Hyaluronic acid is radioprotective in the intestine through a TLR4 and COX-2-mediated mechanism

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Riehl TE, Foster L, Stenson WF. Hyaluronic acid is radioprotective in the intestine through a TLR4 and COX-2-mediated mechanism. Am J Physiol Gastrointest Liver Physiol 302: G309–G316, 2012. First published October 28, 2011; doi:10.1152/ajpgi.00248.2011.—The intestinal epithelium is sensitive to radiation injury. Damage to the intestinal epithelium is dose limiting in radiation therapy of abdominal cancers. There is a need for agents that can be given before radiation therapy to protect the intestinal epithelium. C57BL6 mice were subjected to 12 Gy of total body radiation. Some mice received intraperitoneal hyaluronic acid (HA) before radiation. Mice were killed 6 h after radiation to assess radiation-induced apoptosis in the intestine; other mice were killed at 84 h to assess crypt survival. Total body radiation (12 Gy) resulted in increased expression of HA synthases and HA in the intestine and increased plasma HA (5-fold). Intraperitoneal injection of HA (30 mg/kg) before radiation resulted in a 1.8-fold increase in intestinal crypt survival and a decrease in radiation-induced apoptosis. The radioprotective effects of HA were not seen in Toll-like receptor 4 (TLR4)- or cyclooxygenase-2 (COX-2)-deficient mice. Intraperitoneal injection of HA induced a 1.5-fold increase in intestinal COX-2 expression, a 1.5-fold increase in intestinal PGE2, and the migration of COX-2-expressing mesenchymal stem cells from the lamina propria in the villi to the lamina propria near the crypt. We conclude that 1) radiation induces increased HA expression through inducing HA synthases, 2) intraperitoneal HA given before radiation reduces radiation-induced apoptosis and increases crypt survival, and 3) these radioprotective effects are mediated through TLR4, COX-2, and the migration of COX-2-expressing mesenchymal stem cells.

Intraperitoneal injection of HA (30 mg/kg) before radiation resulted in a 1.8-fold increase in intestinal crypt survival and a decrease in radiation-induced apoptosis. The radioprotective effects of HA were not seen in Toll-like receptor 4 (TLR4)- or cyclooxygenase-2 (COX-2)-deficient mice. Intraperitoneal injection of HA induced a 1.5-fold increase in intestinal COX-2 expression, a 1.5-fold increase in intestinal PGE2, and the migration of COX-2-expressing mesenchymal stem cells from the lamina propria in the villi to the lamina propria near the crypt. We conclude that 1) radiation induces increased HA expression through inducing HA synthases, 2) intraperitoneal HA given before radiation reduces radiation-induced apoptosis and increases crypt survival, and 3) these radioprotective effects are mediated through TLR4, COX-2, and the migration of COX-2-expressing mesenchymal stem cells.

HYALURONIC ACID (HA), a glycosaminoglycan polymer composed of repeating units of the disaccharide D-glucuronic-β1,3- D-acetyl-D-glucosamine-β1,4, is an important constituent of the extracellular matrix. HA, which is secreted by many cell types, is assembled at the plasma membrane by HA synthases (HAS) and extruded into the extracellular space (5, 12, 24). The HA chain can extend up to 2 × 105 monosaccharides and up to 25 μm in length. There are three HAS isoforms (HAS-1, HAS-2, and HAS-3) that synthesize HA of different chain lengths and are subject to differential regulation (8, 9, 18).

The biological activity of HA is a function of its molecular weight (23). In response to injury and inflammation, HA undergoes both increased synthesis through induction of HAS and degradation to smaller molecular weight fragments by hyaluronidases. These lower molecular weight HA fragments are angiogenic, inflammatory, and immunostimulatory. HA expression is increased in many injury states, including Crohn’s disease in humans and dextran sodium sulfate (DSS)-induced colitis in mice. (13, 30). Increased levels of both HA and HAS-1 are seen in a genetic model of ileitis in which TNF-α is overexpressed (4).

HA binds to CD44, which is expressed on the plasma membrane of many cell types, and also binds to the Toll-like receptors TLR2 and TLR4, which are widely distributed in the gastrointestinal tract and are important in mediating the host response to both commensal and pathogenic bacteria (22). Signaling through TLR4 is important in the epithelial response to DSS injury (2, 6, 20). HA binding to TLR2 and TLR4 mediates protective mechanisms in aseptic injury models in the lung and skin (10, 25). We recently demonstrated that HA binding to TLR4 mediates colonic epithelial repair in DSS colitis in mice (30).

