Gastrointestinal, Hepatobiliary, and Pancreatic Pathology

Muc-2–Deficient Mice Display a Sex-Specific, COX-2–Related Impairment of Gastric Mucosal Repair

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Mucus is known to contribute significantly to the prevention and repair of mucosal damage throughout the gastrointestinal tract. Although not normally expressed in the stomach, mucin-2 (MUC-2, encoded by the *MUC2* gene) is expressed in certain disease states. The aim of this study was to determine in a mouse model whether the absence of Muc-2 would result in impaired susceptibility to and healing of gastric mucosal injury. Acute gastric damage was induced in mice deficient in Muc-2 and in wild-type controls, through oral administration of indomethacin. Chronic gastric ulcers were induced by serosal application of acetic acid. The extent of injury and the extent of healing of the damage over time were examined in both models. Indomethacin administration caused similar levels of gastric damage in Muc-2–deficient and wild-type mice, but the erosions healed more slowly in the former. Acetic acid–induced gastric ulcers were initially similar in size in Muc-2–deficient and wild-type mice of both sexes, but ulcer healing was significantly impaired in male Muc-2–deficient mice. Induction of cyclooxygenase-2 in the stomach, in response to indomethacin- or acetic acid–induced ulceration, was significantly reduced in male Muc-2–deficient mice. This phenomenon, and the sex specificity, was also apparent in bone marrow–derived macrophages stimulated with endotoxin. These results demonstrate a marked impairment of gastric mucosal repair in male Muc-2–deficient mice that may be related to an insufficient induction of cyclooxygenase-2, an enzyme known to contribute to mucosal repair. (*Am J Pathol* 2011, 178:1126–1133; DOI: 10.1016/j.ajpath.2010.11.048)

Mucus is a significant contributor to tissue defense and repair in the pulmonary, gastrointestinal, and urogenital systems.¹ In the stomach, mucus helps to reduce bacterial colonization, aids in protecting the epithelium from damage induced by ingested substances, acid, and pepsin, and contributes significantly to repair of epithelial damage.^{1–4} That last effect is attributable to the contribution of mucus in the formation of a microenvironment over sites of mucosal injury, which is conducive to restoration of epithelial integrity.⁴ Disruption of mucus in that circumstance leads to progression of tissue injury to deeper layers of the mucosa, with significant bleeding.⁵

A major component of mucus is mucin, synthesis of which is encoded by 21 different mucin (*MUC*) genes in humans, mouse, and rat.⁶ These genes encode for both membrane-bound and secretory mucins. Mucin expression is cell- and tissue-specific. Normal gastric mucosa expresses MUC-1, MUC-5AC, and MUC-6. The first two are found in the superficial epithelium; MUC-6 is found in the deep glands.⁷ MUC-5AC, a secretory mucin, is the major mucin in the normal stomach, whereas MUC-2, another secretory mucin, predominates in the small and large intestine.⁸ Altered mucin expression patterns have been observed in different digestive disorders. For example, reduced expression of MUC-5AC and MUC-6 has been reported in gastric carcinomas.⁹ MUC-2 is not usually expressed in the stomach, but gastric expression of this mucin has been reported in gastric carcinoma^{9,10} and in *Helicobacter pylori*–infected gastric epithelium.⁹ Studies in mice suggest that deficiency of Muc-2 may contribute to the onset and perpetuation of colitis.¹¹

Given the importance of mucin in health and in different pathological conditions, and the possibility that Muc-2 is expressed in injured or inflamed stomach, we speculated that mice deficient in Muc-2 might display impaired healing of mucosal injury. We investigated this using both acute and chronic models of gastric injury. Acute indomethacin administration results in damage in

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the stomach that is limited to the mucosal layer (so-called erosions). On the other hand, brief application of acetic acid to the serosal wall of the stomach induces the formation of true ulcers (ie, erosions penetrating through the mucosa into the submucosa).¹² Our results suggest that mice deficient in Muc-2 do indeed exhibit impaired healing of both erosions and ulcers, but that this effect is sex-specific (limited to males), appears to be related to impaired up-regulation of mucosal cyclooxygenase-2 (COX-2), and likely is not related (at least not directly) to the absence of Muc-2.

