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Neurokinin-1 Receptor Deficiency Improves Survival in Murine Polymicrobial Sepsis Through Multiple Mechanisms in Aged Mice

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Abstract

Objective: Substance P is a neuropeptide that contributes to a pro-inflammatory state by binding to the neurokinin 1 receptor (NK-1R). Limiting this interaction has been shown to attenuate of acute inflammation. Our hypothesis was that NK-1R activation would contribute to the morbidity and mortality of sepsis in a model using mice genetically deficient in the NK-1R.

Methods: To investigate the role of the SP/NK-1R axis in a murine model of sepsis, cecal ligation and puncture (CLP) in NK-1R deficient and wild type (WT) aged mice was performed. Acute inflammation was assessed by measuring circulating cytokines and clinical parameters.

Results: Deletion of the NK-1R results in improved survival following CLP (NK-1R knock out mice = 100% vs wild type = 14%). A reduction in the inflammatory cytokines IL-6, MIP-2, and IL-1RA, improved hemodynamic parameters and increased neutrophilia were present in the NK-1R deficient mice after CLP compared to WT mice.

Conclusions: These data confirm the hypothesis that eliminating the SP/NK-1R interaction in a highly lethal murine model of sepsis leads to decreased morbidity and mortality through multiple mechanisms.

Keywords

cytokines; inflammation; sepsis; neuropeptides

Introduction

One in every 2–3 deaths in hospitals in the United States is attributed to sepsis (1). Redefined as a “dysregulated host response to infection” in the latest consensus definitions, management of this pathologic process costs upwards of \$17 billion annually (2). Treatment is generally supportive and based on the surviving sepsis campaign guidelines (3). Specifically, timely resuscitation, source control, and broad spectrum antibiotics are integral components of care for the patient with sepsis. No pharmacologic bullet has been discovered to manage the complex inflammatory cascades that result in this dysregulated host response to infection (4).

Of the various pathways that make up the host defense system, neurogenic inflammation, characterized by inflammatory responses activated by the stimulation of sensory neurons and subsequent release of neuropeptides, plays a vital role in response to a noxious or infectious impetus (5, 6). Substance P (SP) is one of these neuropeptides released in the setting of acute inflammation and thus a mediator of neurogenic inflammation. In addition to communicating sensory information from the periphery to the central nervous system, SP when bound to the neurokinin-1 receptor (NK-1R), exerts effects on both the cellular and vascular arms of acute inflammation. This interaction promotes leukocyte release of inflammatory mediators and cytokines as well as endothelial capillary permeability with increased protein extravasation from the intravascular space to the interstitium and subsequent reduction in vascular tone.

Previous work established that SP concentrations increase along with inflammatory cytokine production in the acute septic setting (7, 8). This only establishes correlation and does not show that increased SP with subsequent binding to NK-1R causes the augmented mortality. Our lab recently demonstrated that the SP/NK-1R interaction is associated with hemodynamic instability in the CLP murine model (9). In the current study, we further explore this interaction and its effects on the complex cardiovascular physiology and inflammatory responses using a polymicrobial sepsis model with aged inbred mice genetically deficient in the neurokinin-1 receptor.

Methods

Animals

Experiments were performed on aged matched BALB/c female mice based on our familiarity with this strain (10, 11). Homozygous NK-1R^{-/-} mice lacking the NK-1R gene (NK-1R KO) were bred at the Boston University School of Medicine Laboratory Animal Science Center. The original breeding pair were a gift from Dr. Norma Gerard (Harvard Medical School, Boston, MA) to Dr. Arthur F. Stucchi and were generated on the BALB/c background as described (12). The genetically modified offspring were genotyped to ensure homozygosity at Boston University Medical Center and allowed to mature to an average of 80 weeks before being studied in a humidity and temperature controlled housing with a 12 hour light:dark diurnal cycle. Wild type mice were bred in the same facility. Water and food was provided for the entire study period *ad libitum*. The Institutional Animal Care and Use Committee at Boston University School of Medicine approved all experiments which were performed abiding by NIH guidelines.

Cecal Ligation and Puncture Model

Cecal ligation and puncture (CLP) was performed on mice with modifications to induce polymicrobial peritonitis (9, 11). General anesthesia was induced with 5% isoflurane and reduced to 3% for maintenance. After prepping the skin with Chloroprep antibacterial solution (BiMeda, Le Sueur, MN), a 1 cm midline laparotomy incision was created through the abdominal wall. The cecum was eviscerated, ligated at 50% length, punctured twice with a 25-gauge needle, and a small amount of stool expressed before returning the cecum back to the peritoneal cavity. The abdominal wall was then closed in two layers before

discontinuation of anesthesia. Postoperatively, mice were resuscitated subcutaneously with 1 mL of warm D5 lactated Ringers solution twice daily for five days and received analgesia in the form of buprenorphine 0.5 mg/kg administered every 12 hours for 4 doses. Imipenem 25 mg/kg (Merck, West Point, PA) was administered beginning 2 hours after CLP and continued every 12 hours for a total of 6 doses.

