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TAT-BH4 and TAT-Bcl-xL Peptides Protect against Sepsis-Induced Lymphocyte Apoptosis In Vivo

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Apoptosis is a key pathogenic mechanism in sepsis that induces extensive death of lymphocytes and dendritic cells, thereby contributing to the immunosuppression that characterizes the septic disorder. Numerous animal studies indicate that prevention of apoptosis in sepsis improves survival and may represent a potential therapy for this highly lethal disorder. Recently, novel cell-penetrating peptide constructs such as HIV-1 TAT basic domain and related peptides have been developed to deliver bioactive cargoes and peptides into cells. In the present study, we investigated the effects of sepsis-induced apoptosis in Bcl-xL transgenic mice and in wild-type mice treated with an antiapoptotic TAT-Bcl-xL fusion protein and TAT-BH4 peptide. Lymphocytes from Bcl-xL transgenic mice were resistant to sepsis-induced apoptosis, and these mice had a 3-fold improvement in survival. TAT-Bcl-xL and TAT-BH4 prevented Escherichia coli-induced human lymphocyte apoptosis ex vivo and markedly decreased lymphocyte apoptosis in an in vivo mouse model of sepsis. In conclusion, TAT-conjugated antiapoptotic Bcl-2-like peptides may offer a novel therapy to prevent apoptosis in sepsis and improve survival.

Sepsis is a major growing health problem. Deaths due to sepsis and the resulting multiple organ failure are approaching one quarter million patients per year in the United States (1, 2). Important new insights into the pathophysiology of sepsis have been gained by postmortem studies of patients dying of sepsis. Three independent autopsy studies of adult, pediatric, and neonatal patients who died of sepsis demonstrated profound depletion of T and B lymphocytes (3–7). Studies in both primate models and humans with sepsis have shown that apoptosis is the pathogenic mechanism responsible for the death of lymphocytes (3–5, 8, 9). The extensive apoptosis of lymphocytes and dendritic cells is most likely an important factor contributing to the immunosuppression that is a hallmark of patients with sepsis. Apoptosis-induced lymphocyte depletion occurring during sepsis not only impairs the adaptive immune response, but also compromises the innate immune system because of the cross-talk between these two immune defense systems (10).

The mechanistic importance of apoptosis in sepsis is highlighted by findings from multiple investigative groups showing that therapies that inhibit apoptosis, e.g., overexpression of antiapoptotic proteins, Fas pathway inhibitors, caspase inhibitors, and antiproapoptotic proteins, result in improved survival (11–16). One strategy that has been used is overexpression of the antiapoptotic protein Bcl-2 (11, 17, 18). Sepsis and endotoxemia are known to result in decreased lymphocyte Bcl-2, and three independent investigative groups have demonstrated that transgenic mice that overexpress the antiapoptotic Bcl-2 in their lymphocytes have improved sepsis survival (11, 17, 18).

Recent remarkable studies demonstrate that large cargoes, proteins, and peptides can be delivered intracellularly if conjugated to permeation peptides derived from HIV-1 TAT basic domain or amnotmapedia homeodomain (19–28). Rapid and receptor-independent uptake of TAT-conjugated peptides have been demonstrated to occur in many cell types (24, 26–28). Although TAT-Bcl-2 is insoluble, another member of the antiapoptotic Bcl-2 family, i.e., Bcl-xL, has been conjugated to TAT and is readily soluble. TAT-Bcl-xL has been shown to prevent ischemia/reperfusion-induced apoptosis in brain tissue (20, 21, 23). Given the reproducible and highly beneficial effects of transgenic overexpression of Bcl-2 in sepsis, we hypothesized that TAT-Bcl-xL would inhibit bacterial-induced apoptosis. Production of TAT-Bcl-xL involves bacterial transfection of Bcl-xL and purification of the bacterial extract (19–21). A small amount of endotoxin is invariably present in the resultant purified product, a situation that is not ideal for sepsis studies. Consequently, an alternative approach using solid-phase peptide synthesis of the active antiapoptotic BH4 domain of Bcl-xL was also used. Recently, TAT-BH4 has been reported to be efficacious in decreasing apoptosis in a wide range of models, including irradiation, etoposide treatment, and ischemia/reperfusion (22, 25, 26).

