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Regulation of Colonic Epithelial Repair in Mice by Toll-like Receptors and Hyaluronic Acid

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Abstract

Background and Aims—The protective component of the host response to dextran sodium sulfate colitis in the mouse is mediated through the activation of TLR4, the induction of COX-2 and PGE2 production. TLR4 ligands include bacterial lipolysaccharide and hyaluronic acid a, component of the extracellular matrix. Our hypothesis is that hyaluronic acid, through TLRs, plays a protective role in the host response to dextran sodium sulfate colitis.

Methods—Dextran sodium sulfate (2.5%) was administered for seven days in wild type and MyD88 −/− mice. The mice also received intraperitoneal hyaluronic acid. The expression of hyaluronic acid, COX-2, and MIP2 were evaluated by immunohistochemistry.

Results—Dextran sodium sulfate induced a marked increase in hyaluronic acid in the lamina propria of wild type but not MyD88−/− mice. Treatment with DSS also induced the MyD88-dependent expression of hyaluronic acid synthases 2 and 3, enzymes involved in hyaluronic acid synthesis, in lamina propria macrophages. Exogenous hyaluronic acid induced the expression of TNFα, MIP-2 and COX-2 in the colon in a MyD88-dependent manner. In wild type, but not MyD88 −/−, TLR4−/−, COX-2−/− mice, hyaluronic acid was protective against dextran sodium sulfate colitis. In wild type mice hyaluronic acid was therapeutic in established DSS colitis.

Conclusion—Endogenous hyaluronic acid expression is markedly increased in dextran sodium sulfate colitis and preserves the epithelium through TLR activation and COX-2 expression. Furthermore exogenous hyaluronic acid, through the activation of TLRs and the production of PGE2 through COX-2 has protective effects in dextran sodium sulfate colitis.

Introduction

Hyaluronic acid (HA), a glycosaminoglycan polymer composed of repeating units of the disaccharide [-D-glucuronic-β1, 3-N-acetyl-D- glucosamine-β 1, 4-], is secreted by many cell types and is an important constituent of the extracellular matrix (1,2,3). HA is assembled at the plasma membrane by HA synthases (HASs) and extruded into the extracellular space as

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HA polymers (4). The HA chain can extend up to 2×10^5 monosaccharides and up to 25 μm in length, which makes HA one of the largest extracellular molecules. In mammals there are 3 HAS isoforms: HAS-1, HAS-2 and HAS-3, which synthesize HA of different chain lengths and are subject to differential regulation (5,6).

The biologic activity of HA is a function of its molecular weight. High molecular weight HA (1×10^4 kDa), as is found in synovial fluid, supports tissue hydration and volume expansion (7). In response to injury and inflammation HA undergoes both increased synthesis through induction of HASs, and degradation to smaller molecular weight fragments. Degradation of high molecular weight HA by hyaluronidases generates HA fragments that differ from high molecular weight HA in regards to their biologic activity. HA of 2.5×10^2 kDa induces the expression of inflammatory genes in macrophages (8,9). The biologic relevance of this observation is supported by reports that the HA fragments that induce inflammatory genes *in vitro* are in the same size range as the HA fragments that accumulate in inflammatory states *in* vivo (8,9). Overall lower molecular weight HA fragments are angiogenic, inflammatory and immunostimulatory (10).

The HA receptor CD44 is expressed on the plasma membrane of most cells including fibroblasts, smooth muscle cells, epithelial cells, and immune cells (11). CD44 binding to HA promotes the recruitment, activation, and migration of T cells (12). In addition to binding to CD44, HA also binds to Toll-like receptor-2 (TLR2) and TLR4, components of the innate immune response. TLRs are widely distributed in the gastrointestinal tract and are important in mediating the host response to both commensal and pathogenic bacteria (13). Although TLRs are usually thought of as responding to microbial products, TLR2 and TLR4 also ligate host molecules including HA. In sterile skin and lung injury models, where microbial products are not involved, HA binding to TLR4 promotes the protective component of the host response (14,15). In bleomycin-induced lung injury HA binding to TLR2 or TLR4 results in increased inflammation but decreased apoptosis. Administration of PEP-1, a peptide that blocks HA binding to CD44 and TLR4, results in decreased inflammation but increased apoptosis in this model (14).

Increased expression of HA is seen in Crohn's disease (16). Increased levels of both HA and HAS1 are present in a genetic model of ileitis in which TNFα is overexpressed (17). Incubation of human colonic smooth muscle cells with dextran sodium sulfate *in vitro* results in increased HA production and increased leukocyte adherence (16). The role of HA signaling through TLRs in the host response to gastrointestinal injury has not been explored. We and others have demonstrated a role for TLR4 signaling in the host response to dextran sodium sulfate (DSS) colitis (18,19,20). DSS colitis is more severe in mice deficient in TLR4 or MyD88, a TLR adaptor molecule. In DSS colitis signaling through TLR4 results in increased cyclooxygenase-2 (COX-2) expression and PGE_2 synthesis (18,19). These downstream signaling events promote increased epithelial cell survival and proliferation. One premise of these earlier studies in DSS colitis was that the relevant ligand for TLR4 was the bacterial product lipopolysaccharide (LPS). In this study we sought to determine if a host ligand, HA, could participate in the protective response to DSS colitis through TLR4 signaling.

Materials and Methods

Chemicals and reagents

HA (mw 7.5×10^2 kDa) was from Sigma, St. Louis MO. This product was tested for endotoxin by Associates of Cape Cod, Inc., East Falmouth, MA); it was found to be endotoxin free. High mw HA, Healon (mw 2×10^3 kDa) , was from Physician Sales and Services, St. Louis MO. PEP1 (GAHWQFNALTVR) and scrambled control peptide (WRHGEALTAVNQ) were from Sigma-Genosys (St. Louis, MO) (21).