The small intestine epithelium and the bone marrow are highly sensitive to radiation and are the major sites of injury during radiation therapy (1, 16, 28). Diarrhea induced by radiation injury in the small intestine is a limiting factor in the dosing of radiation therapy for rectal cancer and other abdominal malignancies. There is a need for agents that could be given before radiation therapy that would diminish radiation injury to the small intestine without decreasing the radiation sensitivity of the tumor. Bacterial products can affect the intestinal epithelial response to radiation. Lipopolysaccharide (LPS), a TLR4 ligand, is radioprotective in the mouse intestine through a mechanism that involves prostaglandin E2 (PGE2) synthesis through cyclooxygenase-2 (COX-2) (21). The TLR5 agonist flagellin and flagellin analogs are also radioprotective, but not through a COX-2-mediated mechanism (3, 11, 26).

In view of the induction of HA expression seen in response to other kinds of intestinal injury, we sought to determine whether HA expression serves as a general protective mechanism in aseptic injury models. In addition, in view of the known radioprotective effects of LPS acting through TLR4 and the established ability of HA to bind TLR4, we sought to determine whether HA is radioprotective in the intestine.

MATERIALS AND METHODS

Animals. Adult (6–8 wk old) wild-type (WT), CD44−/−, and TLR4−/− mice on a C57BL/6J background were purchased from Jackson Laboratory (Bar Harbor, ME). COX-2−/− mice on a C57BL/6J background were obtained by backcrossing COX-2−/− mice on B6/129 background (14) to WT C57BL/6J mice for 10 generations. Mice were maintained on a 12:12-h light-dark schedule and fed standard laboratory mouse chow. Animal procedures were in accordance with the Washington University School of Medicine Animal Studies Committee. Mice were given 12-Gy whole body radiation in a Gammacell 40 137Cs irradiator (Atomic Energy of Canada).

Chemical and reagents. HA (molecular mass 750 kDa) was obtained from Sigma (St. Louis, MO). We had previously demonstrated that this HA preparation was not contaminated with LPS (30). HA-
binding peptide (PEP-1: H₂N-GAHWQFNALTVR-0H) (15) and scrambled control peptide (H₂N-NWRHGEALTVNQ-PH) were obtained from New England Peptide (Gardner, MA).

Apoptosis and crypt survival in mouse small intestine. Mice were given intraperitoneal injections of 0.9% saline (controls), 30 mg/kg HA, or 40 mg/kg PEP-1 8 h before irradiation. For apoptosis studies, mice were killed at 6 h after irradiation. The proximal jejunum was divided into a minimum of six 5-mm segments, fixed in 10% formalin, and embedded in paraffin. Apoptosis was scored on a cell positional basis by light microscopic analysis of hematoxylin and eosin-stained cross sections as described by Pritchard (19). One hundred well-oriented half-crypt sections per mouse were counted with at least four mice in each group. All crypts chosen were at least 20 cells in height, with cell position 1 located at the crypt base. Crypt survival was measured in mice killed 84 h after irradiation as described previously using a modification of the microcolony assay (17, 29). Mice received a mixture of 5-bromo-2'-deoxyuridine (BrdU; 120 mg/kg) and 5-fluoro-2'-deoxyuridine (12 mg/kg) by intraperitoneal injection 90 min before death to label S-phase cells. Immunohistochemistry was carried out using goat anti-BrdU, and signal detection was carried out with 3,3'diaminobenzidine tetrahydrochloride (Sigma-Aldrich). The viability of a surviving crypt was confirmed by incorporation of BrdU into five or more epithelial cells within each regenerative crypt.

Plasma HA. Blood was obtained from mice by puncturing the right mandibular vein with a 5.5-mm animal lancet and collecting the blood directly into plasma separator tubes. Plasma HA concentration was determined by ELISA (Corgenix, Broomfield, CO) according to the manufacturer’s directions.

