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Over-Expression of Bcl-2 Provides Protection in Septic Mice by a trans Effect

Akiko Iwata, Vicki Morgan Stevenson, Annie Minard, Michael Tasch, Joan Tupper, Eric Lagasse, Irving Weissman, John M. Harlan, and Robert K. Winn

Transgenic mice that over-express B cell leukemia/lymphomas (Bcl)-2 in myeloid cells under control of the human MRP8 promoter (hMRP8-Bcl-2) or in T lymphocytes under the Eμ promoter (Eμ-Bcl-2) were compared with C57BL/6 control mice following cecal ligation and puncture (CLP). There was a significant difference in outcome between the hMRP8-Bcl-2 and control mice with 100% survival in the hMRP8-Bcl-2 mice vs 25% survival in the control mice. In separate experiments there was a significant difference between Eμ-Bcl-2 and control mice with 87.5 and 22.2% survival, respectively. Adoptive transfer of CD11b-positive bone marrow cells from hMRP8-Bcl-2 or C57BL/6 mice to C57BL/6 mice subjected to CLP resulted in 100 and 0% survival, respectively. Adoptive transfer of CD11b-positive cells from either hMRP8-Bcl-2 or C57BL/6 mice to Rag-1−/− mice (no mature T or B cells) subjected to CLP resulted in survival of 87.5 and 12.5%, respectively. The hMRP8-Bcl-2 mice had significantly more neutrophils and fewer bacteria in the peritoneum compared with C57BL/6 mice 24 h after CLP. These experiments show that Bcl-2 over-expression is protective in CLP and that protection is independent of lymphocytes. We propose that over-expression of Bcl-2 in T cells or myeloid cells induce release of a molecule(s) that protects against death following CLP. The Journal of Immunology, 2003, 171: 3136–3141.

Sepsis, or sepsis syndrome, is a life-threatening illness that continues to be a major health problem despite significant advances in clinical care (reviewed in Refs.1–3). Estimates of the number of cases of sepsis vary from 500,000 to 1 million per year in the United States (reviewed in Ref. 33), and estimates of mortality in sepsis vary from 25 to 80% (reviewed in Refs.2 and 3). In the last decade there have been at least 20 phase III placebo-controlled clinical trials designed to treat sepsis (4, 5). Activated protein C has shown moderate efficacy (6), but all other therapies tested have failed. Most of these trials were directed toward reducing inflammation, and their predominant failure prompts investigation into targeting of the other mechanisms of injury.

A number of recent studies have reported that sepsis induces apoptosis of lymphocytes, epithelial cells in the intestine and lung (7, 10, 11), and endothelial cells (11). These observations led to experimental studies designed to block apoptosis in various cell types. Hotchkiss et al.(12) showed that over-expression of the anti-apoptotic protein Bcl-2 in either B or T lymphocytes improved survival in experimental sepsis induced by cecal ligation and puncture (CLP) in mice. Moreover, they showed that treatment with a broad-spectrum caspase inhibitor reduced mortality in mice subjected to CLP (13, 14). Both the caspase inhibitor and over-expression of Bcl-2 blocked apoptosis of lymphocytes in the spleen and thymus, and they suggested that the mechanism of action of these interventions was through the protection of lymphocytes from apoptosis. Adoptive transfer of T cells that over-expressed Bcl-2 also offered protection from septic death (13). They proposed that immunodepression resulting from lymphocyte apoptosis is a major contributor to mortality in experimental sepsis. More recently, over-expression of Bcl-2 under an epithelial cell specific promoter was also reported to provide protection from death following CLP suggesting an important role for intestinal epithelial cells in sepsis (15).