Blood collection

Facial vein sampling was performed at 24 hours following CLP in 20 μ L quantities to collect blood samples (13, 14). Each aliquot of blood was immediately diluted in 180 μ L of 1:50 PBS:EDTA. Plasma was then separated and used for evaluation of cytokines. Hemoglobin concentrations and a complete blood count with differential was also assessed with a Hemavet instrument (CDC Technologies, Oxford, CT)

Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA was performed using matched antibody pairs for capture and detection (R&D Systems, Inc, Minneapolis, MN) on plasma samples collected at 24 hours following CLP. Cytokines IL-6, MIP-2, and IL-1RA were evaluated by ELISA as described previously (15).

Cardiovascular Function Assessment

Hemodynamic stability was evaluated using the Mouse Ox collar sensor and software (Starr Life Sciences Corp, Oakmont, PA) as used previously in order to determine physiologic parameters heart rate and pulse distension—a surrogate for stroke volume (9, 16). To minimize external stress, mice were allowed to acclimate to the placement of the noninvasive collar for at least five minutes before obtaining vital signs in real time. These physiologic parameters were collected at various time points before and after CLP.

Statistical Analysis

Prism 5 (GraphPad Software, San Diego, CA) was used for statistical analysis. A Kaplan-Meier curve and log rank survival was performed with a 95% confidence interval to evaluate survival. Student's t test was used to compare data between two groups. All values were expressed in mean $\pm$ SE. For repeated measures data, such as the cardiovascular parameters, the data were analyzed using repeated measures ANOVA with Bonferroni correction.

Results

NK-1R knockout mice are resistant to mortality in polymicrobial peritonitis induced by CLP.

Homogenous aged, inbred BALB/c mice genetically lacking the neurokinin-1 receptor (NK-1R) were selected to evaluate the role of the SP/NK-1R axis in survival following a septic challenge. Aged mice were chosen to recapitulate the clinical scenario in which the highest incidence of sepsis occurs in patients who are at least 65 years old as most mortality occurs in either the young or the elderly (1). These clinical observations have been reproduced in the CLP model of sepsis as demonstrated where mice suffered increasing mortality in an age dependent fashion (17). In the current experiments, aged wild type and

KO mice were subjected to CLP and monitored during the first five days of sepsis. It is during this time period that most mice succumb to death, which is associated with a strong inflammatory state (18). The mortality at day 5 following CLP in this inbred population was significantly different for the two groups (Fig 1). The NK-1R KO mice had 100% survival compared to 14% survival in the wild type, age matched group.

Plasma cytokines are lower in NK-1R KO mice compared to wild type mice 24 hours after CLP.

The inflammatory response as determined by plasma levels of cytokines was evaluated at 24 hours following CLP in the BALB/c mice. Blood was sampled from the facial vein and plasma concentrations of the pro-inflammatory cytokine IL-6, the CXC chemokine macrophage inflammatory peptide-2 (MIP-2), and the anti-inflammatory cytokine IL-1 receptor antagonist (IL-1RA) were measured at this time point. A single time point was selected since these cytokine concentrations peak at 24 hour following CLP (18, 19). There were concerns about stressing the aged mice if multiple blood draws were performed. Concentrations of IL-6, MIP-2, and IL-1RA were all significantly diminished in the mice lacking the NK-1R (Fig 2a–c). The lower concentrations of plasma cytokines reflected a decreased systemic inflammatory state following peritonitis in the NK-1R KO mice.

Elimination of NK-1R improves hemodynamic function.

The hemodynamic profile of severe sepsis and septic shock is monitored closely, especially in the early hours after septic challenge. These measurements are critical components in the clinical management of sepsis and guide tailoring of resuscitation. In order to better characterize these physiologic changes, vital signs were measured serially using a non-invasive infrared probe. By placing this collar-like probe around the neck of mice, local blood flow at the level of the carotid artery was monitored to assess pulse distension—a stroke volume surrogate. At baseline, there were no differences in pulse distension in WT and NK-1R KO mice. After CLP, pulse distension was decreased in both groups reaching a nadir in the first six hours with recovery by 24 to 36 hours in both groups (Fig 3A). The reduction in pulse distension was significantly greater in the wild type mice early following CLP, as highlighted at 1 and 6 hours (Fig 3B). Additionally, the wild type mice never recovered completely back to baseline where as the NK-1R KO mice demonstrated a return to baseline by 30 hours.

