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Intestinal Ischemia/Reperfusion Injury Triggers Activation of Innate Toll-Like Receptor 4 and Adaptive Chemokine Programs

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Abstract

Background—Ischemia/reperfusion injury (IRI) is a major problem in intestinal transplantation. Toll-like receptor 4 (TLR4) has been implicated as a possible link between the innate and adaptive immune systems, however little data exists regarding TLR4 in intestinal IRI. The goal of this study is to evaluate the involvement of TLR4 in intestinal IRI and to assess the effect on T cell related chemokine programs.

Methods—C57BL6 mice underwent 100 minutes of warm intestinal ischemia by SMA clamping. Control WT mice underwent laparotomy without vascular occlusion. Separate survival and analysis groups were performed, and intestinal tissue was harvested at 1 hour, 2 hours, 4 hours, and 24 hours post-reperfusion. Analysis included histology, CD3 immunostaining, myeloperoxidase activity, Western blot, and PCR.

Results—Survival was significantly worse in the IRI group vs control (50% vs. 100%). IRI caused severe histopathological injury including mucosal erosions and villous congestion and hemorrhage. Myeloperoxidase activity increased in a time-dependent manner after IRI (2.71 0.25 at 1 hour, 2.92 0.25 at 2 hours, 4 0.16 at 4 hours, 5.1 0.25 at 24 hours vs 0.47 0.11 controls, $P < .05$). Protein expression of TLR4 followed by NF- κ B was increased after IRI. Additionally, mRNA production of IP-10, MIP-2, MCP-1, and RANTES was increased at all time-points, as was mRNA for ICAM-1 and E-selectin.

Conclusion—This study is the first to demonstrate increased expression of TLR4 and NF- κ B after warm intestinal IRI. This detrimental cascade may be initiated by TLR4 via NF- κ B signaling pathways, implicating TLR4 as a potential therapeutic target for the prevention of intestinal IRI.

Ischemia/reperfusion injury (IRI) to the intestine is a major cause of organ dysfunction in surgery and transplantation. Intestinal IRI can result locally in extensive tissue damage and distantly in a systemic inflammatory response. Understanding and ultimately ameliorating IRI is crucial to improving outcomes after this injury.

Historically the innate and adaptive immune systems have been considered separately when examining graft injury after transplantation, with the innate system the focus of antigen-independent IRI and the adaptive system the focus of antigen-dependent rejection. Recently evidence in other organ systems has shown that IRI can facilitate the later development of acute and chronic rejection, implying an overlap between the two immune systems. These historical paradigms are further challenged by evidence for involvement of the adaptive immune response in IRI.¹

Toll-like receptors (TLRs) are transmembrane proteins that recognize pathogen-associated molecular patterns and are present on many different cell types that function in both innate and adaptive immunity.^{2,3} TLRs have been implicated in graft rejection and adaptive immune regulation in transplant models. Studies have also shown that signaling via TLR4 is necessary for the initiation of hepatic IRI.⁴ Together, these data have led to the postulation that TLRs may serve as the bridge between the innate and adaptive immune systems.^{5,6}

The mechanistic links between the innate and adaptive immune responses in intestinal IRI have yet to be elucidated. In this study we investigate the molecular responses to IRI in the murine intestine.

MATERIALS AND METHODS

Intestinal Warm IRI Model

Male C57BL6 wild-type (WT) mice underwent 100 minutes of jejunoileal ischemia as previously described.⁷ Sham control animals underwent the same procedure, but without vascular occlusion. Separate survival and analysis groups were randomly assigned. Survival was assessed at 7 days (n = 6/group). In the analysis group, animals were sacrificed at 1 hour, 2 hours, 4 hours and 24 hours after reperfusion, and tissue from a standard location in the ileum was collected for analysis.

Histopathology and Immunohistochemistry

H & E-stained tissue was blindly graded by a single pathologist. The previously reported grading system⁸ was used: 0 = normal, 1 = superficial epithelial injury, 2 = injury extending into lamina propria, 3 = injury extending into submucosa, 4 = injury extending into muscularis propria, and 5 = full thickness injury.

The primary monoclonal rat anti-mouse CD3 antibody (BD Pharmingen, San Diego, Calif) was used on intestinal tissue cryosections. After blocking with normal serum, bound primary Abs were detected using biotinylated anti-rat IgG and streptavidin peroxidase-conjugated complexes, and developed with DAB Substrate Kit (Vector Laboratories, Burlingame, Calif). Samples were blindly evaluated with the average number of positive-staining cells recorded per high-power field.

Myeloperoxidase Assay (MPO)

Myeloperoxidase activity was detected in snap-frozen tissue as previously described.⁸ One unit of MPO activity was defined as the quantity of enzyme degrading 1 μ mol peroxide per minute per gram of tissue at 25°C.

Western Blot Analysis

Total protein was extracted from frozen tissue as previously described.⁷ Nuclear protein was extracted by resuspending nuclear pellets in 1 mol/L Tris (pH 7.5), 1 mol/L MgCl₂, 2 mol/L KCl, 1% Triton X-100, 5 mol/L NaCl, 0.5 mol/L EDTA, 20% glycerol, 0.2 mmol/L PMSF, and 0.5 mmol/L DTT. Protein in SDS-loading buffer was subjected to 12% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes (Bio-Rad, Hercules, Calif), and blocked in 3% milk. After incubation with polyclonal rabbit anti-mouse TLR4, NF- κ B, and β -actin antibodies (Santa Cruz Biotechnology, Inc, Santa Cruz, Calif), the membranes were developed by Amersham Enhanced Chemiluminescence protocol.

