Collagen degradation and MMP9 activation by Enterococcus faecalis contribute to intestinal anastomotic leak

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Even under the most expert care, a properly constructed intestinal anastomosis can fail to heal, resulting in leakage of its contents, peritonitis, and sepsis. The cause of anastomotic leak remains unknown, and its incidence has not changed in decades. We demonstrate that the commensal bacterium Enterococcus faecalis contributes to the pathogenesis of anastomotic leak through its capacity to degrade collagen and to activate tissue matrix metalloproteinase 9 (MMP9) in host intestinal tissues. We demonstrate in rats that leaking anastomotic tissues were colonized by E. faecalis strains that showed an increased collagen-degrading activity and also an increased ability to activate host MMP9, both of which contributed to anastomotic leakage. We demonstrate that the E. faecalis genes gelE and sprE were required for E. faecalis–mediated MMP9 activation. Either elimination of E. faecalis strains through direct topical antibiotics applied to rat intestinal tissues or pharmacological suppression of intestinal MMP9 activation prevented anastomotic leak in rats. In contrast, the standard recommended intravenous antibiotics used in patients undergoing colorectal surgery did not eliminate E. faecalis at anastomotic tissues nor did they prevent leak in our rat model. Finally, we show in humans undergoing colon surgery and treated with the standard recommended intravenous antibiotics that their anastomotic tissues still contained E. faecalis and other bacterial strains with collagen-degrading/MMP9-activating activity. We suggest that intestinal microbes with the capacity to produce collagenases and to activate host metalloproteinase MMP9 may break down collagen in the intestinal tissue contributing to anastomotic leak.

INTRODUCTION
The most devastating complication after removal of an intestinal segment (resection) and its reconstruction (anastomosis) is an anastomotic leak. The clinical manifestations of an anastomotic leak range from abdominal pain with fever to septic shock. In its extreme form, anastomotic leak can cause peritonitis, sepsis, and even death. Leaks are particularly prevalent in patients undergoing surgery in high-risk regions of the intestine such as the rectum and esophagus (1). In the distal colon and rectal area, the anastomotic leak rate can be excessive (30 to 40%), forcing surgeons to routinely perform a protective diverting stoma (ileostomy and colostomy) to lessen the clinical effects of intestinal content spillage (1). This practice requires a second operation to close the diverting stoma, which itself carries significant morbidity and includes the risk of an anastomotic leak. Consequently, many patients and surgeons elect to leave the stoma as a permanent solution to avoid a second high-risk surgery. Given this, there is little motivation among surgeons to eliminate the routine use of a diverting stoma in lower colorectal surgery because they have accepted that the actual causes of anastomotic leaks remain unknown and hence they are not preventable.

That intestinal microbes play a key causative role in the pathogenesis of anastomotic leak has been suggested for over 60 years. The most direct evidence was first reported in 1955 by Cohn and Rives, who demonstrated that repeated direct topical application of antibiotics onto anastomotic tissues accelerated healing and prevented leak in dogs undergoing colon resection and anastomosis when the supplying blood vessels were divided in a manner that resulted in gross ischemia (2). Remarkably, despite the grossly visible presence of ischemia, direct topical application of antibiotics not only prevented anastomotic leak but also completely reversed the ischemia. Although from 1955 to 1984 oral antibiotics were introduced as a routine part of the preparation of gastrointestinal surgery, they were soon replaced by intravenous antibiotics owing primarily to the convenience of administration and the perception that they were equally efficacious in decontaminating anastomotic tissues of potentially offending pathogens (3, 4). Despite numerous studies demonstrating the benefit of adding oral antibiotics before gastrointestinal surgery to prevent infection and anastomotic leak, most surgeons do not routinely administer oral antibiotics in preparation for gastrointestinal surgery (3, 4). We have recently published work that redresses the role of bacteria in anastomotic leak in a more molecular context (5). We reported that exposure of anastomotic tissues to pathogenic bacteria such as Pseudomonas aeruginosa resulted in selection of a more virulent phenotype characterized by high collagen-degrading activity, which was associated with anastomotic leak (5). We hypothesized that the capacity of intestinal bacteria to degrade collagen may be an important mechanism underlying anastomotic leak. To identify additional and, perhaps, more common bacteria with collagen-degrading activity that might colonize anastomotic tissues after surgery, we next examined the microflora associated with anastomotic tissues using 16S rRNA (ribosomal RNA) and PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) analyses in...
rats after anastomotic surgery (6). Results demonstrated a 500-fold increase in the relative abundance of the genus Enterococcus at the anastomotic site. The PICTRUST functional analysis (7) predicted the predominance of several bacterial virulence factors, one of which, coccolysin [GelE (gelatinase)], is responsible for collagen/gelatin degradation (8, 9). These findings, coupled with the observations that bacterial-derived collagenases are known to play an important role in a variety of intestinal disorders such as those involving inflammation and necrosis, led us to explore the role of Enterococcus in anastomotic leak (10–15).

