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BASIC-ALIMENTARY TRACT

Inducible Nitric Oxide Synthase From Bone Marrow-Derived Cells Plays a Critical Role in Regulating Colonic Inflammation

PAUL L. BECK, YAN LI, J. WONG, CHANG–WEN CHEN, CATHERINE M. KEENAN, KEITH A. SHARKEY, and DONNA–MARIE MCCAFFERTY

Gastrointestinal and Mucosal Inflammation Research Groups, Institute of Infection, Immunity and Inflammation, University of Calgary, Calgary, Alberta, Canada

Background & Aims: **Nitric oxide (NO) is an important mediator of intestinal inflammation. Inducible NO synthase (iNOS) is the main source of NO in inflammation. Because iNOS is ubiquitously expressed, our aim was to determine which cellular source(s) of iNOS plays the central role in mediating intestinal inflammation.** ***Methods:*** **Chimeric lines were produced via bone marrow (BM) transplantation following irradiation. Colitis was induced with dextran sodium sulfate (DSS) or trinitrobenzene sulfonic acid (TNBS). The severity of colitis and markers of inflammation were assessed in standard fashion. Leukocyte recruitment was assessed by intravital microscopy.** ***Results:*** **The irradiated chimeric lines with iNOS−/− BM-derived cells were markedly more resistant to both DSS- and TNBS-induced injury. Resistance to DSS-induced colitis was lost when wild-type (wt) BM was used to reconstitute iNOS−/− mice. Neutrophils were the main source of iNOS in DSS-induced colitis. iNOS−/− chimeric lines had decreased colonic macrophage inflammatory protein 1β and tumor necrosis factor α expression and increased levels of the protective growth factor, keratinocyte growth factor. LPS-mediated leukocyte recruitment was reduced in iNOS−/− mice, and there were marked changes in the inflammatory cell infiltrates between the chimeric lines with iNOS−/− vs wt BM-derived cells. Furthermore, the lamina propria CD4 +ve cells from chimeric lines with iNOS−/− BM-derived cells had reduced intracellular cytokine expression.** ***Conclusions:*** **iNOS produced by BM-derived cells plays a critical role in mediating the inflammatory response during colitis. Cell-specific regulation of iNOS may represent a novel form of therapy for patients with inflammatory bowel disease.**

Nitric oxide (NO) plays critical roles in regulating digestive and defensive functions in the gastrointestinal tract.[1–3] NO is produced by 3 main isoforms of nitric oxide synthase (NOS): neuronal (nNOS) and endothelial (eNOS) are constitutively expressed, whereas inducible NOS (iNOS) can be induced by an array of stimuli, typically in conditions of inflammation or injury. Although iNOS is predominantly regulated at the transcriptional level through nuclear factor (NF)-κB-dependent mechanisms, there are other important factors that mediate iNOS expression including transforming growth factor (TGF)-β, tumor necrosis factor (TNF)-α, interferon (IFN)-γ, and lipopolysaccharide (LPS).[4–7]

NO has been implicated in many pathophysiologic conditions of the gastrointestinal tract. Increased NO production has been described in inflammatory bowel disease (IBD), microscopic colitis, and toxic megacolon as well as in animal models of intestinal inflammation.[3,8–10] The exact role of NO in modulating intestinal inflammation has been difficult to define, with numerous studies reporting apparently conflicting results. Studies using NO donors and NOS inhibitors clearly show that the timing of NO modulation, type, and dose of the inhibitor used as well as the site or tissue in which inflammation occurs all play critical roles in determining the ultimate outcome of altered NO regulation.[4,11,12] Targeted gene disruption has added to our knowledge of the role of NO in modulating intestinal inflammation. We and others have shown that iNOS−/− mice are less susceptible to dextran sodium sulfate (DSS)-induced colitis,[3,13] whereas iNOS deficiency can increase severity of trinitrobenzene sulfonic acid (TNBS)-induced colitis,[12] yet a highly selective iNOS inhibitor can dramatically reduce the severity of TNBS-induced colitis.[14] In our previous studies, we further defined the role of NO in intestinal inflammation by assessing mice deficient in iNOS, nNOS, or eNOS and found that nNOS−/− mice were markedly more susceptible to DSS-induced colitis, whereas eNOS−/− mice were slightly less susceptible than wild-type control ani-

Abbreviations used in this paper: BM, bone marrow; DSS, dextran sodium sulfate; eNOS, endothelial nitric oxide synthase; iNOS, inducible NOS; KGF, keratinocyte growth factor; MPO, myeloperoxidase; nNOS, neuronal NOS; TNBS, trinitrobenzene sulfonic acid.