Materials and Methods

Animals

Six- to eight-week-old C57BL/6 and *Muc2*^{-/-} mice, the latter originally described by Velcich et al,¹³ were purchased from Charles River Laboratories International (Montreal, QC, Canada) or were bred in the animal care facilities at the University of Calgary and McMaster University. The mice were kept in sterilized, filter-topped cages, handled in tissue culture hoods, and fed autoclaved food and water under specific pathogen-free conditions. The experiments were approved by the respective animal care committees of the University of Calgary and McMaster University and were conducted in accordance with the guidelines of the Canadian Council on Animal Care.

Healing of Indomethacin-Induced Gastric Injury

Initially, we compared the effects of indomethacin in male Muc-2-deficient and wild-type (C57BL/6) mice. Subsequently, similar experiments were conducted in female mice. The mice were deprived of food, but not water, for 18 to 20 hours before oral administration of indomethacin (10 mg/kg). Indomethacin was dissolved in 1.25% sodium bicarbonate and was administered at a volume of 0.1 ml/kg. At least five mice from each of the Muc-2-deficient and wild-type groups were euthanized 5, 12, 24, or 48 hours later. The abdomen was opened and the stomach was removed. The extent of gastric erosions was blindly scored. This involved measuring the lengths of all hemorrhagic lesions (in millimeters), then summing these to give a gastric damage score for each mouse.¹⁴ Additional experiments were conducted, as described above, in which the mice were euthanized 5 hours after administration of indomethacin or vehicle, and the stomach was then processed for measurement of gastric synthesis of prostaglandin E₂, as described previously.¹⁵ Gastric tissue samples were collected and processed for COX-1 and COX-2 mRNA expression by quantitative PCR.¹⁴ Other tissues were fixed in neutral-buffered formalin and were processed by routine techniques for immunohistochemical examination of Muc-2 and Muc-5ac expression (see *Immunohistochemistry*).

Healing of Acetic Acid-Induced Gastric Ulcers

Gastric ulcers were induced in groups of at least 10 mice (Muc-2-deficient and wild type; males and females), as

described in detail previously.^{12,16} Briefly, a laparotomy was performed, and acetic acid (200 μ L, 20% v/v) was applied to a 28-mm² area on the serosal wall of the stomach. After 1 minute, the acetic acid was removed and the exposed area was rinsed three times with 200 μ L of sterile isotonic saline. The stomach was then returned to the abdominal cavity, and the abdominal wall and skin were closed with sutures. Three days after this procedure, subgroups of five mice from each of the four groups were euthanized, and the area of ulceration was blindly measured.¹² This involved opening the stomach by an incision along the greater curvature, placing a 25-mm² grid alongside the ulcer, then photographing the stomach. The ulcer area was measured planimetrically, and then the data (in pixels) were converted to units of mm² using the grid as a reference. The remaining mice were euthanized 10 days after the serosal application of acetic acid, and ulcer areas were measured. Gastric tissue was excised from each mouse. COX-1, COX-2, Muc-2, Muc-5ac, and Muc-6 mRNA expression was determined by quantitative PCR. Other tissues, as well as samples of the colon (as a positive control), were fixed in neutral-buffered formalin and were processed by routine techniques for immunohistochemical examination of Muc-2 and Muc-5ac expression (as described in the next section). In additional experiments, samples from the ulcer bed in each mouse were processed for determination of the colonization of the ulcer by aerobic bacteria, as described in detail previously.¹⁷

Immunohistochemistry

Formalin-fixed, paraffin-embedded gastric or colonic segments were sectioned to 5 μ m in thickness, deparaffinized in xylene, and subsequently rehydrated in graded concentrations of ethanol. For histology, sections were processed by routine methods and stained with H&E. For immunofluorescence detection and localization of muc-2 and muc-5AC, the sections were subjected to heat-induced epitope retrieval (Target retrieval solution, pH 9.0; DakoCytomation, Glostrup, Denmark), blocked with 3% bovine serum albumin, and subsequently incubated with either a polyclonal antibody raised against Muc-2 (1:500 dilution; Ab76774; Abcam, Cambridge, MA), or a monoclonal antibody raised against Muc-5ca (1:500 dilution; clone 45M1; Abcam), overnight at 4°C. Sections were washed in PBS/0.5% Tween-20, and incubated with Alexa Fluor 568 F(ab')₂ fragment of goat anti-mouse IgG or goat anti-rabbit IgG (Invitrogen, Carlsbad, CA). After repeated washes, sections were then mounted with ProLong Gold antifade reagent containing DAPI (Invitrogen), and fluorescence was visualized on a Nikon Eclipse 80i microscope equipped with a DS-QiMc monochromatic camera (Nikon) and an X-Cite Series 120Q xenon lamp (Lumen Dynamics Group, Mississauga, ON, Canada). NIS-Elements BR3.1 software (Nikon) was used for all analyses. Images were recorded at identical gain settings, and mean intensity was calculated per image field. Four image fields were taken of each section.