Here, we demonstrate that among commensal microbiota, Enterococcus faecalis strains with enhanced collagen-degrading activity and the capacity to activate intestinal tissue matrix metalloproteinase 9 (MMP9) contribute to the pathogenesis of anastomotic leak.

RESULTS

Clinical outcome of rats after surgery demonstrates that while intestinal devascularization is associated with leak, it does not cause gross ischemia

We created an anastomotic leak model in rats by performing a 1-cm colon resection (at the peritoneal reflection) and primary anastomosis, followed by devascularization of a 2-cm segment of blood supply adjacent to the anastomosis (Fig. 1A). All rats that survived did well after surgery and were healthy-appearing at the time of sacrifice [postoperative day 6 (POD6)] as judged by their feeding pattern, movement in the cage, and stool passage. There were occasional anesthesia-related deaths (<5) within 24 hours. In these cases, additional rats were operated on and added to the groups to achieve equal numbers in each group. All rats were sacrificed on POD6 and underwent laparotomy for gross inspection of the anastomosis and detection of leakage. No leaks were observed in rats subjected to anastomosis alone (Anast). In contrast, 50% of rats subjected to anastomosis with devascularization (Anast + Dvasc) developed an anastomotic leak (Fig. 1B). The devascularization procedure did not cause grossly visible ischemia (Fig. S1A). To confirm this, we performed endoscopy above and below the anastomotic suture line in selected rats that did not demonstrate any gross areas of ischemia (Fig. S1B). Finally, in a separate group of rats, we used confocal laser endomicroscopy after fluorophore injection to examine the microscopic blood supply of the colon just below the anastomosis and demonstrated that the segmental devascularization did not grossly interrupt the blood flow.
Collagen-degrading activity of *E. faecalis* is associated with anastomotic leak

We measured collagen-degrading activity in whole bacterial communities recovered by swabbing anastomotic tissues at the time of sacrifice and performing assays for collagen degradation capabilities on the entire recovered microbiota. Results demonstrated that the collagen-degrading activity of microbes enabled discrimination between leaking versus nonleaking anastomotic sites; microbes with increased collagen-degrading activities predominated in leaking anastomotic tissues (Fig. 1C'). Histologic examination demonstrated visible collagen depletion in rats subjected to anastomosis and devascularization that had anastomotic leakage compared to rats without anastomotic leakage (Fig. 1D). Similarly, collagen content was significantly different between rats with leaking versus nonleaking anastomoses (Fig. 1E'; *P* < 0.05, Student's *t* test), whereas there were no overall differences in collagen content between nondevascularized and devascularized anastomoses (Fig. 1E). There was a clear association between enhanced collagen-degrading activity (Fig. 1C') and attenuated collagen content within the anastomotic leak group (Anast + Dvasc) (Fig. 1E'). In addition, measurements of collagen-degrading activity of whole-tissue extracts demonstrated a significant difference between leaking versus healed anastomotic tissues within the group of rats undergoing anastomosis and devascularization (fig. S2).

To identify the bacterial strains with high collagen-degrading activity, we cultured bacteria recovered from anastomotic tissues by swabbing, *E. faecalis* and *Proteus mirabilis*, members of the commensal microbiota, had the highest collagen-degrading activity among all isolates. *P. mirabilis* strains uniformly had high activity, whereas isolates of *E. faecalis* showed both high and low collagen-degrading activities (Fig. 1F). Notably, the mean collagen-degrading activity of *E. faecalis* strains was significantly increased (*P* < 0.01) in rats with anastomotic leakage compared to those without (Fig. 1G). No such correlation was observed with *P. mirabilis* because all isolates were high collagenase producers. Therefore, we decided to focus on *E. faecalis* because its collagen-degrading activity might be a potential discriminator between anastomotic leakage and nonleakage. We called low collagenase-producing *E. faecalis* strains E1 and high collagenase producers E2.

Time-dependent measurements of collagen-degrading activity revealed that E2 strains started to degrade collagen significantly earlier (*P* < 0.01) and at a higher level compared to E1 strains (fig. S3). To determine other phenotypic differences between E1 and E2 strains, we screened these strains in *Caenorhabditis elegans*–killing assays and observed that the E2 strain was more virulent than E1 (fig. S4). This finding agreed with previous work showing that collagenase plays a key role in the virulence and lethality of *E. faecalis* (16–18). Next, to define the role of collagen-degrading activity among *E. faecalis* strains in the pathogenesis of anastomotic leak, we selected a pair of strains from our isolates from rat anastomoses that were low (E1) and high collagenase producers (E2).

