Femur Fracture Induces Site-Specific Changes in T-Cell Immunity

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INTRODUCTION

Background. Trauma is associated with altered host defense and susceptibility to infection, in part due to cytokine dysregulation and altered T-cell immunity. The gut-associated lymphoid tissue (GALT) provides a defense against infection and contributes to the process of mucosal healing by T-cell activation and cytokine production.

Objective. To determine whether femur fracture induces alterations in Peyer's patch and splenic T-cell phenotype, proliferative response, and cytokine expression following traumatic injury.

Methods. Mice underwent femur fracture or sham procedure and, 48 h later, lymphocytes were isolated from spleen and Peyer's patches. Lymphocytes were cultured, and lipopolysaccharide (10 μg/ml) was added in some cultures. Cells and supernatant were harvested at 48 h. Proliferation was analyzed by [3H]thymidine, and interleukin-10 (IL-10) protein was measured by ELISA in the culture supernatant. T-cell phenotype was determined by flow cytometry.

Results. Femur fracture induced a significant increase in proliferative response in Peyer's patch immunocytes. In contrast, no significant differences were identified in splenocyte proliferative response 48 h after femur fracture injury. Femur fracture induced a significant decrease in IL-10 protein expression of both splenocytes and Peyer's patches. Femur fracture also induced a significant increase in the fraction of CD3⁺, CD4⁺, and T-cell receptor-αβ Peyer's patch immunocytes, whereas splenocytes demonstrated no significant phenotypic change.

Conclusion. Femur fracture is associated with significant alterations in Peyer's patch but not splenic T-cell phenotype and proliferative response early (48 h) after injury. Changes in the GALT immune response may contribute to intestinal mucosal dysfunction and increased susceptibility to gut-derived sepsis after traumatic injury.

The impact of trauma has far-reaching effects beyond local tissue injury. Trauma has been shown to cause changes in intestinal permeability and immunoglobulin production, which may lead to increased susceptibility to infection, systemic inflammatory response syndrome, and multiple organ dysfunction [1–4]. The gut-associated lymphoid tissue (GALT, including Peyer's patch, mesenteric lymph node, intraepithelial, and lamina propria lymphocytes) plays a critical role in providing local defense against intestine-derived infection through interactions between B and T immunocytes, IgA production, and cytokine production. Little is known, however, of the local GALT immune response induced by traumatic injury.

Recent experimental studies have documented significant alterations in GALT immunity in murine models of hemorrhagic shock and trauma. Xu et al. [5] documented a significant decrease in total viable cell yield and an increased apoptosis in Peyer's patch cells 24 and 72 h following hemorrhage or trauma (laparotomy) plus hemorrhage in a murine model. In addition, Abraham and Freitas [6] found decreased numbers of B cells secreting IgM, IgA, and IgG2b as well as total serum Ig levels after hemorrhage, and suggested that these indicators of humoral immunosuppression play a significant role in the intestinal mucosal immune response following trauma and hemorrhage.

It has previously been determined that tissue injury induces significant alterations in systemic immunity...
and cytokine regulation that differ from studies in hemorrhagic shock models. We have previously documented that traumatic injury induces a suppression in systemic interleukin-10 (IL-10) expression, and is associated with increased gut permeability [7, 8]. To our knowledge, no previous studies have been performed to date investigating the alteration in mucosal immune (GALT) function after soft tissue trauma. In these studies we investigated the effect of tissue trauma on intestinal mucosal immunity in a murine femur fracture model by evaluation of Peyer's patch immunocytes.

MATERIALS AND METHODS

Animals. Male Balb/C mice (Taconic Farms, New York, NY), 20 to 30 weeks old and weighing 20–25 g, were used in this study. Animals were maintained on a 12-h light/dark cycle and were fed standard chow and water ad libitum. All procedures, anesthesia, and euthanasia were approved by the Institutional Animal Care and Use Committee of the University of Maryland at Baltimore. All experimental groups (control, sham, femur fracture) included 12 animals per group.

Experimental design. The animals were anesthetized with inhalational methoxyflurane. The right hind leg was shaved and prepped with 70% alcohol. Femur fracture (FFx) animals underwent a force fracture at the midshaft of the left femur with a hemostat, inducing both soft tissue and bone crush injuries, and were resuscitated with 1.0 cc normal saline administered subcutaneously at the base of the neck and maintained on a heating pad until full recovery from anesthesia. Sham controls underwent anesthesia with methoxyflurane and resuscitation alone. Animals were allowed standard chow and water ad libitum in the postoperative period and were sacrificed 48 h after femur fracture.

