by its interaction with voltage-dependent anion channel protein-2 (VDAC-2) [38, 39]. Upon apoptotic stimulation, Bax is inserted into the mitochondrial membrane undergoing conformational changes and homo-oligomerization, while mitochondrial Bak dissociates from VDAC-2 complexes. Activated Bax and Bak heterodimerize [38] and participate in the formation of the putative cytochrome c release (mitochondrial apoptosis-induced) channel, MAC [40].

RNA interference, based on the introduction of small interfering RNA duplexes (siRNA), is an elegant strategy to achieve specific gene silencing via the elimination of mRNA containing homologous nucleotide sequences. Targeted gene "knockdown" at the posttranscriptional level can be initiated at any time during development or tissue growth [41–43]. The technique has not been previously applied in the context of Stx-induced cytotoxicity.

Previous studies in our laboratory suggested that Stx activates the mitochondrial pathway in human renal tubular epithelial (HK-2) cells involving translocation of Bax from the cytosol to the mitochondrium. We hypothesized that the silencing of Bax and/or Bak gene expression will protect HK-2 cells from Stx-induced apoptosis. The aims of this project were (1) to establish that Bax and Bak protein expression can be diminished in renal tubular epithelial (HK-2) cells by RNA interference using sequence-specific siRNA duplexes, and (2) to determine if Bax or Bak silencing decreases Stx-induced apoptosis in these cells.

Materials and Methods

Cell Culture

The human proximal tubular epithelial cell line HK-2 (ATCC # CRL-2190) [44] was grown in RPMI 1640 medium (Life Technologies/Gibco BRL; Grand Island, NY), supplemented with 10% fetal bovine serum (FBS; Cellgro Mediatech; Hendon, VA) and 200 mg/ml L-glutamine, and penicillin/streptomycin, at 37 °C, 5% CO_2 in a humidified incubator. Cell culture dishes were from The Technology Partnership (Royston, UK) and from Corning (Corning, NY).

siRNA and Transfection Experiments

Green fluorescent protein (GFP)-gene sequence specific siRNA duplexes were purchased from Dharmacon (Boulder, CO); siRNA duplexes derived from the open reading frame of published sequences of human Bak (AATGCCTATGAGTACTTCACC-DTDT) [Bak-315] and human Bax (AACTGATCAGAACCAT-CATGG-DTDT) [Bax-391] were designed based on published algorithms and synthesized by Qiagen (Valencia, CA). Glass cover slips, placed into the wells of 12-well tissue culture dishes, were seeded with 1.9×10^5 HK-2 cells per well. Cells were grown for 24 h to reach confluency and rinsed once with warm PBS, followed by the addition of 1 ml serum-free RPMI medium and 0.4 ml transfection mix containing optimized concentrations of siRNA duplexes (3.2 µg), TransMessenger (8 µl) and Enhancer (3.2 µl) reagents (TransMessenger, Qiagen). After 3 h, the transfection medium was replaced with complete RPMI growth medium and gene silencing was allowed to occur over 72 h.

Immunofluorescence Microscopy

siRNA or vehicle-treated HK-2 monolayers on glass cover slips were rinsed in PBS for 3 × 5 min, fixed in 100% methanol for 1 min and rinsed again 3 × 5 min in PBS. Cells were permeabilized with pre-warmed 0.2% Triton-X in PBS at 37 °C for 10 min, washed again in 3 × 5 min in PBS and blocked with 1% crystalline protease- and immunoglobulin-free bovine serum albumin (BSA) (Jackson Immuno Research Laboratories; West Grove, PA) in PBS for 10 min. Cells were incubated in primary antibody, diluted 1:150 in PBS-5%BSA (anti-Bax) and 1:300 (anti-Bak), respectively, in a humidity chamber at 4 °C for 1 h and rinsed 3 x 5 min in PBS, followed by incubation in 25 µg/ml goat anti-rabbit rhodamine-conjugated secondary antibody (Sigma) in PBS-1%BSA for 30 min. Primary antibodies were directed against the N-terminal residues of human Bax (#2772) and Bak (#3792), respectively (Cell Signaling Technologies; Beverly, MA). Cover slips were washed in PBS, postfixed in methanol for 1 min, stained with 0.1 µg/ml 4',6-diamidino-2'-phenylindole dihydrochloride (DAPI) (Sigma) in methanol, rinsed once in methanol and once in PBS and mounted onto slides using VectaShield (Vector Laboratories, Burlingame, CA). All steps were performed at room temperature unless indicated otherwise. Slides were viewed under incident UV light using a Zeiss Axiovert 10 microscope equipped with a filter for ultraviolet excitation (DAPI) and Zeiss Axioplan 2 digital imaging fluorescence microscopy system (Carl Zeiss Microimaging, Inc.; Thornwood, NY). At least five different fields per slide were evaluated. Fluorescence images were captured with standardized parameters to assure uniform assessment of the intensity of the antibody staining. Apoptotic cells were identified by segmented morphology of DAPI-stained cell nuclei. Each slide was read and interpreted by at least two observers.