We introduced live cultures of E1 and E2 (10^6 colony-forming units in 0.1 ml of solution) via enema into rats after colon resection and anastomosis without devascularization and monitored the development of anastomotic leak after sacrifice on POD6 as in previous experiments. Rats were administered systemic cefoxitin and mucolytics (systemic atropine, topical N-acetyl cysteine) to mimic the clinical preparation of the colon for surgery that involves elimination of the normal flora with antibiotics and depletion of mucosal mucus with a purgative bowel preparation (19). Results demonstrated that E2, but not E1, caused anastomotic leak associated with depletion of intestinal collagen (Fig. 1, H and I). Further characterization of E1 and E2 demonstrated that E2 had a greater capacity to degrade gelatin and collagen I (but not collagen IV) (Fig. 1J). We also observed this capacity to degrade collagen I but not collagen IV with the well-characterized human isolate *E. faecalis* V583 (Fig. 1J). Purified GelE from *E. faecalis*, a zinc metalloproteinase, has been shown to cleave numerous substrates including collagen fragments (20). GelE encoded by the *gelE* gene is cotranscribed in a quorum sensing–dependent manner with *sprE* (extracellular serine protease) that encodes the serine protease SprE (21). We therefore used mutants derived from *E. faecalis* V583 including ∆*gelE*, ∆*sprE*, and ∆*gelE*∆*sprE* and observed that both GelE and SprE are involved in the degradation of collagen I (Fig. 1J). Complementation of ∆*gelE* with *gelE* and ∆*gelE*∆*sprE* with *gelE*+*sprE* led to higher collagenase activity compared to the wild-type strain. However, complementation of ∆*sprE* with *sprE* did not lead to increased collagen-degrading activity as compared to the noncomplemented mutant (fig. S5). As others have suggested, it is possible that a critical balance between GelE and SprE is required for their proper function (22). The collagen-degrading activity of E2 and the *E. faecalis* V583 mutant was significantly suppressed by the zinc chelator 1,10-phenanthroline when tested using either gelatin or collagen I as a substrate (fig. S6A), confirming the involvement of a zinc metalloproteinase. However, suppression of gelatin and collagen I degradation by 1,10-phenanthroline was not complete.

To define the role of GelE and SprE in anastomotic leak in our rat model, we introduced the V583 derivative double mutant ∆*gelE*∆*sprE* or ∆*gelE*∆*sprE* complemented with *gelE* plus *sprE* via enema similar to experiments with E1 and E2. Rats were sacrificed on POD6, and anastomotic leak rates were determined by gross inspection. Results demonstrated a significant difference in leak rates between the two groups (*P* < 0.0001), with ∆*gelE*∆*sprE* causing a low rate of leakage of 5% (1 of 20 rats) and ∆*gelE*∆*sprE* complemented with *gelE*+*sprE* causing a high rate of leakage of 70% (12 of 17 rats) (Fig. 1K).

**Activation and cleavage of intestinal MMP9 are associated with anastomotic leak and can be induced by *E. faecalis* in a GeLE/SprE-dependent manner**

In addition to bacterial collagen–degrading proteases, host extracellular matrix–degrading enzymes can also contribute to anastomotic leakage. Among them, intestinal MMP9 has been shown to play an important role in the pathogenesis of anastomotic leak (23). Normally, as part of the response to tissue injury, zinc–dependent MMP9 degrades components of the extracellular matrix including collagen for remodeling and wound healing. Excessive activation of MMP9 could tip the balance during anastomotic healing such that collagen degradation results in leakage instead of healing. Other inflammatory signals could be also triggered in addition to, or as a result of, MMP9 activation (24) and could participate in excessive inflammation at the site of the anastomosis. Zymography analysis of tissue extracts demonstrated higher MMP9 activity in leaking versus healed anastomoses among groups of rats subjected to anastomosis plus devascularization (fig. S7). On the basis of our observation that leaking...
anastomotic tissues were colonized by high collagenase-producing *E. faecalis*, we next hypothesized that *E. faecalis* may activate MMP9 in anastomotic tissues. It has been previously demonstrated that certain bacteria can activate tissue MMP9 (25, 26); however, this does not seem to have been demonstrated for *E. faecalis* to our knowledge. To test this, we incubated rat colon explants isolated from normal healthy rats with live bacterial cells and cell-free supernatants of E1 and E2 strains. Zymography, an electrophoretic method for measuring proteolytic activity, was performed on tissue extracts and revealed that the *E. faecalis* E2 strain induced cleavage of host proteases, resulting in the appearance of an 86-kD band (Fig. 2A). To confirm that this band corresponded to MMP9, we performed Western blot analysis using anti-MMP9 antibodies. Results confirmed that the band was MMP9 (Fig. 2B). To examine direct cleavage of MMP9 by the *E. faecalis* isolates, we used recombinant pro-MMP9 (r-MMP9) as a substrate. Results demonstrated that only the E2 strain was able to activate pro-MMP9 (Fig. 2C). We hypothesized that either GelE or SprE is involved in the proteolytic cleavage of pro-MMP9. To test this, we incubated r-MMP9 with the human *E. faecalis* isolate V583 and its derivative mutants ΔgelE and ΔsprE (8). Zymography demonstrated that the GelE-deficient mutant failed to cleave MMP9, whereas the SprE-deficient mutant cleaved MMP9, leading us to conclude that