Lymphocyte isolation. Lymphocytes were harvested by excising Peyer's patches from the small bowel, mincing, and then rinsing through a 40-μm filter, and centrifuged at 1000 rpm for 5 min. The proper antibody concentrations were added singly or in combination: fluorescein isothiocyanate (FITC) anti-CD3 (clone 145-2C11) (Pharmingen), allophycocyanin (APC) anti-CD8 (clone 53-6.7) (Pharmingen), FITC anti-TCR αβ (clone H57-597) (Pharmingen), FITC anti-TCRγδ (clone GL3) (Pharmingen), R-phycocerythrin (R-PE) anti-CD4 (clone GK1.5) (Pharmingen). All antibodies were in FACS buffer; incubations were for 30 min on ice. Following staining, the cells were washed twice in HBSS containing 1% bovine serum albumin. Three-color flow cytometry analysis was performed on a Becton Dickinson FACSTAR cell sorter for analysis of 10^6 cells per sample. These experiments were repeated on three separate occasions (n = 4 animals per group) to confirm the results obtained.

Statistical analysis. Data are expressed as means ± SEM. Analysis of variance (ANOVA) was performed to assess the difference among all experimental groups. A Fisher protected least significance difference test was used to make post hoc comparisons. A P value of less than 0.05 was considered to be significant.

RESULTS

Lymphocyte Proliferation

Femur fracture induced a significant increase in proliferative response in Peyer's patch immunocytes, with approximately a twofold increase in proliferative index compared with normal and sham controls. In contrast, no significant differences were identified in splenocyte proliferative response 48 h after femur fracture injury (Fig. 1).

IL-10 Protein Expression

Constitutive expression of IL-10 in Peyer's patch and splenic immunocytes was no different in all three experimental groups. Peyer's patch immunocytes from normal controls secrete small amounts of IL-10 in response to LPS, approximately 70 pg/ml. IL-10 protein expression in Peyer's patch immunocytes was significantly downregulated in femur fracture animals, with a decrease of approximately 65% relative to normal and sham controls. Splenocyte IL-10 production was approximately 20-fold greater than Peyer's patch IL-10 production in all experimental groups. Femur fracture also induced a significant decrease in splenocyte IL-10 protein expression 48 h postinjury, approximately a 60% reduction compared with normal and sham controls (Fig. 2).

IL-10 quantitation. IL-10 was measured by ELISA (PerSeptive Biosystems, Framingham, MA) in Peyer's patch and splenic lymphocytes cultured in sterile 96-well flat-bottom microtiter plates with 5 × 10^4 cells per well in RPMI medium and 10 μg/ml LPS in vitro in the culture supernatant.

Flow cytometry. To determine the phenotype of lymphocytes isolated from Peyer's patch and spleen, 10^6 cells were plated in 50 μl of FACS buffer (PBS/2% fetal calf serum/0.08% NaN₃) in V-bottom 96-well plates (Linbro, Aurora, OH). Fe Block (Pharmingen, San Diego, CA) was added at a concentration of 1:100 and kept on ice for 5 min. The proper antibody concentrations were added singly or in combination: fluorescein isothiocyanate (FITC) anti-CD3 (clone 145-2C11) (Pharmingen), allopoycocyanin (APC) anti-CD8 (clone 53-6.7) (Pharmingen), FITC anti-TCR αβ (clone H57-597) (Pharmingen), FITC anti-TCRγδ (clone GL3) (Pharmingen), R-phycocerythrin (R-PE) anti-CD4 (clone GK1.5) (Pharmingen). All antibodies were in FACS buffer; incubations were for 30 min on ice. Following staining, the cells were washed twice in HBSS containing 1% bovine serum albumin. Three-color flow cytometry analysis was performed on a Becton Dickinson FACSTAR cell sorter for analysis of 10^6 cells per sample. These experiments were repeated on three separate occasions (n = 4 animals per group) to confirm the results obtained.