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Animals

Adult (6–8 week-old) female C57BL/6J were purchased from Jackson Laboratory. $CD44^{-/-}$ and $TLR4^{-/-}$ on a B6 background are from Jackson Laboratory. $MyD88^{-/-}$, COX2 $^{-/-}$ mice were backcrossed more than 10 generations on a B6 background. Mice were given 2.5% DSS in their drinking water for 7 days to induce colitis. HA (30 mg/kg) and/or the specific HA blocker, PEP1 (40 mg/kg), were given by intraperitoneal injections at 2 hours before and 3 and 5 days after starting DSS treatment. Bromodeoxyuridine (BrdU) was injected 1–1/2 hours before sacrifice.

Immunohistochemistry

Primary antibodies were goat anti-mouse BrdU (1:100), mouse anti COX-2 (Pharmagen; 1:1000), goat anti-mouse MIP2 (R&D Systems,1:100), HAS-1, HAS-2 and HAS-3 (Santa Cruz, 1:100). Biotinylated secondary antibodies used were donkey anti-mouse, donkey anti-rabbit or donkey anti-goat (Jackson Immunoresearch, West Grove, PA; 1:250). Antibody signal was detected by incubation in strepavidin-POD (Roche, Indianapolis, IN) followed by 3,3'-diaminobenzidine tetrahydrochloride.

For immunofluorescence (IF) we used mouse monoclonal anti-COX-2 (BD Pharmingen 1:50); rat anti-mouse F4/80 (AbD Serotec, Raleigh, NC 1:50); goat anti-mouse MIP2 (R & D System 1:100). Secondary antibodies used were Cy3 labeled (1:500) or FITC-labeled (1:200) donkey anti-rat, donkey anti goat, or donkey anti mouse (Jackson Immunoresearch). For IF for HA sections were incubated with biotinylated hyaluronan-binding protein (Northstar 1:100) followed by AlexaFluor 568 conjugated streptavidin (Invitrogen 1:500).

Isolation of peritoneal macrophages

PBS (5 ml) was injected into the mouse peritoneal cavity. Peritoneal fluid was harvested and centrifuged at 1000x for 10 min. The cell pellet was resuspended in media to obtain 100,000 cells/ml; 2–3 × 10^6 cells were obtained from each mouse.

Measurement of cytokine and chemokine release

TNFα and MIP-2 were measured by ELISA (R &D systems, Minneapolis).

Digestion of HA

Intermediate molecular weight (2×10^2 kDa) HA and high molecular weight HA (Healon 2 × 10^3 kDa) were digested with hyaluronidase. The molecular weights of the hyaluronan fragments were assessed by running the undigested or digested samples in 3% agarose gel and staining with “stains-all” (Fluka).

DSS endocytosis

The fluoresceinated DSS (100μg/ml) incubated with cells for 1 h. Cells were washed with media three times and fixed with 3% paraformaldehyde for 10 min. Cells were washed again with PBS before being stained with DAPI and examined by fluorescence microscopy.

Statistical analysis

One way ANOVA was used to assess differences in measured variables between treatment and the control groups. Data are expressed as the mean+/− SD. Statistical difference was accepted at $p<0.05$.

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Results

DSS induces the expression of HAS2 and HAS3 and the synthesis of HA in a MyD88-dependent manner

In the normal mouse colon HA is distributed in the lamina propria (Fig. 1A). After treatment with DSS there was a progressive increase in HA in the lamina propria of wild type (WT) mice. The increased HA expression is extracellular and located between the crypts. At baseline the distribution of HA in the colons of MyD88$^{-/-}$ mice was similar to that seen in WT mice; however, there was no increase in HA expression with DSS treatment in MyD88$^{-/-}$ mice. DSS induced an increase in plasma HA at 3 days after initiation of oral DSS (Fig 1B). The increases in lamina propria and plasma HA were associated with 11–12 fold increases in mRNA for HAS-2 and HAS-3 in the distal colon. (Fig 1C). Administration of DSS had no effect on mRNA for HAS-1 or the HA receptor CD44. In contrast, in MyD88$^{-/-}$ mice DSS did not increase mRNA for HAS-2 or HAS-3. IF for HAS3 in the distal colon of untreated WT mice shows scattered HAS-3 positive cells in the lamina propria (Fig 1D). After 7 days of DSS the number of lamina propria cells positive for HAS-3 increased substantially. Double IF for HAS-3 and the macrophage marker F4/80 in untreated WT mice showed F4/80 positive cells in the lamina propria (Fig 1E). Rare F4/80 positive cells were also HAS-3 positive. In the untreated colon HAS3- positive cells were also F4/80 positive. After treatment with DSS the number of HAS-3 positive cells in the lamina propria increased. Most, but not all, HAS-3 positive cells were also F4/80 positive. Most F4/80 positive cells were also HAS-3 positive after DSS treatment. Attempts at IF for HAS-2 were unsuccessful.

DSS induces HAS2 and HAS3 in wild type peritoneal macrophages in a MyD88- dependent manner

We next sought to determine the effect of DSS on HAS-2 and HAS-3 expression in macrophages *in vitro*. Incubation of WT peritoneal macrophages with DSS (1%) resulted in a 3–4 fold increase in the expression of HAS-2 and HAS-3 but no increase in CD44 or HAS-1. In contrast, incubation of DSS with peritoneal macrophages from MyD88$^{-/-}$ mice had no effect on the expression of HAS-1, HAS-2, HAS-3 or CD44. (Supplementary Fig 1A)

The demonstration that the effects of DSS on peritoneal macrophages were MyD88 dependent raised the question of whether DSS interacts directly with TLRs. Ligation of TLR4 results in endocytosis of both TLR4 and its ligand (22). Incubation of fluoresceinated DSS with peritoneal macrophages from WT mice, but not MyD88$^{-/-}$ mice, results in incorporation of the fluoresceinated DSS into small round intracellular granules consistent with endocytosis (Supplementary Fig 1B). These experiments suggest that the increase in HA in DSS colitis is the product of DSS ligation of TLRs in intestinal macrophages.