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**Fig. 2.** *E. faecalis* strains with high collagen-degrading activity also activate host intestinal MMP9. (A) Zymography (an electrophoretic technique to determine proteolytic activity indicated by Coomassie staining) of normal rat intestinal tissue exposed to supernatants obtained from high collagenase-producing E2 strains of *E. faecalis* reveals host protease cleavage with the appearance of an 86-kD band. The three bands on the left side (tissue extracts) represent proteins extracted from tissues; the three bands in the middle (tissue-secreted products) represent proteins secreted by tissues; the two bands on the right (no tissue) show negative results for *E. faecalis* supernatants not exposed to tissues. The proteolytic activity appears as clear bands over a deep blue background after Coomassie staining. Vehicle consisted of Todd-Hewitt (TH) broth in which *E. faecalis* is usually grown. (B) Western blots demonstrate that E2 supernatant cleaves MMP9 to its active form. (C and D) Zymography demonstrates the ability of E2 to cleave human recombinant pro-MMP9 (r-MMP9). APMA (p-aminophenylmercuric acetate), an agent known to induce MMP9 cleavage, was used as a positive control. (D) The effect of the human laboratory strain of *E. faecalis* (V583) and its gelE and sprE mutants on human r-MMP9 cleavage is shown. (E and E′) Zymography (E) and Western blot (E′) analyses of MMP9 activation in a murine macrophage cell line J774.1 incubated with *E. faecalis* V583 and its derivative mutants ΔgelE, ΔsprE, and ΔgelEΔsprE. (F) Zymography analysis of MMP9 activation in the murine macrophage cell line J774.1 incubated with *E. faecalis* strains E1 and E2 and *P. mirabilis*. (G to L) Effects of pharmacologic inhibition of MMP9 in rat tissues after colon resection, anastomosis, and devascularization: intestinal tissue MMP9 by Western blot (G), zymography (H), band densities of Western blot (I), anastomotic leak rate (J), collagen-degrading activity (K), and collagen content (L) (n = 3; P < 0.01, Student’s t test). DMSO, dimethyl sulfoxide.
GelE was responsible for the activation of MMP9 (Fig. 2D). To verify the involvement of GelE in the activation of MMP9, we performed ex vivo experiments using the mouse peritoneal macrophage cell line J774. J774 macrophages were incubated with V583 and its derivative mutants ΔgelE, ΔsprE, and ΔgelEΔsprE, and MMP9 was measured by both zymography (Fig. 2E) and Western blot (Fig. 2E′). Surprisingly, we observed the opposite effect with the mutants whereby SprE, not GelE, appeared to be required for MMP9 cleavage (Fig. 2, E and E′). We speculated that the discrepancy between human recombinant GelE, which appeared to be required for MMP9 cleavage (Fig. 2E′), and the opposite effect with the mutants whereby SprE, not GelE, restored MMP9 activation and increased the formation of the truncated form of MMP9 (fig. S8). Complementation of the double mutant ΔgelEΔsprE with gelE+sprE also restored MMP9 activation (fig. S8). Surprisingly, complementation of ΔgelE with gelE enhanced MMP9 activation, suggesting that GelE is important for E. faecalis activation of MMP9 (fig. S8). However, we cannot invoke a specific and independent role for either GelE or SprE in MMP9 activation.