Gastric Acid Secretion

The most common clinical practice for accelerating the healing of gastric ulcers, or for preventing nonsteroidal anti-inflammatory drug-induced gastric damage, is suppression of gastric acid secretion.¹⁸ Although there are no published reports of any link between Muc-2 deficiency and gastric acid secretion, we considered the possibility that any difference in healing of mucosal injury between Muc-2-deficient and wild-type mice could be due to differences in gastric acid secretion. Mice (Muc-2-deficient and wild type) were fasted for 18 to 20 hours, then lightly anesthetized with isoflurane. A laparotomy was performed and the pylorus was ligated.¹⁹ The abdominal incision was closed with sutures, and 3 hours later, the mice were euthanized by an overdose of sodium pentobarbital. The stomach was removed, with care taken to preserve the gastric contents. The gastric contents were collected, and the volume and pH of the gastric juice were measured.¹⁹ Additional studies were performed in which fasted male Muc-2-deficient and wild-type mice were given indomethacin (10 mg/kg orally), and acid secretion studies were performed 12 hours later. Also, gastric juice was collected from wild-type and Muc-2-deficient mice (both sexes) in which gastric ulcers had been induced 10 days earlier, and gastric acidity was determined as above.

Quantitative PCR

Gastric tissues were excised, snap-frozen in liquid nitrogen, and stored at -80°C until required for processing.¹⁴ RNA isolation was performed using TRIzol (Invitrogen) according to the manufacturer's recommendations. cDNA was synthesized from 2 μg of extracted RNA using M-MLV reverse transcriptase (Invitrogen). Quantitative PCR for COX-1 and COX-2 was performed using SYBR Green Supermix (Qiagen, Valencia, CA) in a Rotor-Gene thermal cycler detection system (Corbett Research, Qiagen). Primer sequences used were as follows: COX-1 forward 5'-AAGGAGTCTCTCGCTCTGGTTT-3', reverse 5'-TCTCAGGGATGGTACAGTTGGG-3'; COX-2 forward 5'-ATCCTGCCAGCTCCACCG-3', reverse 5'-TGGTCAATCCTGTGCTCATACAT-3'; and Muc-5ac forward 5'-CAACTGTCCGCAGGGCCAGG-3', reverse 5'-ATGCTGTCCCCGCAGTTGCC-3'. Each quantitative PCR was performed in triplicate and was normalized against GAPDH or β -actin.

Macrophage Studies

Bone marrow macrophages were harvested from male and female Muc-2-deficient mice and were cultured as described previously.²⁰ In brief, femurs and tibia of mice were removed, and bone marrows were flushed with a 26-gauge needle. Red blood cells were lysed with red blood cell lysis buffer (Sigma-Aldrich, St Louis, MO). The remaining cells were washed with RPMI 1640 medium and plated in RPMI 1640 medium supplemented with 10% fetal bovine serum, 10 mmol/L HEPES, 50 mmol/L 2-mercaptoethanol, 2 mmol/L glutamine, 5 mg/ml penicillin/streptomycin sulfate, and 20% L-929 cell condi-

tioned medium. Nonadherent cells were removed at day 4 and day 7, and fresh medium was added to the adherent cells. Cells were plated at $5 \times 10^5/\text{ml}$ in a 24-well plate and were made quiescent overnight in serum-free media. Macrophages were treated with lipopolysaccharide (100 ng/ml) for 20 hours.²¹ The medium was removed, and cells were lysed in TRIzol for RNA extraction. RNA was quantified spectrophotometrically and was reverse-transcribed using Superscript-II (Invitrogen) and oligo-dT primers. The resulting cDNA was analyzed by PCR using a Quantitect SYBR Green PCR kit (Qiagen) and gene-specific primers: COX-1 forward 5'-CACTGTGGATGCCTTCTCT-3', reverse 5'-TCTCGGGACTCCTTGATGAC-3'; and COX-2 forward 5'-CTCCCTGAAGCCGTACACAT-3', reverse 5'-ATGGTGCTCCAAGCTCTACC-3'.