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pression increased from 15% in normal and sham controls to 23% in the experimental femur fracture group (Fig. 4). Peyer's patch immunocytes bearing TCRαβ were also significantly increased in the femur fracture group (Fig. 5). There was no difference in TCRγδ-positive cells in Peyer's patch lymphocytes, which were less than 0.3% of total in all experimen-

**FIG. 1.** Proliferative index of Peyer's patch (A) and splenic (B) immunocytes in normal, sham, and femur fracture mice, determined by [3H]thymidine. Proliferative index was calculated as mean counts per minute (cpm) in LPS-stimulated cultures divided by mean cpm for unstimulated conditions. Femur fracture induced a significant increase in proliferative response 48 h postinjury in Peyer's patch (A) immunocytes, with approximately a twofold increase in proliferative index compared with normal and sham controls (*P < 0.001 vs normal and sham controls). There was no significant differences in splenocyte (B) proliferative response 48 h after femur fracture injury.

**T-Cell Phenotype**

Femur fracture induced a significant increase in the fraction of CD3+ Peyer's patch immunocytes (23% vs 15% in both normal and sham controls, Fig. 3). Similarly, CD4+ Peyer's patch T-lymphocyte ex-

**FIG. 2.** Expression of IL-10 protein in Peyer's patch (A) and splenic (B) immunocytes in normal, sham, and femur fracture mice 48 h after injury. IL-10 protein expression in Peyer's patch (A) immunocytes was significantly downregulated in femur fracture animals, with a decrease of approximately 65% relative to normal and sham controls (*P < 0.05 vs normal and sham controls). Femur fracture induced a significant decrease in splenocyte (B) IL-10 protein expression 48 h postinjury, approximately a 60% reduction compared with normal and sham controls (*P < 0.03 vs normal and sham controls).
FIG. 3. Flow cytometric analysis of T-cell phenotype in Peyer's patch lymphocytes with two-color immunostaining. Expression of CD3+ (lower right quadrant) and CD8+ (upper left quadrant) receptors in normal (A), sham (B), and femur fracture (C) mice. Femur fracture induced a significant increase in Peyer's patch lymphocyte CD3 expression (23% vs 15% in both normal and sham controls). Data shown are representative of three separate experiments.

tal groups. Femur fracture did not induce any significant change in splenocyte CD3, CD4, TCRαβ, or TCRγδ expression, in contrast to the significant alterations demonstrated in Peyer's patch T-cell phenotype (data not shown).

DISCUSSION

GALT is the largest immune cell mass in the body and provides immunological protection against resident microbial flora and potential infectious pathogens [9–11]. The initial site of entry of immature lymphocytes into the gastrointestinal tract is through the Peyer's patches, which are macroscopic lymphoid aggregates that extend from the mucosa into the submucosa of the intestine. The Peyer's patch is a specialized site for initiation of mucosal immune responses. The germinal centers of Peyer's patches are composed primarily of B cells. By 19 weeks of gestational age, the B cells are identifiable in distinct follicular aggregates. Adjacent to the germinal centers are interfollicular zones that contain predominantly CD4+ T cells. Intestinal antigen uptake stimulates naïve B and T cells in the Peyer's patch to migrate to mesenteric lymph nodes, undergo further division, and differentiate into lamina propria and intraepithelial lymphocytes. B lympho-
FIG. 4. Flow cytometric analysis of T-cell phenotype in Peyer's patch lymphocytes with two-color immunostaining. Expression of CD4+ (lower right quadrant) and CD8+ (upper left quadrant) receptors in normal (A), sham (B), and femur fracture (C) mice. Femur fracture induced a significant increase in Peyer's patch lymphocyte CD4 expression (23% vs 15% in both normal and sham controls). Data shown are representative of three separate experiments.

cytes in the lamina propria produce and secrete IgA. The IgA response has also been found to be T cell dependent.

Most T lymphocytes recognize antigen via TCRαβ cell receptor. TCRγδ cells appear in the epithelium by 18 weeks of gestation, when the human gut is sterile. Thus, migration of T cells in the fetal intestine is in part antigen independent. TCRγδ cell function is not well characterized, but these cells have been found in larger quantities in Peyer's patches than in peripheral blood. TCR γδ cells can decrease [12-14] or augment [15-17] immunological response in specific situations and their immunomodulatory role is strongly suggested.