Finally, given that P. mirabilis expressed high collagen-degrading activity, we measured its MMP9 cleavage activity using the macrophage MMP9 assay. As seen in Fig. 2F, P. mirabilis did not cleave MMP9, consistent with its lack of association with anastomotic leak. Thus, pathogens with the dual capacity to degrade collagen and cleave MMP9, such as E. faecalis, appear to be associated with anastomotic leak in this rat model.

To define the role of MMP9, we performed reiterative studies in separate groups of rats using a specific MMP9 inhibitor (MMP9 inhibitor I, Calbiochem, catalog #444278-500UG). All rats underwent colon resection + anastomosis + devascularization with and without the MMP9 inhibitor. Results demonstrated that pharmacologic inhibition of MMP9 suppressed MMP9 production in anastomotic tissues (Fig. 2, G to I) and prevented anastomotic leak (Fig. 2J) in association with suppression of the collagen-degrading activity of whole colonizing microbiota (Fig. 2K) and preservation of intestinal collagen content (Fig. 2L).

We next determined the influence of the intestinal microbiota on key inflammatory mediators present in intestinal tissues in rats 6 days after resection, anastomosis, and segmental devascularization. These mediators included HIF-1α (hypoxia-inducible factor–1α), iNOS (inducible nitric oxide synthase), and MPO (myeloperoxidase) in addition to MMP9 activation (Fig. 3). We directly applied a triple antibiotic solution (ciprofloxacin, metronidazole, and neomycin) to anastomotic tissues via enema both immediately after surgical anastomotic construction and again on POD1. Rats were then treated with and without antibiotic enemas and assigned to the following groups: (i) sham operation; (ii) devascularization alone (Dvasc)—that is, no resection or anastomosis; (iii) resection + anastomosis without devascularization (Anast); and (iv) resection + anastomosis + devascularization (Anast + Dvasc). All rats were sacrificed on POD6, and proteins were extracted from colon or anastomotic segments and analyzed by Western blot for MMP9, HIF-1α, iNOS, and MPO. Data demonstrated that the MMP9 expression in tissues exposed to both anastomosis and devascularization was attenuated when rats were exposed to antibiotics (Fig. 3, A and B, and fig. S9). Zymography confirmed that the highest level of MMP9 activation was in the Anast + Dvasc group and that this increase was attenuated by topical antibiotics (Fig. 3C). HIF-1α expression was similarly induced by devascularization alone or anastomosis alone compared to the untreated tissues. A clear synergistic effect on inflammatory mediators was seen when anastomotic construction was combined with devascularization, an effect that could be markedly attenuated when anastomotic tissues were exposed to the triple topical antibiotics. This synergism was also observed for iNOS and MPO, where topical antibiotic treatment attenuated expression of both inflammatory markers. Together, these results indicate that the intestinal microbiota contributes to the amplification of MMP9, HIF-1α, and inflammation during anastomotic surgery.

**Fig. 3. Antibiotic treatment of rats with anastomosis plus devascularization.** Antibiotic treatment (Abx) of rats with anastomosis plus devascularization (Anast + Dvasc) attenuated MMP9 activation and expression of HIF-1α, iNOS, and MPO. (A) Western blot analyses of rat tissues subjected to devascularization (Dvasc), anastomosis (Anast), or Anast + Dvasc, in the presence or absence of direct topical application of ciprofloxacin, metronidazole, and neomycin (Topical Abx) via enema. Experiments were performed on three rats per group; displayed immunoblot is representative of all results. (B) The evaluation of band intensities using ImageJ software demonstrated the abundance of the active form of MMP9 in the rat group Anast + Dvasc and loss of active MMP9 after topical application of antibiotics. n = 3; *P < 0.01, Student’s t test. (C) Zymography analysis confirmed the abundance of the active form of MMP9 in the Anast + Dvasc rat group and loss of the active form of MMP9 by topical application of antibiotics.

We next compared the topical antibiotic regimen with parenteral cefoxitin, the most common antibiotic used as a single agent for prophylaxis in colon surgery as recommended by the Center for Medical Services Surgical Quality Improvement Project (SCIP) (3). It has been well documented that cefoxitin and related cephalosporins do not eliminate *E. faecalis*. *E. faecalis* has been shown to “bloom” in the intestine after a single parenteral dose of cefoxitin (28). As might be predicted, only the topical antibiotic regimen prevented anastomotic leak (Fig. 4A) in association with suppression of bacterial collagenase (Fig. 4B), preservation of intestinal collagen (Fig. 4C), and lower expression of intestinal MMP9 (Fig. 4D). High collagenase-producing strains of *E. faecalis* were recovered from anastomotic tissues in rats treated with cefoxitin (Fig. S11). No *E. faecalis* strains were recovered from rats treated with the topical antibiotic regimen administered via enema (Fig. S11).