Statistical Analysis

All data are expressed as the mean $\pm$ SEM. Comparisons among groups of data were made using a one-way analysis of variance followed by Dunnett's multiple comparison test. An associated probability (*P* value) of <0.05 was considered significant.

Results

Male Muc-2-Deficient Mice Exhibit Impaired Healing of Gastric Erosions

Male Muc-2-deficient and wild-type mice exhibited similar susceptibility to indomethacin-induced gastric damage, with mean scores of $\sim 15 \pm 3 \text{ mm}^2$ at 5 hours after administration (Figure 1). During the 43 hours after the initial assessment of damage, however, the erosions progressively healed in the wild-type group more quickly than in the Muc-2-deficient group. Thus, at 24 and 48 hours after indomethacin administration, the erosions were significantly more extensive in the Muc-2-deficient group ($P < 0.05$).

When similar experiments were attempted in female Muc-2-deficient mice, it was noted that the females were highly resistant to the damaging effects of indomethacin, with a mean damage score of 4 ± 3 . This increased

Figure 1. Healing of indomethacin-induced gastric damage was delayed in Muc-2-deficient (*Muc2*^{-/-}) mice, compared with wild-type controls (C57BL6). Although the extent of damage induced by oral administration of indomethacin (10 mg/kg) was similar in the two groups, the gastric damage score was significantly reduced in the wild-type mice at 24 and 48 hours after indomethacin administration (**P* < 0.05). Each group consisted of 6 to 8 male mice.

Figure 2. Healing and bacterial colonization of gastric ulcers induced by application of acetic acid to the serosal surface of the stomach. **A:** Healing of ulcers was significantly impaired in male Muc-2-deficient (Muc2^{-/-}) mice, compared with the wild-type controls (C57). The mean ulcer area was similar in the four groups at 3 days after acetic acid application, but by 1 week later (day 10), the ulcers had healed to a significantly greater extent in the wild-type mice (both sexes) and in the female Muc-2-deficient mice (**P* < 0.05 versus the other three groups on day 10). Each group consisted of 6 to 8 mice. **B** and **C:** Colonization of gastric ulcers at day 10 as total aerobes (**B**) and as enterobacteria (**C**). Colonization was negligible in the male Muc-2-deficient mice, in contrast to the other three groups. CFU, colony-forming units.

resistance to indomethacin-induced gastric injury was not related to differences in the degree of suppression of gastric prostaglandin synthesis in female versus male Muc-2-deficient mice. In the male and female Muc-2-deficient mice treated with vehicle, gastric prostaglandin E2 synthesis was 340 ± 65 and 329 ± 63 ng/mg, respectively. In mice treated with indomethacin, gastric prostaglandin E2 synthesis was inhibited by >95% in both sexes: 20 ± 06 and 19 ± 6 ng/mg, respectively (*P* < 0.001).

Male Muc-2-Deficient Mice Exhibit Impaired Healing of Gastric Ulcers

To further investigate the apparent differences in healing of gastric damage between Muc-2-deficient and wild-type

mice, and the sex-related differences in mucosal injury, a series of experiments were performed in which mice with gastric ulcers were monitored to determine healing rates. Serosal application of acetic acid resulted in the formation of gastric ulcers in all mice, with the mean ulcer area being the same in all groups (Figure 2); however, when the mice were left for 7 days and then ulcer areas were examined again, differences in healing were evident. A significantly greater extent of healing was seen in the wild-type (both sexes) and in female Muc-2-deficient mice, compared with the male Muc-2-deficient mice. This effect was consistent with the impaired healing of indomethacin-induced gastric erosions in male Muc-2-deficient mice.

Bacterial Colonization of Gastric Ulcers

Significant bacterial colonization of acetic acid-induced gastric ulcers was observed (Figure 3). The numbers of both total aerobes and enterobacteria were comparable among the wild-type mice and the female Muc-2-deficient mice; however, there was a marked absence of bacteria colonizing the gastric ulcers in male Muc-2-deficient mice.