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mals. Of the 3 NOS-deficient lines studied, clearly the iNOS−/− mice were the least susceptible to DSS-induced intestinal injury, suggesting that the production of NO via iNOS played a critical role in the pathogenesis of intestinal inflammation in this model system.[3]

Of the NOS isoforms, iNOS is the most widely expressed, with high levels of activity in epithelial cells, neutrophils, macrophages, neurons, glia, and endothelial cells.[10,15,16] Because iNOS-derived NO can impact a vast array of tissues and mediate numerous cellular responses, it is thought that the site of expression plays a key role in determining the ultimate physiologic outcome of altered NO expression. In the present study, we sought to define further the role of iNOS-mediated NO production in regulating intestinal inflammation. The aims of the present study were (1) to determine the main cell cellular source(s) of iNOS in DSS-induced colitis, (2) to determine which cell types are critical in regulating the iNOS-dependent events that may be involved in mediating the resistance to DSS-induced colitis seen in the iNOS−/− mice, (3) to assess the role of bone marrow (BM) cell-derived iNOS in TNBS-induced colitis, and (4) to examine the mechanisms by which iNOS-derived NO mediates intestinal inflammation. To address these aims, we developed chimeric mouse lines in wild-type (wt) or iNOS−/− mice by ablating the host BM and replacing it with wt or iNOS−/− BM.

The findings of this study indicate that iNOS-derived NO produced by cells of BM origin plays a critical role in modulating intestinal inflammation. This study also provides further evidence that BM-derived cells can modulate intestinal inflammation. Recently, there have been several reports of IBD patients who have undergone BM transplantation and have experienced complete remission, or marked improvement, of their IBD.[17,18] Thus, further studies on the role of BM-derived cells may directly advance our understanding of the pathogenesis of IBD and may aid in the management and possibly cure of these disease states.

Materials and Methods

Animals

Thy1.1 and Thy1.2 congenic and iNOS−/− mice (all on a C57/BL6 background) were purchased from the Jackson Laboratory (Bar Harbor, ME). All mice strains were bred and housed in microisolator cages in the animal resource center of the University of Calgary. For each study, animals were matched by age, sex, and body weight. Animals were ear tagged, and investigators assessing mice daily were unaware of the genetic background of each group. The care and experimentation of animals were performed in accordance with the guidelines of the Canadian Council on Animal Care, and protocols were approved by the University of Calgary Animal Care Committee.

BM Transplantation

BM cells were isolated from 4- to 6-week-old male Thy1.1 C57/BL6 congenic donor mice as previously described.[19] Recipient female Thy1.2 C57/BL6 mice were irradiated by Gammacell 1000 (Nordion International, Kanata, Ontario, Canada) (^{137}Cs source) twice, at a dose of 5.0 Gy, 3 hours apart. Following the second irradiation, 10 to 20 $\times$ 10^6 donor BM cells were injected into recipients via the tail vein. For the first 2 weeks following BM transplantation (BMT), mice received 0.2% neomycin sulfate in their drinking water,[19] followed by a 6-week engraftment recovery period. To confirm recipient reconstitution by donor cells, fluorescence-activated cell sorter (FACS) analysis on cells isolated from the thymus were labeled for CD4, CD8, Thy1.1, and Thy1.2 (anti-mouse CD4 [L3T4] monoclonal antibody, anti-mouse CD8a [Ly-2] monoclonal antibody, anti-mouse Th1.1 [CD90] monoclonal antibody, and anti-mouse Th1.2 [CD90.2] monoclonal antibody) (BD Bioscience, Mississauga, ON). To further assess reconstitution, donor cell Y chromosomes were assessed by fluorescence in situ hybridization at 4, 6, 8, 12, and 18 weeks following BMT using the CY3-labeled mouse Y chromosome paint probe (Cambio Ltd, Cambridge, United Kingdom) according to the manufacturer's instructions.

Induction and Assessment of Colitis

DSS model. Colitis was introduced by the addition of DSS (2.5% wt/vol added to the drinking water; molecular weight, 40,000; ICN Biomedical, Aurora, OH, Lot No. 3073B) as described previously.[3] Animals were assessed daily and mean DSS/water consumption and body weights were recorded. Fecal blood loss, hematocrit, and diarrhea were assessed as described previously.[3,9,20] Mice were killed at day 3 and day 7 following addition of DSS to the drinking water. The entire colon was removed, opened along the mesenteric border, and fecal material removed. Tissue was removed, fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned, and stained with H&E in standard fashion.

The severity of colitis was scored histologically using 2 different parameters on coded slides in a blinded fashion. An inflammation score was used: 0, no inflammation; 1, increased inflammatory cells noted above the muscularis mucosa only; 2, increased inflammatory cells involving the submucosa and above; 3, increased inflammatory cells involving the muscularis and/or serosa.[20] The extent of ulceration was determined by measuring the amount of ulceration on each section as measured along the muscularis mucosa (expressed as percentage ulcerated mucosa). A myeloperoxidase (MPO) assay was performed to assess colonic granulocyte infiltration as described previously.[3]

TNBS model. Following our studies in the DSS model, we assessed whether the resistance to colitis that was noted in the chimeric mice produced by transferring