In this study, we demonstrate that administration of TAT-Bcl-xL and TAT-BH4 provided highly significant protection both in vitro and in vivo against bacterial-induced lymphocyte apoptosis. We conclude that TAT-conjugated BH4 may offer a novel means to prevent the profound immune cell depletion that is central to the pathophysiology of sepsis.
Materials and Methods

Bcl-xL\textsubscript{L} transgenic mice

Mice that selectively overexpress Bcl-x\textsubscript{L} in T lymphocytes using the lck-proximal promoter were provided by C. Thompson (University of Pennsylvania, Philadelphia, PA) (29). These mice had been backcrossed to C57BL/6 mice for >10 generations. Tail snips were obtained to verify the presence of the transgene via PCR analysis. C57BL6/6 mice purchased from The Jackson Laboratory were used as controls.

Sepsis model: cecal ligation and puncture (CLP)\textsuperscript{1}

C57BL6/6 male mice were housed for at least 1 wk before manipulations. The CLP model was used to induce intra-abdominal peritonitis (30). Previous studies from our laboratory include positive blood cultures for polymicrobial organisms (aerobic and anaerobic bacteria) from CLP, but not sham-operated mice (31). Mice were anesthetized with halothane, and an abdominal incision was performed. The cecum was identified, ligated, and punctured with a 30-gauge needle. The abdomen was closed in two layers, and 1 ml of 0.9% saline was administered s.c. Sham-operated mice were treated identically, except the cecum was not ligated or punctured.

For survival studies, mice received 25 mg/kg imipenem 3 h postoperatively and twice per day for 2 days. Survival was recorded for 7 days.

Quantification of apoptosis

Thymocytes and splenocytes were obtained from CLP and sham-treated mice ~20 h postoperatively, and apoptosis was quantified by flow cytometry using Abs to active caspase 3 (Cell Signaling Technology; catalog 9664) and/or TUNEL assay. The APO-BrdU kit (Phoenix Flow Systems) was used for flow cytometric quantitation of TUNEL, and the manufacturer’s instructions were followed without modification. Lymphocyte B and CD3 T cells were identified using fluorescently labeled mAbs directed against their respective CD surface markers (BD Pharmingen): flow cytometric analysis (25,000–50,000 events/sample) was performed on FACS can (BD Biosciences).

Escherichia coli bacterial-induced human lymphocyte apoptosis

Lymphocytes were harvested from peripheral blood obtained from six healthy volunteers using a Ficoll gradient separation technique. Approximately 1 × 10\textsuperscript{6} lymphocytes were plated in individual Transwell containers. E. coli bacteria (strain ATCC 25922), grown overnight in tryptophase soy broth, were added to a separate compartment of the Transwell chamber separated from direct contact with the lymphocytes by a 0.02-μm pore size filter (25 μl of bacteria at 3 × 10\textsuperscript{5} CFUs added to 1 ml vol). Bcl-x\textsubscript{L}, TAT-Bcl-x\textsubscript{L}, TAT-BH4, or an inactive TAT-BH4(D)\textsubscript{2} (see below) were placed in experimental wells within 20 min after addition of bacteria, and the lymphocytes were incubated for 5 h. The inactive TAT-BH4(D)\textsubscript{2} was identical with TAT-BH4, except 2 aa substitutions (aspartic acid replaced two tyrosines in the BH4 sequence) that rendered the BH4 inactive by simulating the TAT-BH4, except for 2 aa that are essential for the antiapoptotic activity of BH4 were replaced by aspartate to render it inactive.