Immunohistochemical analysis. Proximal jejunums were fixed for 45 min at 24°C in 2% paraformaldehyde, 75 mM lysine, and 75 mM NaPO₄, pH 7.4. Tissue was washed two times in PBS (pH 7.4)-10% sucrose at 4°C, followed by PBS (pH 7.4)-20% sucrose overnight at 4°C, and then frozen in optimal cutting temperature compound (Tissue TEK) in flat sheets to optimize orientation.

Immunofluorescence detection of antigens used unconjugated primary antibodies followed by fluorescently labeled (Alexa Fluor) secondary antibodies (Invitrogen, Carlsbad, CA). Primary antibodies purchased from BD Pharmingen (San Jose, CA) were mouse anti-COX-2 (1:100), rat anti-CD29 (1:50), rat anti-CD31 (1:50), and rat anti-CD44 (1:50). Primary antibodies purchased from eBioscience were rat anti-CD54 (1:50), rat anti-CD105 (1:50), and rat anti-CD106 (1:50). Rat anti-F4/80 (1:50) was purchased from Abcam (Cambridge, MA). Biotinylated hyaluronan-binding protein (1:800; North Star, East Falmouth, MA), followed by Alexa Fluor 594-conjugated streptavidin (1:1,000; Invitrogen), was used to detect HA.

**Fig. 1.** Radiation injury induces increased hyaluronic acid (HA) expression in wild-type (WT) mouse intestine through induction of HA synthases (HAS). Mice received 12 Gy of total body irradiation. A: immunofluorescence staining of HA in mouse jejunum at baseline is shown primarily in the extracellular matrix around the crypts, but by 6 h after 12 Gy irradiation, HA expression had expanded into the villi. Original magnification, ×200. B: radiation injury induced increases in mRNA levels of HAS-1, HAS-2, and HAS-3 in mouse jejunum. Data are means ± SE for 4 mice per group. *P < 0.01; **P < 0.001 compared with unirradiated mice. C: radiation injury induced increased circulating levels of HA. Data are means ± SE for 4 mice per group. *P < 0.02; **P < 0.005 compared with unirradiated mice.
Formalin-fixed paraffin-embedded proximal jejunum long sections were used for counting the number of COX-2-expressing stromal cells by immunofluorescence. The number of COX-2-expressing stromal cells was scored from pictures taken at ×200 magnification and saved as Axiovision zvi files. The length of intestine covered in each picture was 335 μm as determined with a Scalings program from Carl Zeiss Imaging Systems. The number of COX-2-expressing stromal cells was scored separately for the villus and crypt zones. There were 20 pictures and 4 mice per treatment group.

Quantitative RT-PCR. Total RNA was extracted from distal jejunum tissues by homogenization in RiboZol RNA extraction reagent (Amresco, Solon, OH). Quantitative RT-PCR was used to measure gene expression, with actin expression as a control. The final results were expressed as fold differences in gene expression relative to the actin gene. The threshold cycle (Ct) for each amplicon was determined as the PCR cycle at which the fluorescence intensity crossed a user-established threshold. The following primers were synthesized by Integrated DNA Technologies (Coralville, IA): HAS-1, 5′-CTT TCA AAG CAC TGG GCC GAC and 5′-CAG CGC TTC ATA GGT CAT CCC; HAS-2, 5′-ACA GGC ACC TTA CCA ACA GGG TGT and 5′-GCA TGC ATA GAT CAA AGT TCC CAC G; HAS-3, 5′-ACT GCC TTC AAG GCC TGG TG and 5′-AAAT GTT CCA GAT GCG GCC ACC; and actin, 5′-CAACA GGA GGG GTT CCG ATG and 5′-GGC ACA GGA TTC CAT ACC CA.

SDS-PAGE and Western blot analysis. Distal jejunums were homogenized in cold protease inhibitor cocktail containing antipain (25 μg/ml), aprotinin (25 μg/ml), leupeptin (25 μg/ml), chymostatin (25 μg/ml), phenanthroline (50 μM), phenylmethylsulfonyl fluoride (100 μM), pepstatin A (10 μg/ml), and dithiothreitol (2 nM) in N-tris(hydroxymethyl)-methyl-2-aminomethane sulfonic acid (20 mM), pH 7.4. Protein (100 μg) samples were separated by SDS-PAGE on a Criterion TGX polyacrylamide gel (Any kD, 567-1123; Bio-Rad, Hercules, CA) and transferred to polyvinylidene fluoride membrane (Invitrogen). Antibodies used for detecting proteins were mouse monoclonal anti-COX-2 (1:500; BD Pharmingen) and mouse monoclonal anti-tubulin (1:1,000; Santa Cruz Biotechnology). Visualization of protein bands was carried out with horseradish peroxidase-linked secondary antibodies (1:2,000) and ECL reagent (GE Healthcare, Piscataway, NJ) with fluorographic detection on Blue-sensitive autoradiography film (Mrd Sci, St. Louis, MO).