Shiga Toxin and Western Blotting

HK cells were grown on glass cover slips in 12-well dishes as described above (for the detection of nuclear fragmentation), or in 6- or 12-well dishes without cover slips (for Western blotting). Following siRNA silencing for 72 h as described above, monolayers were stimulated for 18 h with 10 pM purified Shiga toxin-2 (Stx2), kindly donated by Dr. M. A. Karmali (Guelph, Ontario). Floating cells were aspirated and kept on ice; residual cells were washed in ice-cold PBS and detached by gentle scraping. Cells were combined, washed in ice-cold PBS at 250 × g for 10 min at 4 °C and extracted in 25 µl Laemmli buffer with protease inhibitors (Cocktail Set II from Calbiochem, San Diego, CA). Samples were sonicated at 70% duty cycle for 10 pulses (Branson Sonifier), centrifuged at 14,000 × g for 10 min at 4 °C, and the supernatants assayed for protein using a detergent-compatible BioRad protein assay (Hercules, CA). Whole cell lysates (50 µg protein/lane)

were separated by 6% SDS-polyacrylamide gel electrophoresis and transferred onto polyvinyldifluoride (PVDF) membrane (Immun-Blot; BioRad) by electroblotting. Following transfer, gels were stained with GelCode Blue (Pierce) to confirm equal loading. Membranes were blocked for 1 h with 5% BSA in Tris-buffered saline (TBS) and rinsed 3 × 20 min in TBS-Tween 20 (0.1%), followed by incubation with polyclonal anti-poly(ADP-ribose) polymerase (PARP) antibody (Cell Signaling Technology #9542) diluted 1:1000 in TBS-5% BSA for 12–15 h at 4 °C. Membranes were rinsed 3 × 20 min in TBS-Tween 20 and incubated for 1 h in 1:8000 diluted horseradish peroxidase-conjugated goat anti-rabbit antibody (Sigma) and developed with luminol/peroxide-based chemiluminescent substrate (SuperSignal West Pico; Pierce, Rockford, IL).

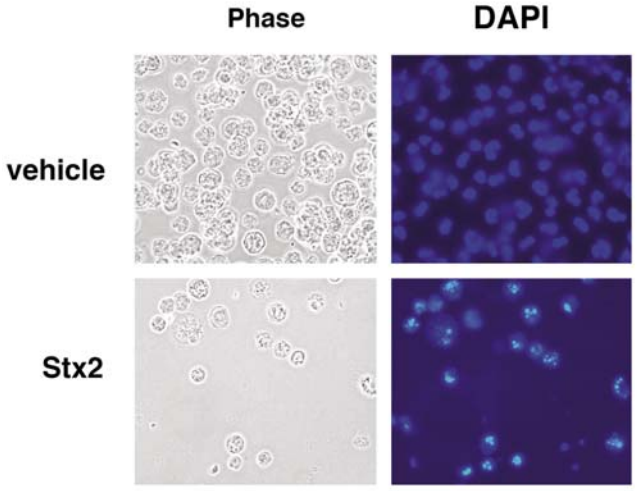

Figure 1. HK-2 cell monolayers were treated with Shiga toxin 2 (100 pM) for 18 h. Floating and adherent cells were combined, fixed and stained with DAPI as described in the Methods section, and examined under phase contrast and fluorescent light (original magnification 40 ×). Nuclear fragmentation, a characteristic feature of apoptosis, is apparent in most Stx-treated cells, but absent in vehicle-treated control monolayers.