In the experiments reported here, we determined the effect of over-expression of Bcl-2 in myeloid cells on survival from sepsis induced by CLP. There was 100% survival of mice over-expressing Bcl-2 in myeloid cells vs 25% in control mice. Moreover, adoptive transfer of myeloid cells from bone marrow of Bcl-2 over-expressing mice into normal C57BL/6 or Rag-1−/− mice also markedly improved survival. Mice over-expressing Bcl-2 in myeloid cells exhibited markedly increased neutrophil accumulation and reduced bacteria counts in peritoneum following CLP, consistent with an increased host response.

Materials and Methods

Materials

All protocols were approved by the University of Washington Animal Care and Use Committee (Seattle, WA) and complied with the National Institutes of Health guidelines for animal use. C57BL/6 mice were purchased from Charles River Breeding Laboratories (Wilmington, MA). Transgenic mice over-expressing human Bcl-2 under the myeloid cells or T lymphocytes were bred in our animal facility. These transgenic mice were on a C57BL/6 background and specific expression was controlled by cell specific promoters. The MRP8 protein is restricted to myeloid cells and the human promoter for this protein was used to drive expression of human Bcl-2 (16). Polymorphonuclear neutrophils (PMNs) and circulating monocytes, but not tissue macrophages, from the human MPR8 (hMRP8)-Bcl-2 mice express the transgene in myeloid cells as expected (16). The Eμ-promoter is active in lymphocytes and mice were produced that over-express human Bcl-2 under control of this promoter. Several strains of transgenic mice have been produced using this
promoter resulting in expression of Bcl-2 in only T lymphocytes (17) or only B lymphocytes (18) or both (19). The site of incorporation of the transgene appears to determine where expression will occur. We used the mice expressing Bcl-2 in T lymphocytes. Bcl-2 expression under the Eμ-promoter was restricted to T lymphocytes (17) for experiments reported here. The transgenic mice were genotyped by PCR using genomic DNA from a small portion of the tail. Rag-1−/− mice on a C57BL/6 background were obtained from The Jackson Laboratory (Bar Harbor, ME). These mice are homozygous for the Rag-1−/− Tml Mom mutation and produce no mature T or B cells (20). Mice were maintained at the University of Washington, Department of Comparative Medicine facility (Seattle, WA).

Experimental sepsis induced by CLP

Mice were anesthetized with halothane, an abdominal midline incision was made, and the cecum was gently removed and ligated below the ileocecal valve without obstruction of the ileum or colon. The cecum was then subjected to a single “through and through” perforation with a 20-gauge needle and a small amount of cecal contents was gently expressed through the needle wound. The bowel was carefully returned to its original position, and then the abdominal incision was closed in layers with 4–0 sutures.

Adaptive transfer of bone marrow cells

hMRP8-Bcl-2 and C57BL/6 mice were killed and the long bones were removed from their legs. Bone marrow was removed by flushing with RPMI containing 1% FBS and 7 U/ml heparin, and the marrow cell suspension was filtered through a 70-μm mesh, producing a single cell suspension. Bone marrow cells were washed with PBS containing 0.5% BSA and 2 mM EDTA. CD11b-positive cells were isolated using magnetic beads as described by the manufacturer (Miltenyi Biotec, Auburn, CA). These CD11b-positive cells were >90% PMNs determined by differential cell counts. Cells were resuspended in PBS, and 107 cells/mouse from hMRP8-Bcl-2 mice or control C57BL/6 mice were injected into the peritoneum of C57BL/6 or Rag-1−/− mice 2 h after CLP.

Survival after CLP

Mice were followed for up to 8 day after CLP. Each animal was scored using an assessment form that evaluated each animal’s health as described by Morton and Griffiths (21), and euthanized if they exceeded a predetermined score that suggested irreversible sepsis. All animals received 0.5 mg of Imipenem 2 h after CLP, and the injection of 0.5 mg/mouse of Imipenem in 1.0 ml of D5W was continued every 12 h for 4 days.