RESULTS

Radiation induces the expression of HA in the intestine. In the normal mouse intestine, extracellular HA is found in the lamina propria surrounding the intestinal crypts. After 12-Gy total body radiation, the amount of HA in the extracellular space surrounding the crypts was increased and, in addition, HA was found in the lamina propria in the villi (Fig. 1A). The increase in HA expression in the intestine in response to radiation was associated with increases in HAS-1, HAS-2, and HAS-3 (Fig. 1B). The levels of HAS-1 mRNA were increased at 6 h after radiation and reached a maximum 5-fold increase compared with unirradiated controls at 24 h after irradiation. The levels of HAS-1 returned to baseline levels by 72 h. In contrast, HAS-2 mRNA levels increased rapidly, peaking at six times the level seen in unirradiated controls by 6 h after irradiation. These levels then fell progressively and reached baseline levels by 72 h after irradiation. The increase in HAS-3 levels was delayed. HAS-3 mRNA levels were up 4-fold by 48 h and 5-fold by 72 h after irradiation.

Radiation induces an increase in plasma HA. Six hours after 12-Gy total body irradiation, plasma HA levels were increased 10-fold in WT mice (Fig. 1C). Plasma HA levels fell rapidly and were near normal by 24 h after irradiation, and they continued at near normal levels through 72 h. There was no difference between the 6-h plasma HA levels in WT and Myd88−/− mice (data not shown), indicating that the induction of HA by radiation is not TLR dependent.

Exogenous HA increases crypt survival and diminishes radiation-induced apoptosis. Mice received 12-Gy total body irradiation and were killed 84 h later. The number of surviving crypts per cross section were counted in the small intestine. In WT mice there were 14 surviving crypts per cross section (Fig. 2A). There were 26 surviving crypts per cross section in WT mice receiving intraperitoneal HA 8 h before irradiation. Co-administration of HA and PEP-1, a peptide that blocks the binding of HA to its receptors (15, 30), resulted in a level of crypt survival similar to that seen in the absence of HA. Crypt

**Fig. 2.** HA is radioprotective in the intestines of WT mice. Mice received vehicle, HA, HA-binding peptide (PEP-1), PEP-1/HA, or scrambled PEP-1/HA 8 h before 12-Gy total body irradiation. A: crypt survival. Mice were killed 84 h after radiation, and the numbers of surviving crypts were counted in 6 proximal jejunum cross sections per mouse. Data are means ± SE for 8 irradiated control, 12 HA-treated, 4 PEP-1-treated, 7 PEP1-treated, and 7 scrambled PEP-1-treated mice. *P < 0.01 compared with HA-treated mice. **P < 0.001 compared with irradiated control mice. +P < 0.001 compared with PEP-1 + HA-treated mice. B: apoptotic index. Mice were killed 6 h after 12-Gy irradiation, and the cell positional distribution of apoptosis in the crypt was scored in 100 half-crypt sections per mouse. Data are means ± SE for 8 irradiated control mice and 4 HA- or HA + PEP-1-treated mice. *P < 0.01; **P < 0.001; ***P < 0.0001 compared with HA-treated mice. +P < 0.05; ++P < 0.01; +++P < 0.01 compared with HA + PEP-1-treated mice.
survival in mice treated with HA and scrambled PEP-1 was the same as in mice treated with HA alone, indicating the specificity of PEP-1 binding for HA. Crypt survival in mice given PEP-1 alone was the same as in irradiated controls, demonstrating that PEP-1 has no activity of its own.