Gryglewski et al. [18] recently reported that GALT phenotype was altered in a murine model of major surgery (partial gastrectomy). These studies documented a significant postgastrectomy Day 3 increase in the percentage of TCRγδ in Peyer's patches (546% of sham control values) and a concomitant decrease in TCRαβ (55% of sham control values). There was a simultaneous decline in both TCRαβ and TCRγδ cells in peripheral blood (45 and 58% of normal values, respectively). In contrast, leg amputation was associated with no change in Peyer's patch TCRγδ or TCRαβ. These authors suggested that major surgical stress (partial gastrectomy) may disturb the normal cell traffic selectively, with increased TCRγδ cell homing in
intestinal Peyer's patches and lymph nodes (GALT) and with cell displacement from peripheral blood to lymphatic organs.

Furthermore, Xu and colleagues [5] determined that hemorrhagic shock alone or trauma coupled with hemorrhage in mice produces decreased cell yield and increased apoptosis in Peyer's patch cells, which was associated with increased Fas expression. These changes were noted to be predominantly in the B-cell lineage. These studies suggest that the induction of apoptosis or "programmed cell death" may also play an important role in intestinal mucosal immune dysfunction after trauma—hemorrhage.

In the studies presented herein we sought to investigate alterations in the gut-associated immune response after traumatic tissue injury instead of hemorrhagic shock. Murine femur fracture induced a significant increase in Peyer's patch lymphocyte CD3, CD4, and TCRαβ expression early (48 h) postinjury, whereas splenocyte phenotype was unchanged. It should, however, be clarified that the studies of T-cell phenotype were performed on unstimulated immunocytes and not in those immunocyte cultures that were exposed to LPS as a stimulant. These data suggest that GALT T-cell phenotype is affected very early after injury, when other constitutive responses such as proliferation and IL-10 production are unaltered.

Proliferative response of Peyer's patch immunocytes stimulated with LPS was significantly increased after femur fracture, also suggesting local lymphocyte acti-
vation in the intestinal microenvironment. Previous studies in our laboratory [7] investigated splenocyte proliferative responses to LPS and two other stimulants (concanavalin A and phytohemagglutinin) 24 and 96 h after femur fracture. A similar response was noted with all three stimulants, documenting a significant increase in splenocyte proliferation at 24 h and a significant decrease in proliferation at 96 h. Additional long-term studies determined that the depressed splenocyte proliferative response persisted for up to 2 weeks following femur fracture in this murine model. There is, therefore, a time-dependent alteration in splenocyte proliferative response after femur fracture injury. We have investigated Peyer’s patch proliferative response at one other time point after injury (72 h) and a significant increase in proliferative response was also identified (proliferative index = 45).

Interleukin-10 expression by Peyer’s patch and splenic immunocytes was also examined after tissue trauma. IL-10 is an important anti-inflammatory cytokine, and plays a critical role in intestinal immunity as shown in IL-10 deficient mice who develop fatal enterocolitis. The primary defect in these mice is a failure to control normal intestinal immune responses to enteric antigens, leading to excessive inflammatory cytokine release in the intestinal milieu. Acquired local IL-10 deficiency in the gut after trauma may also result in cytokine dysregulation and an exaggerated intestinal inflammatory response. Lagoo and colleagues [19] analyzed cytokine gene expression in several murine lymphoid tissues and demonstrated that Peyer’s patches constitutively expressed higher levels of multiple inflammatory cytokine transcripts compared with most other tissues examined. This is consistent with the continuous overexposure of these GALT immunocytes to intestinal luminal antigens including bacterial endotoxin.

We have previously documented that traumatic injury induces a suppression in systemic IL-10 expression, and is associated with intestinal mucosal dysfunction and increased intestinal permeability [7, 8]. We hypothesized that GALT IL-10 expression would also be downregulated after tissue trauma, and confirmed this in the studies presented here. Constitutive IL-10 expression in Peyer’s patch and splenic immunocytes was no different in all three experimental groups. In contrast, Peyer’s patch and splenocyte IL-10 protein expression in response to LPS stimulation was significantly decreased in mice that sustained femur fracture compared with normal and sham controls.

CONCLUSION

Femur fracture (as a model of traumatic tissue injury) induced a significant increase in Peyer’s patch lymphocyte CD3, CD4, and TCRαβ expression and an associated significant decrease in Peyer’s patch IL-10 protein expression, suggesting a greater tendency toward an increased local intestinal inflammatory response early postinjury. Changes in the GALT immune response may contribute to intestinal mucosal dysfunction and increased susceptibility to gut-derived sepsis after traumatic injury.

REFERENCES

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