Expression and purification of rTAT-Bcl-x\textsubscript{L}

The Bcl-x\textsubscript{L} coding sequence was PCR amplified from C57BL6/6 mouse whole-brain cDNA, as previously described (27). Purified PCR fragments were cloned into the XhoI/EcoRI sites of the pTAT-HA vector kindly provided by S. Dowdy (University of California School of Medicine, San Diego, CA). All expression cassettes included a sequence encoding six consecutive histidine residues for purification. TAT-Bcl-x\textsubscript{L} was expressed in E. coli strain BL21(DE3) pLYsS (Novagen) and lysed by sonication. E. coli lysates were denatured in 8 M urea before affinity chromatography. Bacterial debris was pelleted, and the supernatant was subjected to metal-affinity chromatography using a Ni-NTA matrix. TAT-Bcl-x\textsubscript{L} identity was confirmed by Western blotting. Urea and salt were removed by gel filtration using a PD-10 Sephadex G-25M column (Amersham Biosciences).

Peptide synthesis

Amino acid sequences of TAT basic domain and the BH4 peptide used in the present study are similar to those used in earlier studies with two exceptions. First, (d)-amino acids were used for synthesis of TAT basic domain because these are more slowly metabolized, and therefore the effective t\textsubscript{1/2} of the compound is prolonged (32). Second, previous sequence-activity analysis had shown that substitution of ornithine for glutamine enhanced cell permeation of the TAT peptides by ~10-fold (32). The amino acid sequence of TAT-BH4 was the following: (d)-Ac-RKKRR-

On-RRR, Bb-A-(l)-SNRELVVDLSKLYLKSQKGS-COOH, wherein Bb represents β-alanine. Orn is ornithine, and the N terminus is acetylated.

The peptide that was used as a control for TAT-BH4 was identical with the TAT-BH4, except for 2 aa substitutions (aspartic acid replaced two tyrosines in the BH4 sequence) that rendered the BH4 inactive by simulating the native phosphoprotein domain (22). The amino acid sequence of the inactive TAT-BH4(D)\textsubscript{2} was the following: (d)-Ac-RKKRR-Orn- RRR, Bb-A-(l)-SNRELVVDLSKLYLKSQKGS-COOH.

Peptides were generated by solid-phase peptide synthesis using standard Fmoc chemistry by Tufts University Peptide Synthesis Core and purified by HPLC. Identity was confirmed by amino acid analysis and mass spectrometry. Purity was ~95%.

In vivo administration of TAT-BH4 via infusion pumps

To evaluate the antiapoptotic efficacy of TAT-BH4 in an in vivo model of sepsis, miniosmotic pumps (Alzet Model 2010D; Direct) were loaded with 1 mg of TAT-BH4 or the TAT-BH4(D)\textsubscript{2}, inactive analog dissolved in 200 μl of sterile saline and implanted in the s.c. tissues on the dorsum of the mice. The pumps were implanted ~3 h before CLP because it requires ~3 h for pumps to activate and deliver steady state levels of compound. In addition to the TAT-BH4 peptides that were administered by the Alzet miniosmotic pumps, an additional dose of 0.5 mg of TAT-BH4 or inactive TAT-BH4(D)\textsubscript{2} was administered via i.p. injection 2–3 h before sacrifice of the animals, which was ~18 h postprocedure.

 Laser-scanning confocal microscopy of TAT-BH4-treated human lymphocytes

To confirm that TAT-BH4 was internalized by the cells, freshly isolated human lymphocytes were incubated with a fluorescently labeled TAT-BH4 peptide. To prepare the fluorescent labeled TAT-BH4, (d)-Ac-C (FM) RKKRR-Orn-RRR-Bb-A-(l)-SNRELVVDLSKLYLKSQKGS-COOH, an N terminus cyanine, was included in the internal solid state peptide synthesis of the peptide and, following HPLC purification, the peptide was thiol conjugated to fluorescein maleimide (1:2 equivalent; Molecular Probes) at ambient temperature in 50% dimethylformamide/water for 2 h. Quantitative yields were analyzed by C\textsubscript{18} reverse-phase HPLC. For labeling, cells were suspended for 30 min in modified Earls’ balanced salt solution containing 1 μM fluorescently labeled TAT-BH4 (33). Control cells were treated identically, except no labeled TAT-BH4 was added. Following fixation (10 min) in 4% paraformaldehyde, cells were analyzed for peptide internalization via detection of fluorescence by confocal microscopy using an inverted Zeiss Axiore 200 laser-scanning confocal microscope coupled to a Zeiss LSM 5 PASCAL fitted with a 488 nm excitation Ar laser and a 520-nm bandpass emission filter. All images were obtained using a water immersion lens (×40) and identical instrument settings.