NK-1R regulates vascular fluid shifts and changes in body weight and hemoglobin.

NK-1R KO and control, wild type BALB/c mice were resuscitated with crystalloid fluid at the time of CLP and then every 12 hours for the 5 days during the acute septic period. Despite receiving equal volumes of resuscitation, NK-1R KO mice lose more weight than wild type mice (Fig 4A), suggesting that the NK-1R KO mice are better able to maintain intravascular volume potentially by excreting the additional crystalloid. Further support for this concept was found in hemoglobin measurements. Hemoglobin values 24 hours after CLP were more concentrated in the wild type mice compared to the NK-1R KO mice (Fig 4B). This may indicate better cardiovascular function since the wild type mice could have a vascular leak and a fluid shift from the intravascular space. The indiscriminate loss of fluid

from the vasculature would manifest as less weight loss due to edema and increased hemoglobin concentrations.

NK-1R mice develop robust neutrophilia.

Mice who survive CLP sepsis have significantly more circulating neutrophils compared to those who die (18). We measured the number of PMNs within the bloodstream 24 hours after CLP. The NK-1R knockout mice, who had better survival, had significantly more PMNs compared to the WT mice (Figure 5A). Relative to normal values, the NK-1R KO had significant neutrophilia while the WT mice failed to increase the number of neutrophils. Lymphopenia also occurs in CLP induced sepsis, however while the lymphocyte count was reduced there was no difference between the two groups.

Discussion

Modulating the SP/NK-1R axis has been demonstrated in various murine models of acute inflammation. Genetically eliminating the pre-protachykinin-A gene product, the precursor to SP, reduces end organ inflammation in LPS endotoxemia model (20). Similar findings were confirmed in the CLP model where mice deficient in the pre-protachykinin-A gene demonstrated not only reduced inflammation with lower cytokine levels but also a reduction in microvascular permeability and capillary leakage in the lungs (8). These findings were further substantiated in a CLP model of wild type mice who were treated with a neurokinin-1 receptor antagonist (NK-1RA) resulting in a similar pattern with reduction of various inflammatory cytokines (21). More recently, our lab showed that inhibiting the NK-1 in the early interval following CLP improves survival through multiple mechanisms including: attenuating the acute inflammatory response with lower cytokine production, less fluid loss and albumin extravasation in the intestinal tract, and improved cardiovascular function (9). This is the first study to date evaluating the inflammatory effects of polymicrobial peritonitis in a murine model deficient in the NK-1R.

In this study, we have demonstrated that deletion of the NK-1R in mice results in markedly improved survival following CLP. The effects of eliminating the SP/NK-1R axis are manifold, affecting both the cellular and vascular arms of acute inflammation. As such, mice lacking the NK-1R subjected to CLP exhibit similar patterns of reduced systemic inflammation with lower levels of IL-6, MIP-2, and IL-1RA. Additionally, cardiovascular function as a measure of stroke volume was dramatically improved in the NK-1R KO mice early in the first hours following the initial septic insult. This improved cardiovascular function persisted for the duration of the acute phase of sepsis, a finding that was not appreciated when NK-1RA was administered. The pulse distension curves seen in this study follow a similar curve seen in prior studies with a clear reduction initially following CLP and subsequent increase six hours later (9, 22). Unlike Zanotti's work, where they demonstrated a resuscitation dose difference in stroke volume using serial echocardiographic monitoring, our lab has demonstrated that attenuating or even eliminating the SP/NK-1R axis affects stroke volume without the need for additional resuscitation (23). This is a critical finding given that too much resuscitation in the septic patient has been associated with increased risk of mortality (24). Moreover, the stroke volume curves demonstrated in their

study also suggests a rapid decline in cardiovascular function in the early hours following septic challenge despite very aggressive resuscitation. Our studies were designed to demonstrate the differences between the NK-1R KO mice and wild type mice, and fluid resuscitation was adequate in the NK-1R KO mice since pulse distension returned to normal.

Furthermore, it was interesting to note that weight change was significantly different at 24 and 48 hours after CLP. NK-1R KO mice sustained more weight loss relative to the wild type mice following resuscitative efforts. This finding correlates with the hemoconcentration seen in the wild type mice, who have higher hemoglobin concentrations 24 hours after CLP. Both of these measures are likely the result of increased capillary leakage with intravascular volume lost to the interstitium and subsequent fluid retention in the wild type mice. These findings are consistent with evidence shown previously that the SP-NK-1R promoted increased microvascular permeability and capillary leakage of various organ systems including lungs and enteric tract in the setting of noxious stimuli (8, 9, 25).