PMN and bacteria counts in peritoneal lavage fluid

In a separate set of experiments, mice were killed 24 h after CLP and the peritoneal cavity was washed with sterile saline. Peritoneal lavage was performed by making a small incision in the midline of the abdomen and injecting 1–2 ml of sterile saline. The saline was removed by aspiration and the process was repeated until a total of 5 ml of lavage fluid was obtained. The total number of leukocytes in a 0.1-ml aliquot was determined after methylene blue nuclear staining. A cytospin preparation was made using Diff-Quick staining (Dade Behring, Deerfield, IL). Aliquots of serial 1–10 dilutions of the peritoneal lavage fluids were plated on tryptic soy agar dishes (PML Microbiologicals, Tuatilin, OR), and the number of colonies was counted at 24 and 48 h after CLP.

Thymus cellularity determination

Twenty-four hours after CLP, C57BL/6 and hMRP8-Bcl-2 mice were sacrificed by cervical dislocation. Thymi were harvested and single cell suspensions were prepared by mechanical disruption and maceration between frosted glass slides, as described by Cooke et al. (22). Viable cells, as assessed by trypan blue exclusion, were enumerated by hemocytometer counting.

TUNEL assay

Transgenic and non-transgenic mice were sacrificed 24 h after CLP, and their thymus and entire intestine was removed. The intestine was opened along the length of its cephalocaudal axis and washed in 4% neutral buffered formalin to remove luminal contents. Then the tissue was rolled from the proximal to the distal end and fixed with 10% neutral buffered formalin. Paraffin embedded sections from thymus and intestine were examined for DNA strand breaks TUNEL (In Situ Cell Death Kit; Roche Molecular Biochemicals, Indianapolis, IN) as described by the manufacturer.

Cytokine assay

Whole blood was drawn by cardiac puncture at 12 or 24 h after CLP and plasma prepared. Blood was centrifuged for 10 min at 1200 rpm to separate plasma from cells. Concentrations of IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12(p40), IL-12(p70), IL-17, G-CSF, GM-CSF, IFN-γ, KC, MIP-1α, RANTES, and TNF-α were measured by Bio-Plex protein assay system (Bio-Rad, Hercules, CA) and read by Luminex 100 system (Mirai Bio, Alameda, CA) according to manufacturer specifications.

Statistical analysis

The data are presented as the means ± SEM. Statistical analysis was performed using the two-tailed Student’s t test. Survival was evaluated using Fisher’s exact test. Differences associated with a value of p < 0.05 were considered statistically significant.

Results

Over-expression of Bcl-2 in myeloid cells promotes survival after CLP

The survival curve of hMRP8-Bcl-2 mice compared with C57BL/6 mice is shown in Fig. 1. The hMRP8-Bcl-2 mice showed dramatic improvement in survival compared with C57BL/6 mice. Survival following CLP was 100% in both groups at 24 h but by 72 h survival in the C57BL/6 group decreased to 50% and by 144 h post CLP it was 25%. Survival in the hMRP8-Bcl-2 group was 100% at the end of the experiment (p < 0.02). The experiment was repeated twice for a total of 12 animals in each group. Apoptotic death of lymphocytes occurs following CLP in mice (7), and over-expression of Bcl-2 in T and B lymphocytes prevents lymphocytes apoptosis and improves survival (12, 14). Therefore, we determined whether over-expression of Bcl-2 under a myeloid promoter would protect lymphocytes. To this end, we determined cell number in thymus 24 h after CLP in hMRP8-Bcl-2 and C57BL/6 mice. Total viable cell number in thymus from both hMRP8-Bcl-2 and C57BL/6 following CLP was significantly decreased compared with hMRP8-Bcl-2 and C57BL/6 mice that were not subjected to CLP (86.9 and 83.7% reduction from control thymus, respectively) (Fig. 2), consistent with loss of lymphocytes due to apoptosis (23). There was no difference in cell number between the C57BL/6 mice and the hMRP8-Bcl-2.