HA induces TNFα, macrophage inflammatory protein 2 (MIP-2), and COX-2 expression in peritoneal macrophages in a TLR-4 dependent manner

Incubation of WT peritoneal macrophages with HA (100 μg/ml) induced 3–5 fold increases in TNFα, MIP-2 and COX-2 (Supplementary Fig 2A, 2B, 2C, 2D, 2E). These increases were not seen in peritoneal macrophages from MyD88$^{-/-}$ or TLR4$^{-/-}$ mice. The induction of TNFα by HA in CD44$^{-/-}$ macrophages was similar to that seen in WT macrophages (Supplementary Fig 2B) a TLR4 blocking antibody reduced TNFα production with HA stimulation; whereas a CD44 blocking antibody had no effect (Supplementary Fig 2C). Administration of HA to WT, but not MyD88$^{-/-}$, peritoneal macrophages also induced a 2 fold increase in proliferation (Supplementary Fig 2F). To block interactions between HA and WT peritoneal macrophages, we used the HA blocking peptide PEP-1, which blocks HA dependent leukocyte recruitment *in vivo* (14,21). PEP-1 (125μg/ml) had no effect on the proliferation of WT macrophages;

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however when HA and PEP-1 were administered together, PEP-1 blocked the proliferative effects of HA (Supplementary Fig 2F).

The biologic effects of HA are dependent on its molecular weight. We incubated wild type peritoneal macrophages with HA of various molecular weights (MWs) and measured TNFα production. The greatest production of TNFα was induced by HA of MW 7.5×10^2 kDa. Low MW (25kDa) HA and high MW (1×10^3 kDa) HA induced lesser amounts (Supplementary Fig 3A). Treatment of HA (MW 7.5×10^2 kDa) with hyaluronidase results in lower molecular weight forms of HA and eliminates the ability of HA to stimulate TNFα production (Supplementary Fig 3B). Healon is a commercially available HA of high molecular weight (2×10^3 kDa). This product does not stimulate TNFα production by wild type peritoneal macrophages; however, treatment with hyaluronidase generates lower molecular weight HA fragments which do have biologic activity. These data support the importance of molecular weight in determining the biologic effects of HA.

We wanted to determine if the biologic effects of HA were a consequence of LPS contamination. Incubation of wild type peritoneal macrophages with either LPS or HA induces TNFα production (Supplementary Fig 3C). Co-administration of the LPS blocking agent polymycin B sulfate with LPS eliminates almost all of the ability of LPS to stimulate TNFα. Administration of polymycin B sulfate has a much smaller effect on the stimulation of TNFα by HA. Administration of the HA blocking agent PEP-1 with LPS has no effect on the ability of LPS to stimulate TNFα production; however, PEP-1 eliminates 90% of the induction of TNFα by HA. Similarly treatment of HA with hyaluronidase eliminates 90% of the ability of HA to induce TNFα production but treatment of LPS with hyaluronidase has no effect on its biologic activity.

Intraperitoneal administration of HA induces TNFα, MIP-2 and COX-2 in the distal colons of WT but not MyD88$^{-/-}$ mice

We next sought to determine the effects of exogenous intraperitoneal HA on plasma HA. Intraperitoneal HA induced an increase in plasma HA at 12 hours in both wild type and MyD88$^{-/-}$ mice (Fig 2A). However, the increase in plasma HA persisted for 48 hours in the wild type mice and was gone after 12 hours in the MyD88$^{-/-}$. The sustained increase in plasma HA after intraperitoneal HA may be a product of endogenous synthesis of HA by HAS-2 and HAS-3. Intraperitoneal HA induces HAS-2 and HAS-3 in distal colons of wild type mice (Fig 2B).

Having demonstrated that HA induces the production of TNFα, MIP-2 and COX-2 in peritoneal macrophages in a MyD88-dependent manner, we next sought to determine if peritoneal administration of HA would have the same effects on the distal colon *in vivo.* WT and MyD88$^{-/-}$ mice received intraperitoneal injections of HA on days 1, 3 and 5 and were sacrificed on day 7. HA (2×10^2 kDa) resulted in a 3 fold increase in TNFα in WT mice but not in MyD88$^{-/-}$ mice. (Fig 2C). The increase in TNFα with HA was blocked by co-administration of PEP-1. HA caused 3.0–3.5 fold increases in MIP-2 and COX-2 in the distal colons of WT mice (Fig 2D). Co-administration of PEP-1 blocked the ability of HA to induce COX-2 and MIP-2.

MIP-2 co-localized with the macrophage marker, F4/80, in colons from both untreated and HA treated WT mice (Fig 2D). All of the MIP-2 positive cells in the distal colon were also F4/80 positive, indicating that at baseline MIP-2 is expressed only in macrophages and that treatment with HA induces MIP-2 expression only in macrophages.

The number of COX-2 expressing cells in the distal colon was increased by HA treatment in WT but not MyD88$^{-/-}$ mice (Fig 3A, 3B). Moreover the increase in COX-2 expressing cells

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induced by HA in WT mice was blocked by coadministration of PEP-1. The MyD88-dependent increase in COX-2 expressing cells led us to investigate whether COX-2 and TLR4 co-localized in the colon. In untreated mice there were scattered COX-2 positive cells and scattered TLR4 positive cells in the lamina propria; however no cells were both COX-2 and TLR4 positive (Fig 3C). HA increased the number of COX-2 expressing cells and these cells were localized around the base of the crypts. After HA treatment some, but not all, COX-2 expressing cells also expressed TLR4; however, all of the TLR4 expressing cells also expressed COX-2. We next sought to determine if the COX-2 expressing cells, either at baseline or after the administration of HA, were macrophages. In the distal colons of untreated mice there were scattered COX-2 positive cells in the lamina propria and scattered F4/80 positive cells in the lamina propria but there was no co-localization of COX-2 and F4/80 (Fig 3D). After treatment with HA the number of COX-2 positive cells in the lamina propria increased and some, but not all, of the COX-2 positive cells were also positive for F4/80. These co-localization studies indicate that in the distal colons of untreated wild type mice there is a population of macrophages that express F4/80 and TLR4 and untreated mice there is a population of COX-2 expressing cells which are not macrophages. The increase in COX-2 expressing cells in the distal colon of HA treated mice reflects the induction of COX-2 expression in macrophages through TLR4 activation.