A robust neutrophilia response was appreciated 24 hours after CLP was appreciated in NK-1R KO mice. This increase in circulating neutrophils is certainly not a surprise given the inflammatory response's ability to eradicate infection. Likewise there was a reduction in neutrophil count in the wild type mice. This may be explained by the fact that attenuating the SP/NK-1R response does in fact result in reduced adhesion molecule activation (26). Moreover, the lower circulating neutrophil count in wild type mice may be reflected by the higher local neutrophil recruitment to the peritoneum as seen previously (27, 28).

Interestingly, the findings observed in this study do not correlate with all models of sepsis. For example, Verdrengh And Tarkowski evaluated the SP/NK-1R axis in a model of bacteremia(29). Staphylococcal aureus was injected into the tail vein of mice lacking the NK-1R demonstrated an increase in bacterial load in the kidneys, decreased phagocytic capabilities, and increased mortality despite no difference in interleukin-6 plasma concentrations. These disparate findings may be partially explained by the fact that a different pathogen was used to induce sepsis with a different method of inoculation.

Apart from the obvious difference in our prior study where the NK-1R was inhibited pharmacologically following CLP, another difference is that aged mice were used. Turnbull et al demonstrated that the inflammatory response in the setting of an acute septic challenge varies depending on age (17, 30). Aged mice tend to have increased plasma cytokines and decreased survival in the face of similar injury severity compared to young mice. One reason for utilizing aged mice in this study was to demonstrate the exaggerated difference of knocking out the NK1R initially hypothesized.

Our study used aged mice since prior publications have shown that in patients age was an independent predictor of mortality (31). We found similar results when comparing our current results with prior publications. In BALB/c mice using CLP with a 25 gauge needle there is typically 5% mortality over 7 days in young mice (32) while the current study with aged mice has greater than 80% mortality in the same time frame. Other investigators have reported that aged mice, compared to young mice, have increased mortality after CLP (17, 30).

This study reaffirms that neuropeptides play a critical role in the inflammatory response in the septic setting. Mice subjected to peritoneal sepsis experience an attenuated inflammatory response as depicted by a reduction in circulating cytokines, smaller cardiovascular hit and improved survival with elimination of the SP/NK-1R. The results are considerably more dramatic than we described when the SP/NK-1R axis was attenuated with a neurokinin-1 receptor antagonist. Further work is warranted to elucidate the mechanisms responsible for these findings.

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Figure 1: NK-1R knockout mice are resistant to mortality in polymicrobial peritonitis induced by CLP.

All NK-1R KO mice (n = 9) that underwent 25-gauge double puncture CLP survived. Age matched wild type mice (n = 7) that underwent CLP had a 14% survival rate. ***p < 0.001 by log rank survival.

Figure 2: Plasma cytokines are lower in NK-1R KO mice compared to wild type mice 24 hours after CLP.

Blood was sampled via facial vein 24 hours after CLP. NK-1R KO mice ($n = 9$) had lower concentrations of (A) pro-inflammatory IL-6, (B) chemokine MIP-2, and (C) anti-inflammatory IL1-RA relative to aged matched wild type mice ($n = 7$). Each symbol is an individual mouse and the mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.001$ comparing wild type mice to the NK-1R knockout mice.

Figure 3: Hemodynamic instability is attenuated when NK-1R is absent.

Using a non-invasive infrared cervical probe, pulse distension (a surrogate for stroke volume) and heart rate were obtained following CLP. (A) In the NK-1R knock out mice, the decrease in pulse distension was less than the decrease observed in wild type mice (NK-1R KO n = 9, WT n = 7). (B) To highlight the early changes, the pulse distension is significantly reduced in the WT group at 1 hour and 6 hours post CLP. **p < 0.001 comparing wild type mice to the NK-1R knockout mice.

Figure 4: Physiological measurements indicate that mice lacking NK-1R experience less fluid shifts.

Both groups of mice were resuscitated with equal volumes of crystalloid during the acute phase of sepsis. (A) NK-1R deficient mice (n = 9) experienced more weight loss in the 48 hours following CLP suggesting fluid administered was excreted more efficiently, likely the result of improved intravascular volume, renal perfusion with subsequent urine loss compared to wild type mice (n = 7). (B) Hemoglobin concentration was less in NK-1R KO compared to wild type suggesting less hemoconcentration. **p < 0.01 by repeated measures ANOVA with Bonferroni correction (panel A) or test (Panel B).

Figure 5: Circulating blood count differentials following CLP.

(A) The neutrophil count in the blood is significantly elevated after CLP in NK-1R deficient mice ($n = 9$) compared to wild type mice ($n = 6$). (B) Lymphopenia occurs in both NK-1R KO mice and wild type mice, but there was no significant differences between the two groups. * $p < 0.05$ comparing wild type mice to the NK-1R KO mice for the PMN count.