Leukocyte populations in peritoneal lavage fluid

The total number of leukocytes and PMNs in peritoneal lavage fluid from hMRP8-Bcl-2 and C57BL/6 mice at 24 h after CLP was determined in separate experiments. Total leukocyte (Fig. 3A) and PMN and bacteria counts in peritoneal lavage fluid (Fig. 3B) were increased compared with hMRP8-Bcl-2 and C57BL/6 mice that were not subjected to CLP (86.9 and 83.7% reduction from control thymus, respectively) (Fig. 2), consistent with loss of lymphocytes due to apoptosis (23). There was no difference in cell number between the C57BL/6 mice and the hMRP8-Bcl-2.

FIGURE 1. Over-expression of Bcl-2 in myeloid cells improves survival in sepsis. Twelve hMRP8-Bcl-2 mice and 12 C57BL/6 mice underwent CLP. Mice were given antibiotics at 2 h after CLP and repeated doses every 12 h until day 4, as described in Materials and Methods. Two separate studies were performed and the results were combined. Mice over-expressing Bcl-2 had improved survival compared with C57BL/6 controls (p < 0.02).
Bcl-2 PROTECTS SEPSIS IN MICE BY TRANS EFFECT

PMNs in peritoneal lavage after CLP. Four hMRP8-Bcl-2 mice and four C57BL/6 mice underwent CLP and sacrificed 24 h after CLP. Peritoneal lavage fluid was obtained and total leukocyte (A) and PMN (B) counts were determined. Total leukocytes were increased in hMRP8-Bcl-2 mice and 86.3% of them were PMNs. *, p < 0.05

Bacteria in the peritoneal lavage fluid. The number of bacteria in the peritoneal lavage fluid from hMRP8-Bcl-2 and C57BL/6 mice was determined at 24 h after CLP. Colonies on soy agar plates were counted 24 and 48 h after being plated. The number of colonies formed in the hMRP8-Bcl-2 mice was significantly lower than in the C57BL/6 mice (*, p < 0.05). Note: the y-axis is a log scale.

Adoptive transfer of CD11b-positive bone marrow cells to Rag−/− mice
We transferred CD11b-positive bone marrow cells from hMRP8-Bcl-2 or C57BL/6 mice into Rag−/− mice 2 h after CLP to determine whether lymphocytes were necessary for the protection observed in the hMRP8-hBcl-2 mice. These Rag−/− mice are totally deficient in mature T and B cells. Survival of Rag−/− mice that received CD11b-positive bone marrow cells from hMRP8-Bcl-2 mice was 87.5%, whereas Rag−/− mice that received CD11b-positive bone marrow cells from C57BL/6 control mice was 12.5% at day 8 (Fig. 6).

Over-expression of Bcl-2 in myeloid inhibits sepsis-induced intestinal apoptosis
DNA strand breaks were determined by TUNEL staining of intestine and thymus taken from mice 24 h after CLP. TUNEL-positive cells from bone marrow of hMRP8-Bcl-2 mice (○) and C57BL/6 mice (○) were significantly reduced at 24 h after CLP compared with normal C57BL/6 (△) and hMRP8-Bcl-2 (△) mice.

Adoptive transfer of CD11b-positive bone marrow cells from hMRP8-Bcl-2 to C57BL/6 mice
We performed adoptive transfer of CD11b-positive bone marrow cells from hMRP8-Bcl-2 mice or C57BL/6 mice into C57BL/6 mice to determine whether the protection seen in the hMRP8-Bcl-2 mice could be transferred to normal mice. Transfer of control or Bcl-2 over-expressing bone marrow cells was performed 2 h after CLP. The results of these experiments are shown in Fig. 5. All mice that received CD11b-positive bone marrow cells from hMRP8-Bcl-2 mice survived 6 day following CLP, whereas none of the mice receiving CD11b-positive bone marrow cells from C57BL/6 mice survived past day 4.