Results

Stx2 Causes Apoptosis of HK-2 Cells

Exposure of HK-2 cell monolayers to various concentrations of Stx2 (0.1 to 100 pM) over 18 h resulted in increasing degrees of microscopically evident cytotoxicity and detachment of the cells from the plastic support. Figure 1 depicts a typical example of Stx-induced nuclear fragmentation. Exposure to Stx2 also led to substantial cleavage of the nuclear enzyme PARP (see below). Nuclear fragmentation and PARP cleavage are strong indicators of apoptosis.

Effect of siRNA Targeting on Bax and Bak Protein Expression

HK-2 cells were grown on glass cover slips and transfected with vehicle or with siRNA directed at (non-mammalian) green fluorescent protein (GFP) as control, Bax or Bak sequences. Cells were probed for decreased target protein expression by immunofluorescence using polyclonal anti-Bax or anti-Bak antibodies. As shown in figure 2, Bax siRNA duplexes failed to interfere with Bax protein expression. In contrast, the siRNA duplex targeting Bak revealed a substantial signal reduction under otherwise identical experimental conditions (Figure 3). Similar results were obtained after transfection with (irrelevant) GFP siRNA instead of transfection reagent only (results not shown). We then used Western blotting to confirm the results obtained with immunofluorescence. As shown in figure 4, siRNA targeting of Bak mRNA reduced the relative amount of Bak protein, while Bak protein expression was unchanged in cells treated with Bax siRNA or control duplexes.

siRNA-Mediated Silencing of Bak Reduces Stx2-Induced PARP Cleavage in HK-2 Cells

HK-2 cells were grown in multi-well dishes and transfected with vehicle or siRNA duplexes targeting Bax or Bak (alone and in combination), or GFP. Monolayers were subsequently exposed to vehicle or 10 pM Stx2 for another 18 h. Apopto-

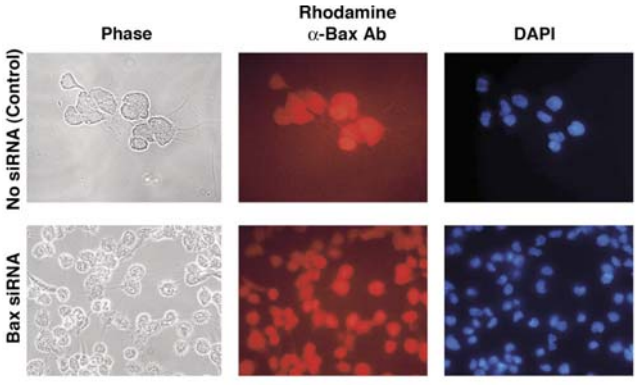

Figure 2. HK-2 cells grown on glass cover slips in 12-well dishes were transfected with or without sequence-specific siRNA for Bax mRNA. Bax protein expression was detected by immunofluorescence using polyclonal anti-Bax and rhodamine-conjugated secondary antibody. Cells were counterstained with DAPI (original magnification 63 ×).

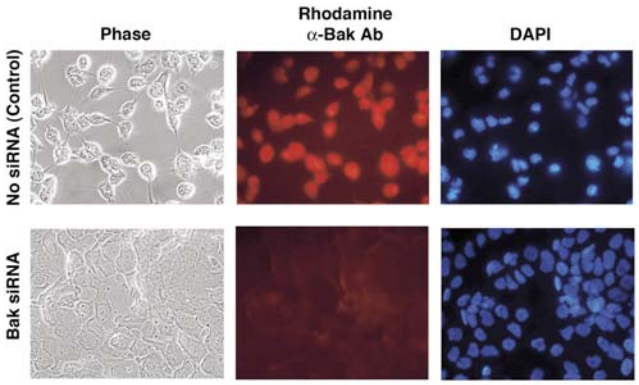

Figure 3. HK-2 cells grown on glass cover slips in 12-well dishes were transfected with vehicle or sequence-specific siRNA for Bak and probed for Bak protein expression by immunofluorescence using polyclonal anti-Bak and rhodamine-conjugated secondary antibody and counterstained with DAPI (original magnification 63 ×).