**Bacterial strains present on human colon tissues at the time of anastomotic construction are capable of degrading collagen and activating MMP9**

We next determined whether human colon anastomotic segments harbored microbial organisms that expressed the “leak phenotype” defined by their ability to degrade collagen and activate MMP9. Eleven consecutive patients undergoing colon surgery were studied under Institutional Review Board (IRB) protocol approved by the University of Chicago. During surgery, once the colon segment was resected, the distal and proximal ends were immediately swabbed for microbial analysis. Swabs were used to isolate DNA for 16S rRNA analysis and, in addition, were cultured to test individual growing organisms for collagen-degrading activity and their ability to cleave human r-MMP9. All 11 patients received intravenous cefoxitin prophylaxis according to the SCIP recommendations, and all recovered from surgery and were discharged home (3). One patient (patient #10) was readmitted because of complications. Of 64 cultured strains, only two species, *P. aeruginosa* and *E. faecalis*, showed significant collagenase activity (Fig. 5A) and cleaved MMP9 (Fig. 5B and C). 16S rRNA analysis demonstrated disruption of the normal microbial community structure and membership distribution in anastomotic tissues among the 11 patients. This is not surprising given that patients received purgative intestinal cleansing solutions and antibiotics before surgery. Normally, the dominant phyla in the colon are Bacteroidetes and Firmicutes, which are known to protect the underlying intestinal epithelium from invasion by pathogenic bacteria as well as to induce a health-promoting immune response. Under normal conditions, Proteobacteria represent less than 1% of the indigenous microbiota in the colon; however, when they predominate, they are often associated with intestinal pathology. Several of our patients displayed a reversal in the ratio of health-related Bacteroidetes to disease-related Proteobacteria (Fig. 4, A and B), the opposite of what would normally be expected. Of particular note was the ratio of Proteobacteria to Bacteroidetes in the swab from the distal end of the colon of patient #10 that showed a ratio of Proteobacteria to Bacteroidetes of 3:1, indicative of a highly imbalanced microbiota (Fig. 6, A and C). In this patient, the phylum Proteobacteria (with an abundance of 50.12%) comprised mainly the class Gammaproteobacteria (44.47%) (Fig. 6, B and D) and the family Enterobacteriaceae (43.1%), which encompasses the greatest number of human pathogens. Thus, among all 11 patients, patient #10 had the most pronounced reversal of the ratio of Bacteroidetes to Proteobacteria (3:1) and was culture-positive with a collagenolytic *P. aeruginosa* strain that cleaved MMP9. This patient was discharged after surgery and then readmitted for an intestinal obstruction and a suspected abdominal abscess. Subsequently, the patient developed a clinical course characteristic of an anastomotic leak including small-bowel obstruction up to the point of the ileocolic anastomosis, perianastomatic inflammation and free air, and fluid collection. The patient was treated with percutaneous drainage, nasogastric tube decompression, and broad spectrum antibiotics. An anastomotic leak, however, was not confirmed with a contrast enema because this was a right hemicolecotomy, and a full-contrast enema or colonoscopy would have been risky and difficult. The patient’s course gradually resolved with conservative management alone. Finally, although 1 of the 11 patients harbored a collagenolytic *E. faecalis* strain, this patient did not develop a clinical leak, perhaps owing to less disruption in this patient’s indigenous microbiota.

**DISCUSSION**

Data from the present study offer the possibility that members of intestinal commensal microflora, such as *E. faecalis*, may contribute to anastomotic leak. Here, we show in rats that the current method of antibiotic prophylaxis used in humans (that is, systemic administration