Six hours after 12-Gy total body irradiation, there was a marked increase in apoptosis in the intestine. This increase was positional and peaked at positions 4–6 in the crypt (Fig. 2B). The frequency of radiation-induced apoptosis diminished in cells further up the crypt. Administration of HA 8 h before irradiation resulted in a decrease in radiation-induced apoptosis. At position 4, the apoptotic index was 18% in mice receiving HA before irradiation compared with 42% in mice not receiving HA. Administration of PEP-1 in addition to HA reversed almost all of the antiapoptotic effects of HA. At position 4, the apoptotic index in mice receiving both PEP-1 and HA was 44%, similar to the 42% seen in mice receiving irradiation alone and much higher than the 18% seen in mice receiving HA before irradiation.

Radioprotective effects of HA are mediated through binding to TLR4. HA binds to CD44 and TLR4. To determine the contributions of the binding of HA to CD44 and TLR4 to the radioprotective effects of HA, we irradiated CD44<sup>−/−</sup> and TLR4<sup>−/−</sup> mice in the presence and absence of HA and measured crypt survival and positional apoptosis. Crypt survival was measured in CD44<sup>−/−</sup> mice and TLR4<sup>−/−</sup> mice 84 h after 12-Gy irradiation. In CD44<sup>−/−</sup> mice, crypt survival was similar to what was seen in WT mice and there was a significant increase in crypt survival in the CD44<sup>−/−</sup> mice given HA just as there was an increase in crypt survival in HA-treated WT mice (Fig. 3A). Crypt survival in the TLR4<sup>−/−</sup> mice was slightly increased compared with the WT mice; however, administration of HA before irradiation had no effect on crypt survival in the TLR4<sup>−/−</sup> mice.

CD44<sup>−/−</sup> mice had a pattern of positional irradiation-induced apoptosis similar to what was seen in WT mice (Fig. 3B). In CD44<sup>−/−</sup> mice, there was a decrease in radiation-induced apoptosis when the mice were given HA 8 h before irradiation.
irradiation (Fig. 3C). This pattern was similar to that seen in WT mice. In TLR4−/− mice, the pattern of radiation-induced apoptosis was similar to that seen in WT mice (Fig. 3B); however, the decrease in radiation-induced apoptosis induced by the administration of exogenous HA was more modest than that seen in WT or CD44−/− mice (Fig. 3D). Together, the positional apoptosis studies and the crypt survival studies suggest that the radioprotective effects of HA are mediated in part through TLR4.

**Radioprotective effects of HA are mediated through COX-2.**

We have previously demonstrated that the radioprotective effects of LPS are mediated through TLR4 and COX-2. To determine whether the radioprotective effects of HA are also mediated through COX-2, we performed radiation-induced crypt survival studies and apoptosis studies in COX-2−/− mice in the presence and absence of exogenous HA. Crypt survival in COX-2−/− mice was similar to that seen in WT mice; however, there was no increase in crypt survival with the administration of exogenous HA in COX-2−/− mice (Fig. 3A). Radiation-induced apoptosis in COX-2−/− mice (Fig. 3E) was substantially diminished compared with that in WT mice, CD44−/− mice (Fig. 3C), or TLR4−/− mice (Fig. 3D). Moreover, the administration of exogenous HA to COX-2−/− mice had no effect on radiation-induced apoptosis (Fig. 3E). The apoptosis studies and the crypt survival studies suggest that the radioprotective effects of HA are mediated through COX-2.

**Administration of exogenous HA is associated with an increase in COX-2 expression and an increase in PGE2 production.** Having demonstrated that the radioprotective effects of HA are COX-2 dependent, we next sought to determine whether administration of HA results in an increase in the number of COX-2-positive cells in the intestine. The number of COX-2-positive cells was similar in the intestines of HA-treated and untreated mice (Fig. 5A), but the distribution of the COX-2-positive cells was different (Fig. 5B). At baseline, 80% of the COX-2-expressing cells were in the lamina propria in the villi and 20% were in the lamina propria near the crypts; 8 h after administration of HA, 60% of the COX-2-positive cells were associated with the villi and 40% were in the lamina propria near the crypts (Fig. 5C).