TAT-BH4-induced gene expression determined by microarray analysis

To determine potential mechanisms of protection afforded by TAT-BH4, microarray analysis was performed. Fresh peripheral blood was obtained from healthy human volunteers (n = 5). Cells were isolated by ficoll density gradient separation in combination with RosetteSep (StemCell Technologies), which negatively selects for total lymphocytes. Approximately 4 × 10\textsuperscript{6} lymphocytes/well were incubated for 5 h either untreated or treated with E. coli, E. coli + 1 μM TAT-BH4, or E. coli + 1 μM TAT-BH4(D)\textsubscript{2}. Following incubation, lymphocytes were lysed on QiShredder spin columns and RNA was isolated using RNeasy Mini Spin Columns (Qiagen). Quality and yield of RNA were determined using 2100 Bioanalyzer (Agilent). Target was prepared from isolated RNA using the Nugen Ovation Biotin System and hybridized to Affymetrix Human Genome U133 Plus 2.0 Genechip Arrays. Arrays were stained and washed using an Affymetrix fluids station and scanned for signal intensity per manufacturer’s protocol.

Statistical analysis

Data are reported as the mean ± SEM. Data were analyzed using the statistical software program Prism (GraphPad). Data involving two groups only were analyzed by Student’s t test, while data involving more than two groups were analyzed using one-way ANOVA with Tukey’s multiple comparison test. Significance was accepted at p < 0.05.

Statistical analysis for microarray data

Normalized expression values were calculated using Robust Multichip Average software. A two-way ANOVA with a Bonferroni correction for multiple test groups and pairwise comparisons were performed for gene discovery. A false discovery rate (significance = 0.05) was used to identify

\textsuperscript{1} Abbreviation used in this paper: CLP, cecal ligation and puncture.
genes with significantly altered gene expression. Ingenuity Pathways Analysis evaluated potential mechanisms of cell death.

Animal studies were approved by the Animal Studies Committee at Washington University School of Medicine. The use of volunteer blood donors for lymphocyte studies was approved by the Human Studies Committee at Washington University School of Medicine.

Results

Overexpression of Bcl-xL decreases sepsis-induced apoptosis and improves survival

Sepsis caused a marked increase in CD3 T cell apoptosis in thymus and in both CD3 T and B cell apoptosis in spleen in wild-type mice as determined by increased active caspase 3 and TUNEL-positive cells (Figs. 1 and 2). In contrast, transgenic overexpression of Bcl-xL totally prevented the increase in CD3 T cell death in both organs (Figs. 1 and 2) (n = 8 each for wild-type and transgenic groups).

Significantly, Bcl-xL overexpression not only prevented sepsis-induced lymphocyte apoptosis, but it also caused a >3-fold improvement in long-term survival from sepsis, thereby confirming the key role of lymphocyte death in this disorder (Fig. 3) (n = 8 wild-type and 8 transgenic mice).

TAT-Bcl-xL and TAT-BH4 decrease E. coli-induced CD3 T cell apoptosis in vitro

Human lymphocyte apoptosis as determined by active caspase 3 immunohistochemical staining and flow cytometry increased from 6.0 ± 1.1% in control to 30.1 ± 8.2% with E. coli treatment (p < 0.01) (n = 6) (Fig. 4). TAT-Bcl-xL fusion protein at 0.5 and 1.0 μM caused a decrease in E. coli-induced apoptosis to 13.7 ± 2.7% and 9.8 ± 2.6%, respectively (p < 0.05). There was no decrease in E. coli-induced apoptosis by treatment with free Bcl-xL (Bcl-xL that was not conjugated to TAT).