HA rescues the DSS colitis model in WT but not MyD88$^{-/-}$ mice

WT and MyD88$^{-/-}$ mice given DSS (2.5%) lose similar amounts of weight. Intraperitoneal HA in addition to the oral DSS eliminated the weight loss seen with oral DSS alone (Fig 4A). Intraperitoneal PEP-1 plus oral DSS resulted in weight loss similar to DSS alone. Intraperitoneal HA plus oral DSS in MyD88$^{-/-}$ mice resulted in weight loss at the same level as seen with oral DSS alone. The patterns for colonic disease severity score and histology scores in the DSS model followed the pattern seen with weight loss (Fig 4B, 4C). In each case intraperitoneal HA in addition to oral DSS resulted in diminished disease severity compared with DSS alone in WT mice; however, the addition of intraperitoneal HA to oral DSS had no effect on the disease activity in MyD88$^{-/-}$ mice.

HA treats established DSS colitis

Having demonstrated that HA blunts the development of DSS colitis if HA is given at the start of DSS administration, we sought to determine if administration of HA to a mouse with established DSS colitis would be therapeutic. In this experiment mice were given DSS for 7, 14, or 21 days. In some mice HA therapy was started on day 7 with intraperitoneal injections of HA every other day. DSS induced progressive weight loss and progressively worsening clinical and histologic scores. Administration of HA with DSS reversed the weight loss induced by DSS and improved histologic and clinical scores (Fig 5 A, B, C).

HA reverses DSS colitis in CD44$^{-/-}$ mice but not TLR4$^{-/-}$ mice

Administration of DSS to either CD44$^{-/-}$ or TLR4$^{-/-}$ resulted in colitis with weight loss, increased clinical score and increased histologic score (Fig. 6 A, B, C , D). DSS colitis was more severe in CD44$^{-/-}$ compared to WT mice based on clinical score and histologic score although weight loss was similar. The severity of the colitis was somewhat worse in the TLR4$^{-/-}$ mice compared to WT or CD44$^{-/-}$ mice as assessed by clinical and histologic score but the degree of weight loss induced by DSS was similar. However, the major distinction between DSS colitis in the CD44$^{-/-}$ and TLR4$^{-/-}$ mice is that HA rescues the colitis in the CD44$^{-/-}$, just as it does in the wild type mice; whereas HA does not rescue DSS colitis in the TLR4$^{-/-}$, just as it fails to rescue DSS colitis in the MyD88$^{-/-}$. The effects of HA on DSS rescue were identical as assessed by weight loss clinical score or histologic score. This indicates

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that signaling through TLR4, but not signaling through CD44 are required for the rescue of DSS colitis by HA.

We also measured plasma HA levels in WT, CD44$^{-/-}$ and TLR4$^{-/-}$ (Fig 6E). Seven days of DSS induced a 20 fold increase in plasma HA in the WT mice, a three fold increase in the CD44$^{-/-}$ and no increase in the TLR4−/−. Thus the induction of plasma HA by DSS is universally proportional to the severity of the colitis. These findings are consistent with the suggestion that the induction of endogenous Ha is part of the protective host response to DSS and that signaling through both TLR4 and CD44 are required.

HA fails to rescue the DSS colitis model in COX-2$^{-/-}$ mice

To determine if the induction of COX-2 expression mediates the protective effects of HA in DSS colitis we administered HA and DSS to COX-2$^{-/-}$ mice. COX-2$^{-/-}$ mice given oral DSS (2.5%) lose weight during 7 days of treatment. Administration of intraperitoneal HA in addition to the oral DSS in COX-2$^{-/-}$ mice results in weight loss similar to that seen with DSS (Fig 7A). Similarly administration of DSS to COX-2$^{-/-}$ mice resulted in a progressively worsening histologic score over a period of seven days. In COX-2$^{-/-}$ mice administration of intraperitoneal HA in addition to oral DSS resulted in histologic scores similar to those seen in mice given DSS alone (Fig 7B). The rescue of the DSS colitis model by the administration of HA is both MyD88 and COX-2 dependent.

Discussion

DSS colitis is more severe in mice deficient in TLR4 or MyD88, a TLR adaptor molecule; this suggest that TLR4 plays an important role in the host defense response to DSS colitis (18,19, 20). It has been assumed that LPS is the TLR ligand that mediates the host defense response in DSS colitis. However, HA also binds to TLR4 and HA binding to TLR4 promotes the protective component of the host response in sterile skin and lung injury models where LPS is not involved (14,15). In this study we sought to determine if DSS colitis resulted in an upregulation of HA expression and if HA could induce a protective response in DSS colitis.

Oral administration of DSS results in increased expression of HAS-2 and HAS-3 in the distal colon and increased extracellular HA in the affected colon. In the distal colons of DSS treated mice HAS-3 co-localizes with F4/80, a macrophage marker. Moreover extracellular HA surrounds lamina propria macrophages. These data suggest that DSS induces HA synthesis and the macrophage is the major cell type involved in the increased HA synthesis. In peritoneal macrophages *in vitro* and distal colons *in vivo* the induction of HAS-2 and HAS-3 and the increase in HA expression after DSS exposure are all MyD88 dependent. Activation of peritoneal macrophages *in vitro* by DSS is TLR4 dependent. Moreover fluorescinated DSS is take up by wild type macrophages but not by peritoneal macrophages from MyD88 deficient mice. This is the first suggestion that DSS directly activates TLRs. Ligation of TLR4 is associated with subsequent endocytosis of both TLR4 and its ligand. The endocytosis of fluorescinated DSS in peritoneal macrophages from wild type but not MyD88$^{-/-}$ mice supports the direct ligation of TLR4 by DSS. The central role for macrophage TLR signaling in the regulation of the host response to DSS colitis is consistent with our previous study in which we used adoptive transfer of peripheral blood leucocytes from Rag$^{-/-}$ into MyD88$^{-/-}$ mice to rescue the MyD88$^{-/-}$ phenotype in DSS colitis (18).