Muc-2 and Muc-5ac Expression

In the absence of Muc-2, it was possible that other mucins were up-regulated in the stomach, as has been reported to occur in the small intestine.²² The expression of mRNA for Muc-5ac, the most abundant gastric mucin, was therefore examined. There were no significant differences in expression of Muc-5ac between the Muc-2-deficient and wild-type mice, either in males or females; that is, in Muc-2-deficient mice, the expression of Muc-5ac ranged between 60% and 160% of that observed in the corresponding wild-type mice (not significant).

Mucin expression might also have been altered as a consequence of induction of mucosal injury. To explore that possibility, Muc-2 expression in the stomach was examined using immunohistochemistry. Muc-2 was not detected in the stomach of wild-type mice under control conditions, but was present in the colon (Figure 3). After administration of indomethacin, Muc-2 staining was evident

Figure 3. Indomethacin induces expression of Muc-2 in the gastric mucosa. Muc-2 was expressed in the colon, but not the stomach, of vehicle-treated C57Bl6 mice. At 12 hours after oral administration of indomethacin (10 mg/kg), Muc-2 immunoreactivity was evident (arrowheads) in the gastric mucosa. No Muc-2 immunoreactivity was detected in the stomach of Muc-2-deficient mice (Muc2^{-/-}), nor in the isotype controls.

Figure 4. Muc-5ac expression in the stomach was not affected by indomethacin administration. Muc-5ac immunoreactivity was evident in the gastric mucosa of C57Bl6 and Muc-2-deficient (*Muc2*^{-/-}) mice, and was not changed by administration of indomethacin (10 mg/kg 12 hours before harvesting of tissue).

in the gastric mucosa of wild-type mice. As expected, Muc-5ac was abundantly expressed in the gastric mucosa of both wild-type and Muc-2-deficient mice (Figure 4), with no obvious differences in the intensity of expression between the two strains. Administration of indomethacin had no marked effect on Muc-5ac expression in the stomach. In mice in which gastric ulcers had been induced 10 days earlier via serosal application of acetic acid, Muc-5ac staining was evident in both sexes of both Muc-2-deficient and wild-type mice, particularly in the region of the ulcer margin (Figure 5). Moreover, staining for Muc-2 was evident in the same regions in the wild-type mice, although expressed much less intensely than for Muc-5ac.

Gastric Acid Secretion

After 3 hours of pylorus ligation, significant gastric juice (~0.5 ml) with a pH of <2 accumulated in the stomach of wild-type mice, irrespective of sex. In sharp contrast, although both male and female Muc-2-deficient mice

secreted approximately the same volume of gastric juice as the wild type, the pH was significantly higher than in the wild type (range, 4 to 5; no significant difference between sexes). Reduced gastric acidity (ie, higher pH) would be expected to result in an enhancement of healing of mucosal injury.¹⁸ These results suggest that the male Muc-2-deficient mice exhibited impaired mucosal healing despite very low levels of gastric acid secretion. Histological examination of gastric tissue from the Muc-2-deficient mice did not reveal any significant morphological differences, compared with the wild-type mice. The parietal cell mass appeared to be similar in the two strains.

In mice treated with indomethacin, the differences in acid secretion between the Muc-2-deficient and wild-type mice were no longer evident. Both the volume of secretion (average, ~0.7 ml) and the pH of gastric juice (range, 2.5 to 3.5) were similar in both sexes of both strains of mice. Likewise, when gastric ulcers had been induced in the mice, there were no significant differences in the pH of gastric juice between the wild-type and Muc-2-deficient mice, or between the sexes within either strain of mice (pH of 3.8 ± 0.3 and 3.6 ± 0.6 in male and female Muc-2-deficient mice, respectively, and 3.8 ± 0.6 and 4.0 ± 0.4 in male and female wild-type mice, respectively).