**Identification of the COX-2-positive cells in the intestine.** To determine the identity of the COX-2-expressing cells, we performed colocalization studies of COX-2 with a number of surface proteins. Immunohistochemistry for COX-2 and surface proteins was performed on normal mouse intestine at baseline and 8 h after intraperitoneal HA. The results of the baseline study are shown in Fig. 6. COX-2 colocalized CD29 (Fig. 6A), CD44 (Fig. 6B), CD54 (Fig. 6C), CD105 (Fig. 6D), and CD106 (Fig. 6E). COX-2 is a cytoplasmic protein, whereas the others are surface proteins, so colocalization is visible as a red center with a green rim. CD29, CD44, CD54, CD105, and CD106 are all surface proteins associated with mesenchymal stem cells (2, 27). The colocalization of COX-2 with CD29, CD44, CD54, CD105, and CD106 suggests that the COX-2-expressing cells are mesenchymal stem cells. Some of these proteins, including CD44, are also expressed on non-stem cells. As a result, all the COX-2-expressing cells also express CD44, CD54, CD105, and CD106 proteins, including CD44, are also expressed on non-stem cells. As a result, all the COX-2-expressing cells also express CD44.

**Administration of exogenous HA is associated with the redistribution of COX-2 expressing cells from the villi to the crypts.** Having found that the radioprotective effects of HA are mediated through COX-2 and that administration of HA results in a small but significant increase in COX-2 expression, we next sought to determine whether administration of HA results in an increase in the number of COX-2-positive cells in the intestine. The number of COX-2-positive cells was similar in the intestines of HA-treated and untreated mice (Fig. 5A), but the distribution of the COX-2-positive cells was different (Fig. 5B). At baseline, 80% of the COX-2-expressing cells were in the lamina propria in the villi and 20% were in the lamina propria near the crypts; 8 h after administration of HA, 60% of the COX-2-positive cells were associated with the villi and 40% were in the lamina propria near the crypts (Fig. 5C).

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**Administration of exogenous HA is associated with an increase in COX-2 expression and an increase in PGE2 production.** Having demonstrated that the radioprotective effects of HA are COX-2 dependent, we next sought to determine whether administration of HA results in an increase in the number of COX-2-positive cells. A Western blot for COX-2 (Fig. 4A) showed a 1.5-fold increase in COX-2 expression in the intestine in WT mice given intraperitoneal HA 8 h before death compared with untreated mice (Fig. 4B). Administration of HA also resulted in a 1.5-fold increase in intestinal PGE2 (Fig. 4C).

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CD44, but only some CD44-expressing cells also express COX-2. CD31 is an endothelial cell marker and is included as a negative control. We also performed all these colocalization studies in intestines from mice given intraperitoneal HA 8 h before death. Although the distribution of the COX-2-positive cells was different in the HA-treated mice compared with the untreated mice (Fig. 5), the results of COX-2 colocalization studies were identical in the intestines of the HA-treated and the untreated mice (data not shown).

**DISCUSSION**

In this study we found that radiation injury induces HA synthesis in the intestine through increased expression of HAS and that exogenous HA, given before irradiation, reduces radiation-induced apoptosis and increases crypt survival. The radioprotective effects of HA are mediated through TLR4 and COX-2. The increase in intestinal HA through increased HAS expression after radiation injury is similar to the increase in colonic HA expression seen in DSS colitis (30). Increased HA expression is part of the tissue response to injury in other organs in a variety of types of injury (10, 25). In humans, increased HA is seen in response to Crohn's disease (13). Having demonstrated that radiation induces increased HA production, we next sought to determine whether exogenous HA is radioprotective in the intestine. Intraperitoneal HA decreases radiation induced apoptosis and increases crypt survival when given before radiation. These effects are mediated through TLR4, COX-2, and PGE2. PGE2 is radioprotective in the intestine (7). LPS, a bacterial ligand for TLR4, is radioprotective in the intestine when given intraperitoneally (21). This effect is mediated through COX-2 and PGE2 as demonstrated by the failure of LPS to be radioprotective in COX-2−/− mice.

Radioprotection refers to the prevention of radiation-induced injury rather than the enhancement of repair. The beneficial effects are only seen when the radioprotective agent is given before irradiation, as was demonstrated with TLR4-dependent radioprotection by LPS (21). Thus the conditions relevant to radioprotection, in terms of COX-2 expression and PGE2 levels, are those that are present at the time of radiation.