Having demonstrated that DSS induces HA expression in the distal colon through the induction of HAS-2 and HAS-3, we next sought to determine if exogenous HA would affect the course of DSS colitis. If HA is administered at the initiation of treatment with DSS, it blocks the development of DSS colitis. If HA is administered after DSS colitis is already established it diminishes the severity of colitis as measured by weight loss, clinical activity scores and

histology scores. The beneficial effects of HA on DSS colitis are TLR4 dependent and COX-2 dependent. Intraperitoneal HA induces HAS-2 and HAS-3 in the distal colon and causes a sustained rise in plasma HA. This suggests that there is a feed forward loop in which exogenous HA induces endogenous HA synthesis.

Intraperitoneal HA results in an increase in COX-2 expression in the distal colon. The increase in COX-2 expression is associated with an increase in the number of COX-2 expressing cells which are primarily macrophages. HA does not rescue DSS colitis in COX-2 mice. These data support the suggestion that the beneficial effects of HA in DSS colitis are mediated by prostaglandins produced through COX-2. This is consistent with the previous demonstration that the endogenous epithelial protective response to DSS is mediated through COX-2 (18, 19).

HA is typically secreted by cells as a high molecular weight (2×10^3 kDa) polymer. However TLR activation by HA and the wound healing effects as described by here are associated with lower molecular weight HA (3,7,8). Peritoneal macrophage activation by HA of various molecular weights demonstrated peak production of TNFα by HA of 7.5×10^2 kDa and almost none by HA of 1×10^3 kDa. This suggests that the secreted HA needs to be broken down into smaller fragments for the observed biologic effects. The generation of lower molecular weight HA fragments is typically associated with the activation of hyaluronidases. A recent study demonstrated that platelet hyaluronidases may participate in HA degradation in inflammation (23).

HA binds to CD44 in addition to binding to TLRs (3). We induced DSS colitis in $CD44^{-/-}$ mice and found that the colitis was more severe than in wild type mice. HA was able to prevent DSS colitis in the $CD44^{-/-}$ mice just as it did in wild type mice. This suggests that signaling through CD44 is required for the endogenous protective response to DSS colitis but is not involved in the rescue effects of exogenous HA. A recent study demonstrated that a mediated ileitis model was more severe in $CD44^{-/-}$ mice (17) T cell adhesion to HA through CD44 is important in T cell migration. DSS colitis is not a T cell mediated injury model.

Studies in the skin and lung demonstrated that HA binding to TLR4 is an important component of the protective host response to noninfectious injury (14,15). Here we find that HA, through a TLR4 mediated mechanism, is an important component of the protective host response to DSS colitis. Previous studies demonstrating the protective role of TLR4 signaling in DSS colitis assumed that LPS was the relevant ligand for TLR4 in inducing these protective events (18, 19,20). The demonstration that HA expression is induced in the colon in DSS colitis and that exogenous HA also diminishes the severity of DSS colitis suggests that endogenous HA plays a role in diminishing the severity of DSS colitis. HA production is increased in DSS colitis; moreover, HA treatment of mice with established DSS colitis markedly improves the colitis. This raises the possibility that either HA or agents that would induce HA synthesis may have preventive and therapeutic effects in other animal models of colitis and in human inflammatory bowel disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

COX, cyclooxygenase; DSS, dextran sodium sulfate; HA, hyaluronic acid; HAS, hyaluronic acid synthase; IF, immunofluorescence; MIP, macrophage inflammatory protein; MyD, myeloid differentiation primary response gene; TLR, Toll-like receptor; WT, wild type.

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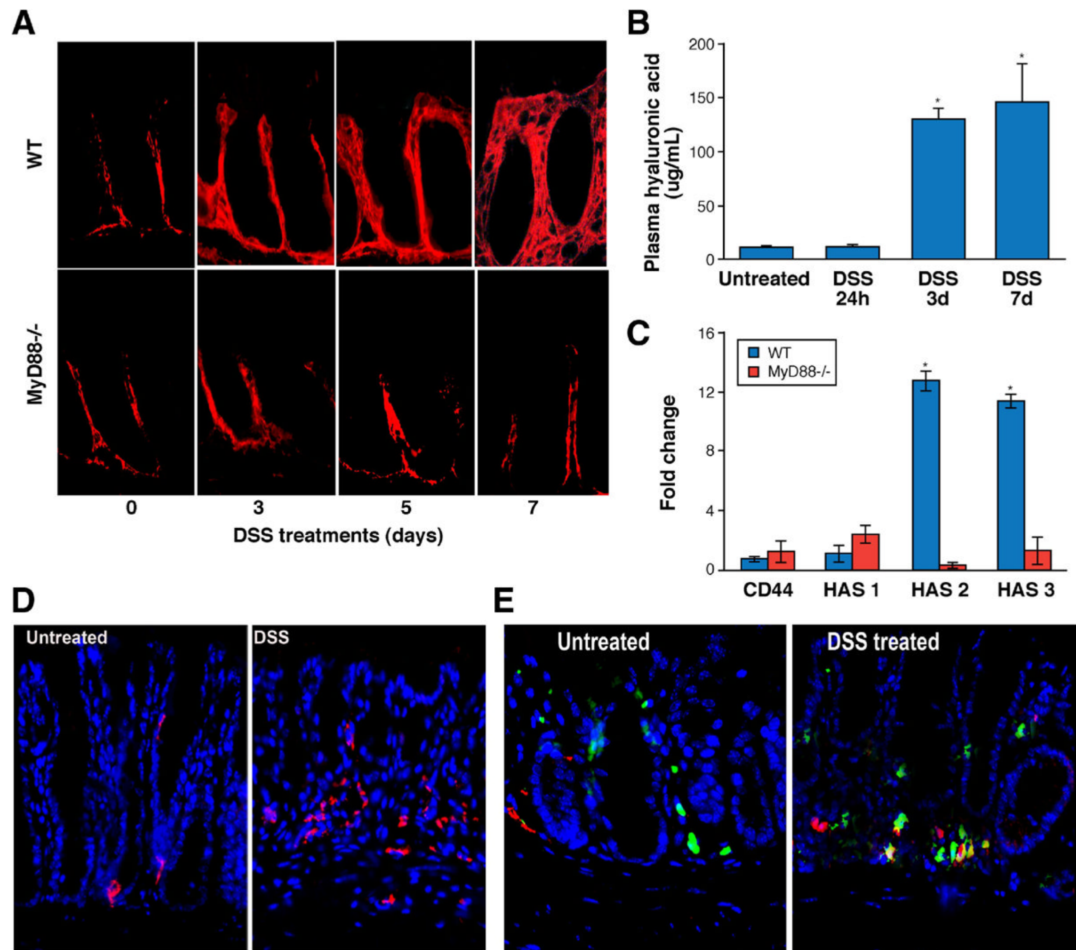