TAT-BH4 peptide at 0.5 and 1.0 μM (but not 0.2 μM) caused a decrease in E. coli-induced apoptosis in CD3 T cell human lymphocytes. No decrease in apoptosis occurred in cells treated with the TAT-BH4(D)2 peptide that was inactivated by the 2-aa substitutions (n = 6) (Fig. 5). The TUNEL method confirmed the active caspase 3 findings in an independent, small cohort of cells (our unpublished data).

TAT-BH4 administered in vitro localizes intracellularly as determined by laser-scanning confocal microscopy

To verify that the TAT-BH4 peptide was taken up and internalized within the cells, laser-scanning confocal microscopy was performed on freshly isolated human lymphocytes loaded with a fluorescein-conjugated TAT-BH4. Serial sectioning of cells incubated with fluorescein-conjugated TAT-BH4 demonstrated a homogeneous uptake of the compound throughout the cell (Fig. 6, A and B). No fluorescence was demonstrated in cells not incubated with the fluorescein-tagged TAT-BH4.

TAT-BH4 administered in vivo localizes intracellularly as determined by flow cytometry

To determine whether TAT-BH4 was taken up and internalized within cells when administered in vivo, mice were injected with 1 mg of FITC-conjugated TAT-BH4, and ~4 h later PBLs and splenocytes were harvested. Flow cytometry demonstrated uptake of the FITC-labeled TAT-BH4 into the cells compared with cells from mice that had no injection of labeled compound (Fig. 6C).

In vivo administration of TAT-BH4 decreases sepsis-induced lymphocyte apoptosis

Infusion of the active antiapoptotic TAT-BH4 peptide caused a significant decrease in sepsis-induced splenic CD3 T cell (p < 0.05) and B cell apoptosis (p < 0.01) compared with mice infused with inactive TAT-BH4(D)2 peptide (Fig. 7). There was a similar trend toward decreased thymic and blood CD3 T cell apoptosis in septic mice treated with TAT-BH4, but the differences were not statistically significant (n = 6 control, 8 TAT-BH4, and 8 inactive TAT-BH4(D2)).

Gene expression analysis

Negative selection yields a population of cells that were 90.4 ± 0.6% CD3 T cells by flow cytometric analysis (n = 20). The 330
probe sets showed significantly altered levels of expression >2-fold in E. coli-treated cells compared with control. A complete list of these probe sets that were up- or down-regulated is provided on the following link: www.ebi.ac.uk/arrayexpress/. The accession number is: E-MEXP-555. Cell death, cell-cell interaction/signaling, and cell cycle were three high-level functions identified as significantly altered in E. coli-treated cells compared with control. All 55 of the genes with known involvement in apoptosis were up-regulated. Among these are genes involved in both the receptor and mitochondrial-mediated pathways. Only two genes, the zinc-finger transcription factor egr3 and the NR4A nuclear receptor family member nr4a3 (nor-1), showed a significant change in gene expression in cells treated with E. coli plus TAT-BH4 compared with E. coli plus TAT-BH4(D)2. Although the change in expression of these two genes was statistically significant, it was <2-fold. There were no significant changes in gene expression between the E. coli plus TAT-BH4(D)2- and E. coli alone-treated groups.

Discussion

Apoptosis is a major pathophysiologic event in sepsis, leading to depletion of lymphocytes and dendritic cells (3–5, 8, 9, 13, 15). The findings from the Bcl-xL transgenic mice documenting that overexpression of Bcl-xL prevents sepsis-induced lymphocyte apoptosis and improves survival are important because they strongly support the hypothesis that lymphocyte apoptotic death is a critical element in the disorder. The results in the Bcl-xL transgenic mice are highly consistent with three previous publications showing that transgenic mice that overexpress Bcl-2 in lymphocytes have decreased sepsis-induced apoptosis and decreased mortality (11, 17, 18). Bcl-xL transgenic mice responded to sepsis in a manner comparable to Bcl-2 transgenics, although both proteins have different functions in development (29).