Adoptive transfer of CD11b-positive bone marrow cells from hMRP8-Bcl-2 to C57BL/6 mice

Survival curves for Eμ-Bcl-2 mice compared with C57BL/6 control mice are shown in Fig. 7. Survival in the Eμ-Bcl-2 mice was significantly greater than for the control mice. There was 87.5% survival in transgenic mice compared with 22.2% survival in the control mice.
cells were seen in the intestinal villi in the control mice (Fig. 8A). In contrast, hMRP8-Bcl-2 mice had fewer of these TUNEL-positive cells (Fig. 8B). There was no difference in TUNEL staining in the thymus of C57BL/6 control mice and hMRP8-Bcl-2 mice and both showed extensive TUNEL positive staining. (Fig. 8, C and D).

Cytokine analysis

Plasma from transgenic and nontransgenic littermate animals was measured for 18 different cytokines (IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12(p40), IL-12(p70), IL-17, G-CSF, GM-CSF, IFN-γ, KC, MIP-1α, RANTES, and TNF-α). There is no difference between transgenic and control mice at baseline or at 12 h post CLP (transgenic mice: n = 6; littermate control mice: n = 6). At 24 h, IL-1α, IL-1β, IL-2, IL-3, IL-4, and IL-6 were significantly reduced in hMRP8-Bcl-2 mice compared with littermate controls (transgenic mice: n = 6; littermate control mice: n = 5).

Discussion

The experiments reported in this study show that survival of transgenic mice over-expressing Bcl-2 in myeloid cells or T lymphocytes was greater than in control mice following CLP. In addition, in this model of severe septic peritonitis, adoptive transfer of CD11b-positive bone marrow cells from hMRP8-Bcl-2 mice resulted in increased survival in both C57BL/6 and Rag-1−/− mice compared with adoptive transfer of CD11b-positive bone marrow cells from C57BL/6 mice.

Protection in Rag-1−/− mice, indicates that mature lymphocytes are not necessary for survival, since they are deficient in mature T and B lymphocytes. Also, there was a significant reduction in lymphocyte number in the thymus of both C57BL/6 and hMRP8-Bcl-2 mice following CLP and the reductions in both groups were equivalent. These data suggest that over-expression of Bcl-2 in T lymphocyte under the Eμ-promoter and in myeloid cells under the hMRP8-promoter, may exert their protective effect through a pathway other than by blocking apoptosis of the over-expressing cell (7, 12–14).

Previous experiments by Hotchkiss and colleagues (12–14) showed that over-expression of Bcl-2 in T cells or B cells, or adoptive transfer of Bcl-2 over-expressing T lymphocytes, or injection of caspase inhibitors reduced lymphocyte apoptosis and resulted in improved survival in sepsis. Consistent with these studies, we show here that over-expression of Bcl-2 in T cells in a different transgenic line (Eμ-Bcl-2) afforded protection (see Fig. 7). These results would seem to suggest a major role for lymphocytes in eliminating pathogens through an unknown mechanism. Hotchkiss et al. (13) proposed that prevention of lymphocyte apoptosis might result in increased production of cytokines such as IFN-γ or IL-2 as a first step in a protective pathway since IFN-γ and IL-2 have been shown to be efficacious in models of sepsis (24). However, our results show that lymphocytes are not necessary since adoptive transfer of myeloid cells over-expressing Bcl-2 protected Rag-1−/− mice, which do not have mature T and B cells. Moreover, the result of TUNEL staining shows that thymocytes of hMRP8-Bcl-2 and C57BL/6 mice had an equivalent amount of apoptosis. In addition, our measurement of cytokines suggests that they did not significantly alter the outcome. There was no specific cytokine increase (or decrease) in transgenic mice that can explain the animal survival.

Furthermore, Cooper et al. (16) have shown that over-expression of Bcl-2 in epithelial cells affords protection in CLP. Therefore, we propose an alternative explanation for the protection seen following CLP in mice that over-express Bcl-2 in various cell lineages. We propose the release of cyto-protective or anti-
inflammatory molecule(s) by cells of multiple lineages (i.e., T, B, epithelial, and myeloid) when they over-express Bcl-2.