Fig 1.

HA expression is increased in the DSS model of colitis. (A) Immunofluorescence (IF) staining of HA in distal colons of wild type and MyD88−/− mice during 7 days of treatment with 2.5% DSS. In wild type mice DSS treatment induced a marked and progressive increase in intestinal HA in the lamina propria of the distal colons, but no increase occurred in MyD88−/− mice. (B) Mice were given DSS in the same schedule as in "A" and plasma was collected at 1, 3 and 7 days. Plasma HA levels were measured by ELISA. n=6, *p<.01 compared with untreated. (C) DSS treatment increased the mRNA levels of HAS2 and HAS3 by 10 fold in the distal colons of wild type mice compared to untreated mice, but had no effect in MyD88$^{-/-}$ mice. The mRNA levels of HAS1 and CD44 did not change with DSS treatment. Data are the means +/− SE for 4–7 mice *p<.001 for HA treated compared to untreated mice. (D) IF staining for HAS3 in mouse distal colon. There were only scattered HAS3 expressing cells in untreated mice, the DSS treatment of over 7 days increased the number of HAS3 expressing stromal cells. (E) Immunofluorescence for F4/80 (green) and HAS-3 (red). (Original magnification 400X)

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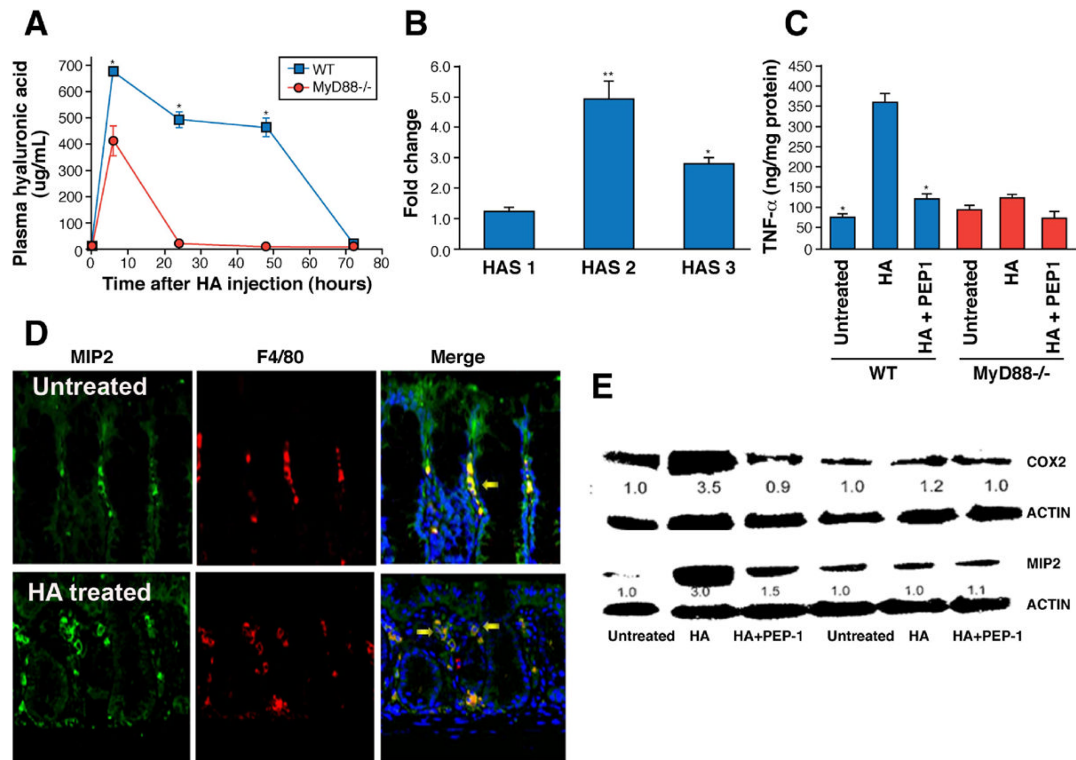

Fig. 2.