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**Fig. 4. Topical antibiotic treatment applied to rat anastomotic tissues prevents leak.** Effect of intramuscular (IM) cefoxitin (systemic antibiotic treatment) versus direct topical antibiotic treatment with ciprofloxacin, flagyl, and neomycin via enema on POD 0 and 6. (A to C) Effects of systemic and topical antibiotic treatment on leak rate, collagen-degrading activity of whole microbial communities, and intestinal collagen content in rats that underwent surgical resection, anastomosis, and devascularization (*n* = 10; *P* < 0.01, Student’s *t* test). (D and D’) Effect of intramuscular cefoxitin versus topical antibiotic treatment on intestinal tissue MMP9 activity (*n* = 3; *P* < 0.05, Student’s *t* test).
of cefoxitin) fails to eliminate *E. faecalis* in association with anastomotic leak. A recent study examining the fecal microbiota of patients undergoing colorectal surgery for cancer before and after surgery demonstrated that, despite the use of both intravenous (that is, a second-generation cephalosporin) and oral antibiotics (kanamycin and metronidazole), *E. faecalis* and *P. aeruginosa* were among the most dominant pathogens remaining in feces after surgery with a several log-fold increase in their concentration and prevalence over the course of recovery and hospitalization (29). Others have also demonstrated the high prevalence of *Enterococcus* after colon surgery despite adequate antibiotic prophylaxis (30). Here, we found that *E. faecalis* contributes to anastomotic leak through its collagenolytic and MMP9-activating functions. Our rat model suggests that there may exist a leak phenotype among intestinal microbes that colonize anastomotic tissues. This phenotype appears to be a function of a given bacterial strain’s collagen-degrading activity and its ability to cleave MMP9 to its active, extracellular matrix−degrading form (31). MMPs, and in particular MMP9, are associated with inflammation (32, 33) in general and anastomotic leak (34–37) specifically. We show that *E. faecalis* can activate MMP9 in a manner that is dependent on GelE and SprE, both of which are present in *E. faecalis* and *P. aeruginosa* isolates from patients undergoing colon surgery despite adequate anti-biotic prophylaxis (30).
Although the prevalence of vancomycin-resistant strains of *E. faecalis* has been examined in light of our results, the role of this organism in anastomotic leak is unknown. Perhaps it should be considered in the screening of patients undergoing colorectal surgery. To better understand the role of anaerobic bacteria in anastomotic leak, we performed a microbial analysis of resected colon samples from 11 patients who underwent elective colon surgery. (Proteobacteria (disease-related) to Bacteroidetes (health-promoting) in the 11 patients (Pt) who underwent colorectal surgery.)

We plan to directly swab anastomotic tissues during the serial endoscopic surveillance of anastomotic leak over the entire course of healing (21 days). The observation that *E. faecalis* degrades collagen I and activates MMP9 to its active form suggests a dual mechanism by which *E. faecalis* might complicate anastomotic healing. Some evidence for this was provided by the observation that either bacterial elimination with topical antibiotic treatment or MMP9 inhibition prevented anastomotic leak. It is important to recognize, however, that parenteral cefoxitin did not kill *E. faecalis* in our rat model nor did it prevent leakage. It is noteworthy that the most commonly used antibiotics in colon surgery (that is, second- and third-generation cephalosporins) do not eliminate *E. faecalis* in the gut but in fact allow it to proliferate and predominate (28, 30, 49). Although many have advocated the use of non-absorbable oral antibiotics in colon surgery including the use of a combination of oral kanamycin and erythromycin (50), whether they actually eliminate collagen-degrading *E. faecalis* and other such organisms from anastomotic tissues remains unknown.

Our results from patients undergoing colon surgery were not powered to determine the positive predictive value of the collagenase/MMP9-cleaving phenotype of bacterial strains on anastomotic leak. However, our rat data suggest that it may be worth testing the predictive value of the collagenase/MMP9-cleaving bacterial phenotype on anastomotic leak in a clinical trial of patients undergoing colorectal surgery. How the disruption of normal microbiota community structure due to prolonged illness and antibiotic treatment affects proliferation of bacteria expressing the leak phenotype remains to be defined. Our preliminary survey of 64 bacterial strains isolated from human anastomotic tissues demonstrated that only *P. aeruginosa* and *E. faecalis* expressed the collagen-degrading/MMP9-cleaving phenotype. However, the background microbial composition in the patient harboring *P. aeruginosa* was highly disrupted, and this was the only patient with a suspected anastomotic leak. Given these findings, we are currently planning a clinical trial in which we will use serial endoscopic surveillance of anastomotic tissues after lower colon surgery over the entire course of healing (21 days). We plan to directly swab anastomotic tissues during the serial endoscopic exam and identify tissue-associated microbial composition and phenotype; the microbial community structure, phenotype, and species that develop over time will be investigated.

There are some limitations to our study. First, we did not culture obligate anaerobes at anastomotic sites, which may play a contributory role in anastomotic leak. It is known that anaerobic bacteria such as *E. faecalis* and *P. aeruginosa* might complicate anastomotic leakage. Some evidence for this was provided by the observation that either bacterial elimination with topical antibiotic treatment or MMP9 inhibition prevented anastomotic leak. It is important to recognize, however, that parenteral cefoxitin did not kill *E. faecalis* in our rat model nor did it prevent leakage. It is noteworthy that the most commonly used antibiotics in colon surgery (that is, second- and third-generation cephalosporins) do not eliminate *E. faecalis* in the gut but in fact allow it to proliferate and predominate (28, 30, 49). Although many have advocated the use of non-absorbable oral antibiotics in colon surgery including the use of a combination of oral kanamycin and erythromycin (50), whether they actually eliminate collagen-degrading *E. faecalis* and other such organisms from anastomotic tissues remains unknown.