The mechanism of this protection is not known, however, in vitro studies have shown that over-expressing of Bcl-2 or two of its anti-apoptotic homologues reduces inflammatory signaling in endothelial cells (25, 26) or macrophages (27). Although the mechanism of protection by the cyto-protective molecules is unknown and under investigation in our lab, our pathological observations indicate that the protection might result from a modification of apoptosis. Others have observed increased intestinal apoptosis in septic animals (7, 8, 28). Consistent with these observations, TUNEL-positive cells were seen in the epithelial villi of control mice, whereas, hMRP8-Bcl-2 mice had a reduced number of these TUNEL-positive cells. This observation is consistent with another report (15) showing intestinal epithelial cell apoptosis peaking at 24 h after CLP.

The increased accumulation of PMNs in the peritoneum of the hMP8-Bcl-2 mice suggests that this could account for protection in the hMRP8-Bcl-2 mice. The importance of PMN accumulation at the site of infection is best shown by patients that suffer from severe leukocyte adhesion deficiency type I (LAD-1) syndrome (reviewed in Refs.29–31). These patients have a deficiency of β2-integrin receptors, and as a consequence, their PMNs fail to emigrate to the site of infection. This defect in PMN emigration results in early death from recurrent infections unless bone marrow transplantation is performed early in life (30). Clearly, PMNs are required to control infections; however, an excessive neutrophil response might cause damage to the host as a result of undesired release of toxic substances (reviewed in Refs.32–34).

The potential for inflammatory injury by PMNs raises the question as to whether increasing PMN delivery to the site of inflammation would be helpful or harmful. There have been only a few studies examining the consequences of an increased neutrophil response. We showed that there were more PMNs in peritoneal lavage fluid 24 h after CLP in hMRP8-Bcl-2 mice compared with C57BL/6 control mice. This was accompanied by a reduction in bacteria counts in the hMRP8-Bcl-2 mice compared with C57BL/6 mice at this time-point.

In a recent study, administration of LPS 4 day before infectious challenge also enhanced bacterial clearance and improved survival in septic peritonitis (35). LPS priming resulted in an accumulation of peritoneal PMNs and these cells had a normal ability to produce reactive oxygen metabolite, and they had elevated surface density of Mac-1 and FcyR. These factors are thought to act in concert to improve the total anti-microbial activity and the bacterial clearance in the infected peritoneal cavity. In another study of septic peritonitis, the administration of oligodeoxy nucleotides increased the number of PMNs at the site of inflammation and likewise improved outcome (36). These studies and the present study are consistent with a beneficial effect of an increase in PMN number at the site of infection, i.e., PMNs promote host defense and thus increasing their number enhances host defense.

We observed more peritoneal PMNs in hMRP8-Bcl-2 transgenic mice and it is reasonable to ascribe the increase to prolonged survival of PMNs. However, Lagasse and Weissman (37) noted that, although neutrophils from hMRP8-Bcl-2 mice showed prolonged survival ex vivo, PMN accumulation and clearance from the peritoneum following thioglycollate-induced inflammation was not different from controls. In addition, phagocytosis of aged PMNs isolated from hMRP8-Bcl-2 mice was not different from phagocytosis of PMNs from control mice. They suggested that the signal for phagocytosis was not apoptosis, but was instead related to the age of the PMN (16). Thus, the increased number of PMNs may have resulted from increased recruitment to the site of inflammation rather than from decreased apoptosis.

In summary, this study shows that transgenic over-expression of Bcl-2 in myeloid or lymphoid cells, or adoptive transfer of Bcl-2-expressing myeloid cells to Rag-1−/− mice, improves survival in CLP-induced sepsis. This effect might be attributable to PMN survival and/or to the release of a cyto-protective molecule(s) from the over-expressing cells that might alternate the pathway of apoptosis in intestinal epithelium as the mechanisms of protection.

References