(A) Wild type and MyD88−/− mice were given intraperitoneal HA at time 0 and plasma HA levels were measured at 12, 24, 48 and 72 hours. n=4, *p<.001 compared with MyD88$^{-/-}$ .. (B) Wild type mice were given intraperitoneal HA on days 1,3 and 5. The mice were sacrificed on day 7 and mRNA was extracted from the distal colon. RT-PCR was done for HAS-1, HAS-2, and HAS-3. n=4, *p<.01, **p<.001 compared with untreated. (C) TNFα production in the distal colon increased over 3 fold in WT mice treated with HA (30mg/kg), as measured by ELISA. Pretreating with PEP1 (40mg/kg) for 1 hour blocked HA induction of TNFα production. The data are the means +/− SE for 9 mice. *p<0.001 compared with HA treated mice. (D) IF for MIP2 (green) and F4/80 (red) in distal colons of WT mice shows that most of the MIP2 positive cells were macrophage as indicated by MIP2 colocalization (arrows) with the macrophage marker F4/80 (original magnification 400X). E. Western blot analysis shows that administration of HA induced 3 fold increases in the expression of COX-2 and MIP2 in the distal colons of WT but not MyD88−/− mice. PEP1 blocked the induction of COX2 and MIP2 by HA in WT but not MyD88−/− mice.

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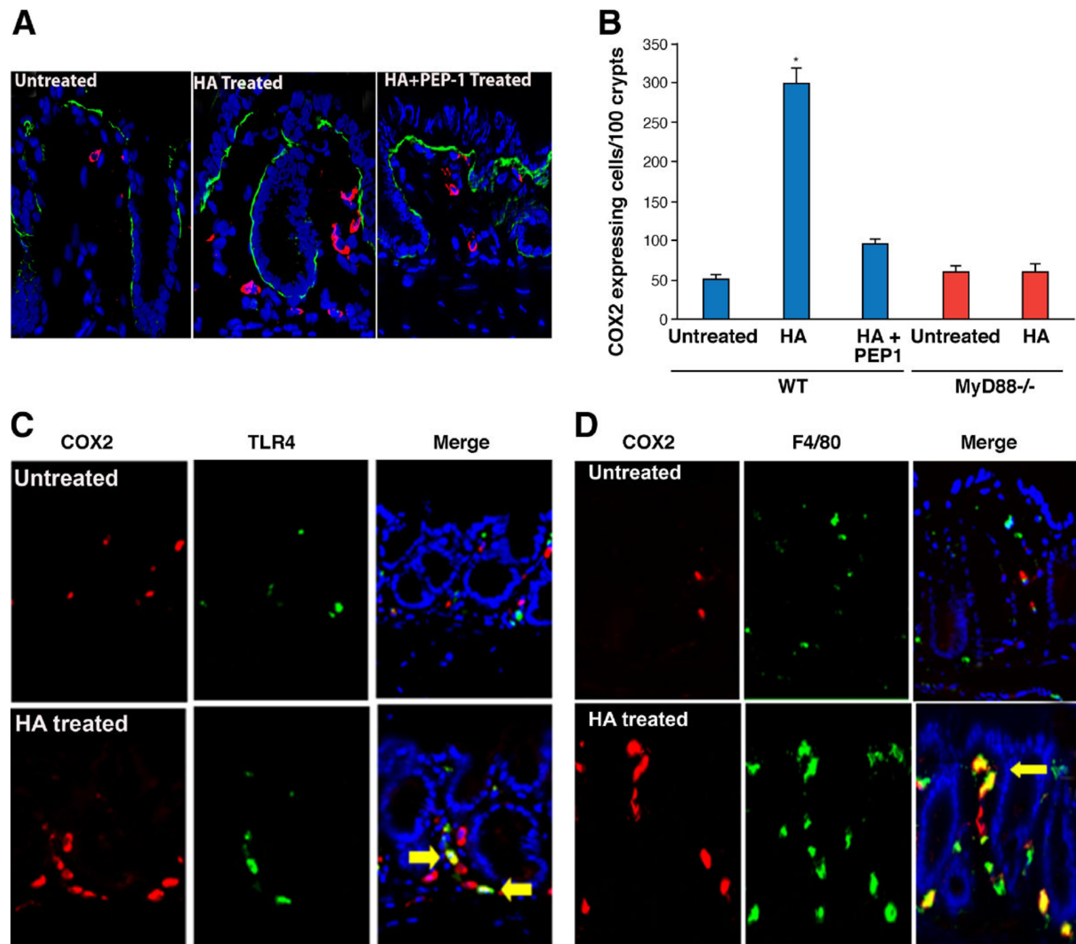

Fig. 3.
Immunofluorescence for COX-2 (red) in the lamina propria of the distal colon of WT mice. Mice were untreated or received HA on days 1,3 and 5 or received HA and PEP1 on days 1,3 and 5. Mice were sacrificed on day 7. (original magnification 400X). B) The number of COX-2 expressing cells Data are the means +/−SE for 4 mice. * $p< 0.001$ comparing HA treated with untreated or HA+PEP1 treated mice. C) Immunofluorescence for COX-2 (red) and TLR4 (green) in mouse distal colons. Mice were untreated or treated with HA on days 1,3 and 5 and sacrificed on day 7. (original magnification 400X). D) Immunofluorescence for COX-2 (red) and F4/80 (green) in the colons of untreated and HA treated mice (Arrow: yellow cell with colocalization of COX-2 and F4/80).

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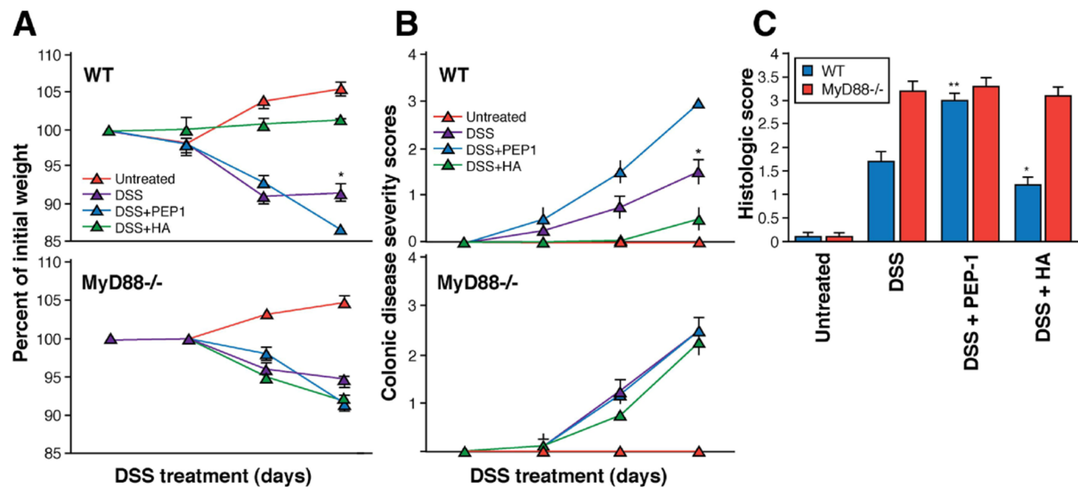

Fig 4.