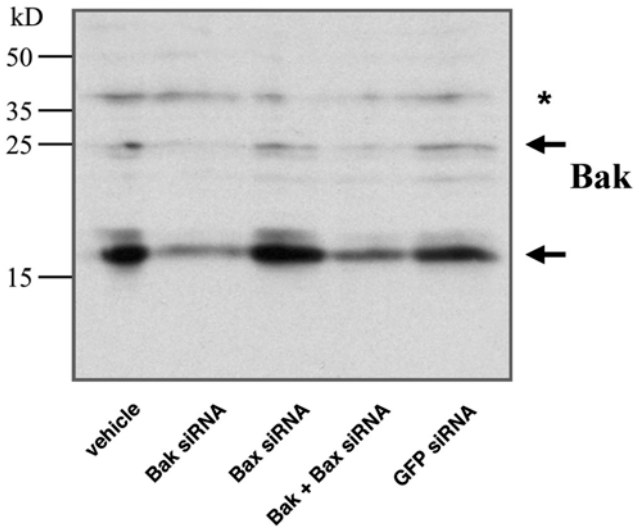

Figure 4. HK-2 cells grown to confluency in 6-well dishes were transfected with or without siRNA duplexes targeting Bak, Bax, combined Bak and Bax, or green fluorescent protein (GFP) as described in the Methods section. After 72 h, monolayers were incubated with 10 pM Stx2 for another 18 h, followed by whole cell protein extraction and SDS-polyacrylamide gel electrophoresis. Western blots were probed for Bak using a polyclonal antibody. This antibody recognizes intact Bak (25 kD) and an approximately 18 kD Bak-related protein (arrows). The asterisk denotes a nonspecific band.

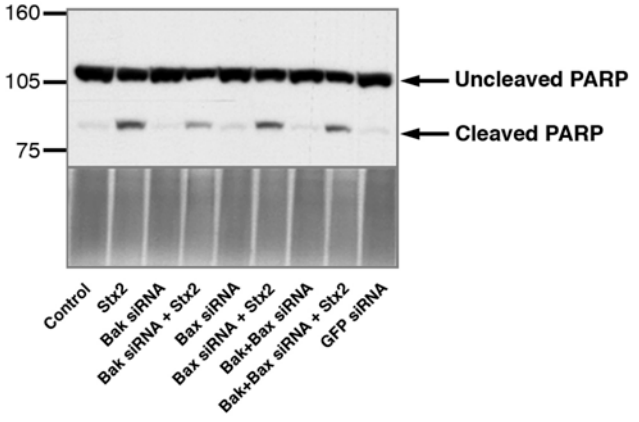

Figure 5. Confluent HK-2 cells grown in 12-well dishes were transfected with or without siRNA duplexes targeting Bak, Bax, (combined) Bak and Bax, or GFP as described in the Methods section. After 72 h, cells were exposed to 10 pM Stx2 for another 18 h, followed by protein extraction and SDS-gel electrophoresis as noted in the legend of figure 4. Western blots were probed for PARP cleavage using a polyclonal anti-PARP antibody, which recognizes the intact (116 kD) and the large PARP cleavage fragment (89 kD). The gel was stained after transfer to confirm equal loading (lower panel).

sis was assessed by the degree of PARP cleavage as determined by Western blotting using whole cell protein extracts. Treatment of HK-2 cells with Stx2 alone induced cleavage of PARP, as evidenced by the appearance of the 89 kD band (Figure 5). In contrast, PARP remained intact when cells were transfected with siRNA duplex in the absence of toxin. Transfection of HK-2 cells with siRNA targeting Bak (alone and in combination with Bax siRNA) reduced the amount of PARP cleavage product induced by Stx2 (Figure 5). In contrast, Bax siRNA – which previously failed to reduce expression of its target protein (see Figure 2) – also failed to prevent Stx-mediated PARP cleavage. These results are taken to indicate that silencing of Bak affords partial protection against Stx2-induced apoptosis.