Up-Regulation of COX-2 Expression Is Impaired in Male Muc-2-Deficient Mice

We and others have previously reported that COX-2 expression in the stomach is rapidly up-regulated after non-steroidal antiinflammatory drug administration.^{23,24} This is particularly so at the margin of gastric ulcers (both in humans and in rodent models),^{25,26} and contributes significantly to healing.^{27,28} In the present study, we observed that male Muc-2-deficient mice had significantly reduced up-regulation of COX-2, compared with female

Figure 5. Expression of Muc-2 and Muc-5ac in mice with gastric ulcers induced by serosal application of acetic acid (10 days before harvesting of tissue). Muc-5ac was expressed in all four groups of mice, with no marked differences in intensity of staining. Muc-2 was expressed, albeit at a low level (arrowheads), in both male and female wild-type mice (C57Bl6), but not in the Muc-2-deficient mice. The Muc-2 staining was localized mainly to the epithelium at the ulcer margin.

male Muc-2 mice. The reasons for this surprising finding are not clear, and require further investigation.

Healing of gastric ulcers involves the re-establishment of a connective tissue foundation at the base of the ulcer, growth of new blood vessels (angiogenesis) and re-establishment of glands through proliferation and differentiation of epithelial cells.³³ It has been known for many years that prostaglandins are important mediators of many of the processes involved in ulcer healing.^{33,34} Moreover, prostaglandins derived from COX-2 appear to be particularly important in ulcer healing.^{26,34} COX-2 is important both in re-epithelialization and angiogenesis, and has been shown to be expressed primarily at the ulcer margin.²⁷ The impaired induction of COX-2 in male Muc-2-deficient mice (compared with the female counterparts and wild-type controls) may therefore explain the impaired ulcer healing in the former. Furthermore, rapid induction of COX-2 in the gastric mucosa after administration of nonsteroidal anti-inflammatory drugs has been shown to play an important role in limiting mucosal damage.^{15,23} In the case of aspirin, this appears to be related to the generation, via COX-2, of gastroprotective lipoxins.¹⁵ Thus, inadequate up-regulation of COX-2 in male Muc-2-deficient mice after oral challenge with indomethacin could contribute to impair healing of the ensuing damage. We noted that the defective up-regulation of COX-2 in male Muc-2-deficient mice was not limited to the stomach. Studies of bone marrow-derived macrophages stimulated with endotoxin also revealed a markedly reduced induction of COX-2 in the cells from male Muc-2-deficient mice.

The underlying reasons for the sex-related differences in COX-2 induction in the Muc-2-deficient mice are not clear. Given the importance of Muc-2 and of COX-2 in mucosal defense and repair, it is not surprising that an increased role of one of these (COX-2) may be seen as a compensatory response to the absence of the other (Muc-2). Our results, however, suggest a lack of appropriate induction of COX-2 only in the male Muc-2-deficient mice. From a review of studies in which Muc-2-deficient mice were used, we could not find any mention of sex-related differences in physiological or pathophysiological functions. One study of the human conjunctiva reported much higher levels of Muc-2 mRNA expression in females than males, but it was not known whether this difference extended to protein expression or to altered function.³⁵ Several studies have suggested a link between Muc-2 expression and COX-2 activity, particularly in the context of cancer. For example, in a study of the epithelium in patients with esophageal adenocarcinoma, a close correlation was found between COX-2 expression and proliferation, and COX-2 expression was strongest in cells that were also MUC-2-positive.³⁶ In human pulmonary epithelial cells, expression of MUC-2 in response to stimulation with IL-1 β was found to be COX-2-dependent.³⁷

Another surprising observation in the present study was the resistance of female Muc-2-deficient mice to indomethacin-induced gastric damage. This may have been related to the stronger induction of COX-2 in these mice, compared with the males. Rapid induction of COX-2 has been shown to limit the damaging effects of nonsteroidal anti-inflamma-

tory drugs in the stomach.³⁸ Increased resistance of female mice to experimental gastritis has been reported,³⁹ but the underlying mechanism was not identified.

In summary, the results of the present study demonstrate a marked impairment of mucosal healing in Muc-2-deficient mice. This impairment was observed only in males, and appeared to be related to a markedly reduced up-regulation of COX-2, which is known to play an important role in healing of mucosal injury throughout the gastrointestinal tract. Our findings are consistent with those of other studies that suggest a close interplay between MUC-2 and COX-2 in many tissues. Our study also demonstrated that Muc-2, although absent from the healthy stomach, was expressed in the mouse stomach after administration of indomethacin or after induction of an ulcer through serosal application of acetic acid. These observations suggest that induction of Muc-2 may represent an adaptive response to mucosal injury.

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