A. HA administration reduced colitis in the DSS model in WT but not MyD88−/− mice. (A). HA (30mg/kg) prevented weight loss in DSS-treated WT mice. *p<0.001 comparing DSS treated with DSS+HA treated WT mice. Lower panel: HA had no effect in MyD88$^{-/-}$ mice. Data are means +/− SE for 9 mice. (B). Upper panel: colonic disease severity scores of DSS-treated WT mice were improved with the addition of HA. Administration of PEP1 (40mg/kg) to DSS treated WT mice resulted in more severe scores than mice treated with DSS alone. *p<.001 comparing DSS treated with DSS+HA or DSS+PEP-1 treated WT mice. Lower panel: HA and Pep1 had no effect on colonic disease severity scores in DSS treated MyD88$^{-/-}$ mice. The data are means +/−SE for 9 mice. (C). In WT mice, the addition of HA to DSS reduced histological scores in the distal colon compared with DSS alone. The addition of PEP1 to DSS increased histological scores. HA and PEP1 had no effect in DSS treated MyD88$^{-/-}$ mice. Data are the means +/−SE for 9 mice. *p<0.01 comparing DSS treated with DSS+HA or DSS+PEP-1 treated mice on day 7.

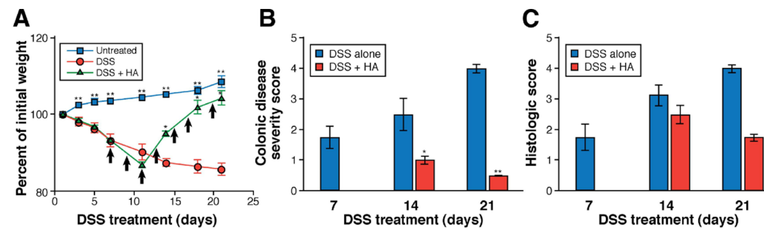

Fig. 5.

A. HA treatment of preexisting DSS colitis. WT mice were treated with oral DSS for 7, 14 or 21 days. HA therapy was initiated after 7 days of oral DSS administration. HA therapy consisted of intraperitoneal HA on alternate days (arrows). A) Weight change. n=4, *p<.001 comparing DSS + HA treated compared with DSS alone. **p<.001 comparing untreated with DSS treated. B) Colonic disease severity score. n=4,*p<.01, **p<.001 compared with DSS alone for 14 days. n=4, **p<.001 compared with DSS alone for 21 days. C) Histologic score . n=4,*p<.001 compared with DSS alone for 21 days.

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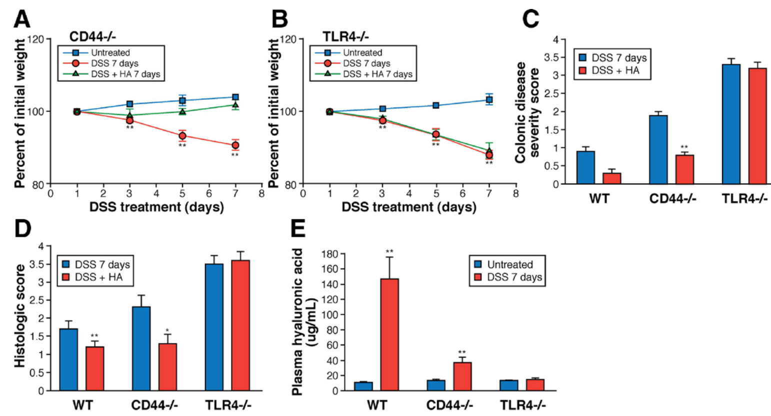

Fig 6.

HA rescues DSS colitis in CD44$^{-/-}$ put not TLR4$^{-/-}$ mice. A) Weight loss in CD44$^{-/-}$ mice n=4, **p<.001 compared with untreated. B) Weight loss in TLR4$^{-/-}$ mice n=4,**p<.001 compared with untreated. C) Colonic disease severity score n=4, **p<.001 compared with DSS alone. D) Histologic scores n=4, **p<.05 compared with DSS alone. E) Plasma HA levels n=6, **p<.001.

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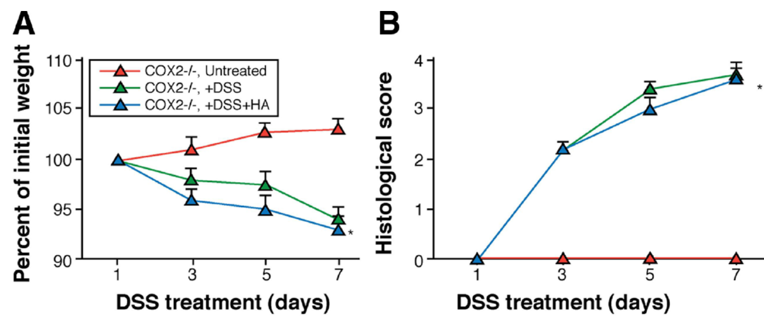

Fig. 7.

HA treatment failed to rescue the DSS-induced weight loss and histologic score in COX2$^{-/-}$ mice. (A) Weight loss in COX2$^{-/-}$ mice. *p <0.01 compared with untreated. (B). Histologic scores in COX-2−/− mice. *p<0.001 compared with untreated.