Discussion

HC and HUS are tightly associated with intestinal infections by high-level Shiga toxin producing the bacteria, mostly *E. coli* (STEC) and *S. dysenteriae* type 1 [9–11]. Stx, which exhibits biological similarities to the plant lectin ricin [17, 45], is a potent inducer of apoptosis. However, its dependence on a specific glycosphingolipid receptor directs – and limits – toxicity to Gb3-expressing tissues, such as microvascular endothelial and renal epithelial cells [46, 19, 23]. The aims of the present study were (1) to further characterize the apoptotic pathway by which Stx induces apoptosis; specifically, to examine the involvement of the pro-apoptotic Bcl-2 family proteins Bax and Bak; and (2) to determine if RNA interference can be successfully employed in our tubular epithelial cell model of Shiga toxin-induced apoptosis.

Since its inception only a few years ago [42], siRNA (RNA interference) technology has been applied to a wide variety of cultured cells as well as to whole organisms [43, 47]. Effective gene silencing in mammalian cells using short (19–23 nucleotide), gene-specific double-strand RNA fragments [42, 43] has been proven a powerful tool to evaluate specific gene functions. In the present work, we employed siRNA silencing in the context of Stx cytotoxicity to analyze the components of the apoptosis pathway(s) induced by Stx in renal tubular epithelial cells. While Bak expression was successfully diminished, the selected siRNA duplex targeting Bax mRNA proved ineffective under our experimental conditions. As a first step, we demonstrated that silencing of Bak reduced apoptosis by Stx as evidenced by diminished cleavage of the nuclear poly-A ribose polymerase (PARP), a useful marker of irreversible apoptosis and nuclear fragmentation [35]. While this result is consistent with a role for Bak in Stx-induced apoptosis, silencing of Bak alone proved insufficient to prevent apoptosis by Stx in these cells.

We have previously reported that Stx predominantly activates the mitochondrial pathway of apoptosis in epithelial cells [48]. Additional work from our laboratory showed that Stx-induced apoptosis is associated with mitochondrial translocation of Bax [49]. While the latter observation lets us postulate that apoptosis induction by Stx in HK-2 cells involves both Bak and Bax, the current results need to be complemented using an alternative set of (Bax) siRNA duplexes.

The specific role and contribution of Bak has recently garnered renewed interest [38, 39, 50, 51]. Our finding that

Bak knockdown partially protected HK-2 cells from Stx-induced apoptosis is consistent with other models of intrinsic apoptotic pathway induction in mammalian cells [38]. *Chandra* et al. [38] recently reported that ablation of Bak by RNA interference impaired the sensitivity of epithelial colon cancer (HCT116) cells to staurosporine and other intrinsic pathway inducing agents, while Bak silencing in isogenic, Bax-deficient cells rendered them completely resistant to apoptosis induction. Similarly, double-knockout mouse embryonic fibroblasts deficient in Bax and Bak were resistant to apoptosis induction by various stimuli that act through disruption of mitochondrial function as well as to endoplasmic reticulum stress stimuli, while deletion of either gene alone failed to confer resistance to the same pro-apoptotic stimuli in these cells [51]. While the degree of functional overlap between Bax and Bak remains controversial, differential apoptotic and non-apoptotic functions have recently been described [38, 40]. Furthermore, quantitative differences have been noted in the expression levels of these proteins in different tissues and cell lines. For example, reduced Bak expression and somatic *bak* mutations have been observed in prostate and gastrointestinal cancer cells, respectively (for references, see [38]). On the other hand, proinflammatory stimuli, such as IFN-gamma, were noted to increase Bak expression in endothelial cells [52], which may tilt the balance of pro- and anti-apoptotic Bcl-2 family proteins.

Additional studies are needed to further define Stx-induced apoptosis pathway(s) and to more definitively establish the critical components of the biological cascade leading to cell and tissue injury by this toxin. Stx-mediated HUS provides an interesting model of a multisystem disease, which is caused, at least in part, by apoptotic events triggered by an infectious agent.

Current treatment of HUS is supportive [12, 5]. New therapeutic strategies should be aimed at the prevention or interruption of early intracellular events leading to toxin-induced organ failure. The goal is to identify suitable intracellular targets, such as key apoptosis related proteins, that are amenable to nontoxic drug therapy and that can be administered during the window between the onset of diarrhea and the development of HUS and other systemic complications.

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