HA administration is associated with a small but real increase in total intestinal COX-2 expression and a similar increase in intestinal PGE2. In addition, HA administration is associated with a change in the distribution of COX-2-expressing cells in the intestine, with fewer COX-2-expressing cells associated with the lamina propria in the villi and more COX-2-expressing cells associated with the lamina propria surrounding the crypts. Immunohistochemistry for surface...
markers demonstrates that those COX-2-positive cells also express CD29, CD44, CD54, CD105, and CD106 but not CD31; this pattern of surface marker expression is consistent with mesenchymal stem cells (2, 27). Although the expression of CD29, CD44, CD54, CD105, and CD106 is characteristic of all mesenchymal stem cells, only colonic and intestinal mesenchymal stem cells express COX-2. The expression of COX-2 in these cells is driven by Fgf9 released by intestinal or colonic epithelial cells (27).

The number of COX-2-expressing cells is the same in the intestines of untreated and HA-treated mice. The COX-2-positive cells in the intestines of untreated and HA-treated mice express the surface markers of mesenchymal stem cells. There is no proof that the redistribution of COX-2-expressing cells is due to cellular migration. It is possible that HA induces COX-2 in a population of crypt-associated mesenchymal stem cells and represses COX-2 expression in a population of villus-associated mesenchymal stem cells, but it seems more likely that HA induces the migration of mesenchymal stem cells from villus to crypt.

This repositioning of COX-2-expressing intestinal mesenchymal stem cells in response to HA is similar to the repositioning of colonic mesenchymal stem cells in DSS colitis (2). In both cases, the COX-2-expressing mesenchymal stem cells move toward the proliferating epithelial cells at the base of the crypt. COX-2 catalyzes the synthesis of PGE2, a potent antiapoptotic agent. PGE2 has a short half-life and operates over very short distances. The physiologically relevant PGE2 concentration is not the concentration averaged over the entire intestine, but rather the concentration in the immediate neighborhood of the target cell of interest, in this case the crypt epithelial cell. The migration of the COX-2-expressing mesenchymal stem cells to a place adjacent to crypt epithelium is likely to dramatically increase the PGE2 concentration in the neighborhood of the crypt epithelium.

The migration of colonic mesenchymal stem cells in DSS colitis and the migration of intestinal mesenchymal stem cells in response to HA are TLR4 dependent. The cell type in which the key TLR4-mediated signaling occurs is not clear. The migration of colonic mesenchymal stem cells toward the epithelium at the base of the colonic crypts in DSS colitis and the migration of intestinal mesenchymal stem cells toward the epithelial cells at the base of the crypts in response to HA could be driven by a chemotactic factor made by the crypt epithelial cells, by adjacent stromal cells such as myofibroblasts, or by macrophages near crypt epithelial cells. It may be that the production of this chemotactic factor is TLR4 dependent. Intestinal and colonic epithelial cells, myofibroblasts, and macrophages all express TLR4. It is less likely that the intestinal mesenchymal stem cells themselves are the site of key TLR4-mediated signaling; although they express TLR4, they do not express CD14, which is important in mediating TLR4 signaling (27).

In summary, this work expands on three observations. The first is that increased HA expression is seen in response to radiation injury. The second observation is that exogenous HA acting through TLR4 binding protects the epithelium and preserves epithelial proliferation in the face of intestinal injury. This protective effect, previously demonstrated in DSS colitis (30), is mediated through COX-2 and PGE2. The third obser-
HA IS RADIOPROTECTIVE

ization is that the protective effects on the epithelium are mediated by the migration of COX-2-expressing mesenchymal stem cells. In response to HA, these cells migrate from the lamina propria in the villi to the lamina propria near the crypt epithelium. A similar migration of COX-2-expressing mesenchymal stem cells also occurs in the colon in response to DSS-induced colitis (2, 27). In radiation injury, the most important interaction is probably that seen between the epithelial stem cells and PGE2 production by mesenchymal stem cells. Evaluation of the specific cell types affected by HA action using stem cell markers would be an important future direction.

This work raises the possibility that HA or other TLR4 agonists may be practical agents for protecting the intestinal epithelium during radiation therapy of abdominal malignancies. Additional studies demonstrating that orally administered HA is radioprotective and that HA does not provide radioprotection to abdominal malignancies would be required before HA could be viewed as a clinically useful radioprotective agent.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

REFERENCES