RNA Extraction and Reverse-Transcriptase PCR Analysis

Total RNA was extracted from frozen intestinal tissue with RNeasy Mini Kit (Qiagen, Valencia, Calif). RNA was reverse-transcribed into cDNA using Superscript First Strand Synthesis System (Invitrogen, Carlsbad, CA). PCR was performed with 35 cycles at the annealing temperature for each primer pair: 53°C (IP-10), 55°C (MIP-2), 55°C (MCP-1), 60°C (RANTES), 58°C (ICAM-1), 58°C (E-selectin), and 60°C (β -actin). PCR products were analyzed in ethidium bromide-stained 2% agarose gels and photographed with Kodak Digital Science DC120 camera. Band density was determined using Kodak Digital Science 1D Analysis Software (Eastman Kodak, Rochester, NY). To compare the relative levels of mRNA, each sample was normalized against its respective β -actin template cDNA.

Statistical Analysis

All data are expressed as mean $\pm$ standard deviation. Statistical comparisons were made using the student's *t*-test.

RESULTS

Survival

The 7-day survival in the group undergoing warm ischemia was 50% compared to 100% in the sham-operated group ($P < .05$). All deaths occurred within 48h of IRI.

Histopathology and CD3 Immunostaining

There was significantly increased tissue damage at 4 hours (3.1 ± 0.4) and 24 hours (3.3 ± 0.5) as compared to sham (0.8 ± 0.4). There was increased staining at both 4h (23.5 ± 6.8) and 24h (28.5 ± 7.4) as compared to sham (0.3 ± 0.5), indicating increased tissue infiltration by CD3+ lymphocytes after IRI.

Myeloperoxidase (MPO)

MPO activity was used as an index of neutrophil accumulation in the intestine. There was a time-dependent increase in MPO activity in the treatment group (2.7 ± 0.25 at 1 hours, 2.9 ± 0.25 at 2 hours, 4.0 ± 0.16 at 4 hours, and 5.1 ± 0.25 at 24 hours) vs. the sham group (0.47 ± 0.11). The increase in MPO was statistically significant at each time point ($P < .05$).

Toll-like Receptor 4 and NF- κ B

A time-dependent increase in TLR4 expression was seen by Western blot, beginning at 1h post-reperfusion. A time-dependent increase in NF- κ B activation was similarly seen, beginning slightly later than TLR4 activation, at about 2 hours post-reperfusion (Fig 1).

Chemotactic Cytokines and Adhesion Molecules

Compared with sham-operated controls, there were significant increases in mRNA production for chemokines IP-10 (CXCL10), MIP-2 (CXCL2), MCP-1 (CCL2), and RANTES (CCL5) at 1 hour, 2 hours, 4 hours, and 24 hours after intestinal IRI ($P < 0.05$). There was significant up-regulation of both ICAM-1 (CD54) and E-selectin (CD62-E) at all four time-points with the highest levels seen at 24 hours ($P < .05$). The kinetics of both chemokine production and adhesion molecule up-regulation appear to parallel the increase in MPO activity after IRI.

DISCUSSION

Although TLR4 has been shown to play a crucial role in IRI of other organ systems, it has not been specifically investigated in the intestine. Here we show for the first time that TLR4 is

activated after warm intestinal IRI. These data demonstrate that intestinal IRI is associated with up-regulation of TLR4, NF- κ B, chemokine programs, adhesion molecules, and infiltration of neutrophils and T cells, associated with a severe intestinal injury by 24 hours postreperfusion.

The intestine is unique among transplanted solid organs. It is quite immunogenic, containing the largest aggregate of lymphoid tissue in the human body. The intestine is renowned for its sensitivity to ischemia, largely due to a natural abundance of xanthine oxidase, producing substantial amounts of oxygen free radicals after ischemia. It was not known whether this distinctly unique and complex organ incurs IRI through mechanisms similar to other organ systems. And, as many of the signals for TLR4 activation are bacterial products and normally exist within the intestine,² it was not a certainty that signaling via TLR4 would play a role in intestinal IRI.

Importantly, we also found that transcription factor NF- κ B is up-regulated after TLR4 activation. NF- κ B activation is induced by infections, oxidative stress, and proinflammatory stimuli, and is a major target of the TLRs. It regulates the expression of immunomodulatory genes, as well as those that function in apoptotic processes, and acts as a principal mediator of systemic inflammation in response to IRI. This multi-functional transcription factor plays a varied and complex role in IRI, simultaneously mediating both systemic inflammation and local tissue protection.⁹ Though NF- κ B has previously been shown to play an important role in intestinal IRI, a link between TLR4 and NF- κ B in intestinal IRI has not been shown.

From these studies, we hypothesize that intestinal warm IRI results in activation of TLR4 and subsequent nuclear translocation of NF- κ B. Target genes for various chemokines and adhesion molecules are then expressed leading to infiltration of tissue by neutrophils and T cells. Once activated, these mediators propagate the cascade of events that leads to tissue injury and cell death. Thus TLR4 may function as an important mediator of intestinal IRI, and may also act as a critical link between the innate and adaptive immune responses.

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Fig 1.

Total cellular protein was analyzed for TLR4 and nuclear protein extracts were analyzed for NF-κB. Expression of both TLR4 and NF-κB is increased after intestinal IRI. Up-regulation of TLR4 is seen at 1 hour postreperfusion, and translocation of NF-κB to the nucleus is seen beginning at 2 hours post-reperfusion.