An interesting and important point is the contrasting effect of sepsis on lymphocyte vs neutrophil apoptosis. Sepsis accelerates lymphocyte apoptosis, but delays neutrophil apoptosis. The normal t1/2 of the neutrophil is 24 h, but sepsis increases this t1/2 significantly. Although investigators have speculated that the delayed neutrophil apoptosis could be responsible for tissue injury, particularly in the lung, there is currently no convincing data to support this.
this hypothesis. Studies in patients treated with G-CSF, which increased the number of activated neutrophils in patients several-fold and delayed apoptosis in neutrophils, showed no detrimental effects on lung or organ function. Nevertheless, this effect of sepsis to accelerate lymphocyte apoptosis, but delay neutrophil apoptosis, demonstrates the complexity of the cellular response to sepsis and may play a yet unrecognized role in the host response.

In addition to findings in animal models of sepsis, clinical studies have shown decreased bcl-2 gene expression and/or Bcl-2 protein concentration in patients with sepsis (9, 34). Bilbault et al. (34) noted enhanced lymphocyte apoptosis and a 10-fold down-regulation of bcl-2 in patients dying of sepsis vs survivors of sepsis. Our laboratory noted an increase in lymphocyte apoptosis associated with a marked decrease in lymphocyte Bcl-2 protein concentrations in patients with sepsis (9). Furthermore, patients with septic shock had the highest degree of lymphocyte apoptosis and the lowest concentrations of Bcl-2. Overall, the Bcl-xL/Bcl-2 animal sepsis studies together with clinical studies of septic patients demonstrating decreased bcl-2 expression and protein content provide a convincing rationale for efforts to deliver antiapoptotic Bcl-2-like compounds intracellularly to immune cells during sepsis.

The ability to deliver biologically active compounds into the intracellular compartment via conjugation to cell-penetrating peptides creates exciting new therapeutic approaches. Investigators have used cell-penetrating peptide constructs such as TAT basic domain and antennapedia to deliver intracellularly a host of diverse molecules, including proteins, peptides, oligonucleotides,
TAT-BH4 decreases sepsis-induced apoptosis in vivo. Miniosmotic infusion pumps containing 1 mg of TAT-BH4 or inactive TAT-BH4(D)2 were implanted in s.c. tissues on the dorsum of the mice 3 h before CLP. The pumps are not activated until ∼3 h after implantation. Mice received an additional 0.5 mg dose of TAT-BH4 or inactive TAT-BH4(D)2 via i.p. injection 2–3 h before sacrifice. Spleens, thymi, and blood were harvested and examined for apoptosis via staining for active caspase 3. TAT-BH4 ameliorated the increase in sepsis-induced CD3 T and B cell apoptosis in the spleen.

FIGURE 7. TAT-BH4 decreases sepsis-induced lymphocyte apoptosis in vivo. Miniosmotic infusion pumps containing 1 mg of TAT-BH4 or inactive TAT-BH4(D)2 were implanted in s.c. tissues on the dorsum of the mice 3 h before CLP. The pumps are not activated until ∼3 h after implantation. Mice received an additional 0.5 mg dose of TAT-BH4 or inactive TAT-BH4(D)2 via i.p. injection 2–3 h before sacrifice. Spleens, thymi, and blood were harvested and examined for apoptosis via staining for active caspase 3. TAT-BH4 ameliorated the increase in sepsis-induced CD3 T and B cell apoptosis in the spleen.

In conclusion, TAT-BH4 peptide is readily internalized into human lymphocytes and has potent antiapoptotic activities against bacterial-induced lymphocyte apoptosis both in vitro and in vivo. Given the extensive animal and clinical studies demonstrating an important role for apoptosis in sepsis and the protective effect of...
Bcl-2 overexpression, these peptides may offer a novel therapy for this highly lethal disorder.

Disclosures
The authors have no financial conflict of interest.

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