Our results from patients undergoing colon surgery were not powered to determine the positive predictive value of the collagenase/MMP9-cleaving phenotype of bacterial strains on anastomotic leak. However, our rat data suggest that it may be worth testing the predictive value of the collagenase/MMP9-cleaving bacterial phenotype on anastomotic leak in a clinical trial of patients undergoing colorectal surgery. How the disruption of normal microbiota community structure due to prolonged illness and antibiotic treatment affects proliferation of bacteria expressing the leak phenotype remains to be defined. Our preliminary survey of 64 bacterial strains isolated from human anastomotic tissues demonstrated that only *P. aeruginosa* and *E. faecalis* expressed the collagen-digesting phenotype. However, the background microbial composition in the patient harboring *P. aeruginosa* was highly disrupted, and this was the only patient with a suspected anastomotic leak. Given these findings, we are currently planning a clinical trial in which we will use serial endoscopic surveillance of anastomotic tissues after lower colon surgery over the entire course of healing (21 days). We plan to directly swab anastomotic tissues during the serial endoscopic exam and identify tissue-associated microbial composition and phenotype; the microbial community structure, phenotype, and species that develop over time will be investigated.

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**Fig. 6. Microbial composition of resected intestinal specimens from 11 patients undergoing elective colon surgery.** (A and B) 16S rRNA analysis at the phylum (A) and class (B) levels of the microbial community composition of the proximal (p) and distal (d) ends of resected colon samples from 11 patients. (C) Ratio of Proteobacteria (disease-related) to Bacteroidetes (health-promoting) in the 11 patients (Pt) who underwent elective colon surgery. (D) Gammaproteobacteria was the most abundant class in patient #10.
Porphyromonas (Bacteroides) gingivalis (51), Bacteroides fragilis (52), and Clostridium histolyticum (53) produce collagenases. Application of metagenomics/metatranscriptomics complemented with proteomics and metabolomics would be an ideal approach to more completely define the roles that microbial community structure, membership, and function play in the pathogenesis of anastomotic leak. Although we attempted to do this in our human samples using 16S rRNA analysis, our clinical study was not set up nor powered to fully use this approach. We are planning a clinical trial in which the microbes present on anastomotic tissues can be comprehensively analyzed over the full course of healing and their role in leak determined. Second, our rat model has certain limitations in terms of recapitulating the anastomotic leak that occurs in humans. Most anastomotic leaks are discovered on computed tomography (CT) scans and show up as inflammation or fluid collections adjacent to the anastomotic site. Leaks are not directly visualized during surgery because most patients do not undergo reoperation. The precise definition of anastomotic leak is thus debated, and both contrast enema and CT scanning are known to carry significant false-negative rates (54). Our approach was to directly examine all anastomoses in rats for evidence of dense adhesion, dehiscence, inflammation, and purulence that clearly contrasted with a healthy non-inflamed anastomosis without adhesions. We believe that this assessment is in line with what might be seen clinically in patients.

In summary, we present evidence that microbial pathogenesis may underlie anastomotic leak and may involve pathogens, such as E. faecalis, that break down collagen and cleave host tissue MMP9.

MATERIALS AND METHODS

Study design
The rationale for the study was based on performing a low colon anastomosis at the junction of the rectum and colon where the highest incidence of anastomotic leak occurs clinically. On the basis of preliminary data, power analyses were carried out to determine the number of animals in each group required for statistical significance. Rules for stopping the experiment were to be enacted if rats appeared moribund or in any distress whereby they were sacrificed immediately and excluded from results. However, this was not the case for any animals in the study. There were five anesthetic deaths that occurred shortly after the initial surgery (within 6 hours). These animals were excluded from the study analysis. Devascularization was performed to divide the blood vessels supplying the removed segment of colon to mimic the clinical performance of intestinal surgery. The overall objectives of the study were to develop a model in which a significant (that is, 50%) leak rate was observed to analyze tissues and determine the molecular mechanisms of anastomotic leak. Standard measurements (that is, histology and gross observation) were used to determine leak, and Western blotting, microbial analyses, and zymography were used to determine molecular markers of leak. Randomization of rats to the various groups was performed by randomly picking rats housed in groups of five to one group or another. All analyses, including the gross determination of leak, were performed in a blinded fashion where the analysis was performed by a person blinded to treatment. Sample size and replicates are included in the figure legends. End point selection was based on using gross inspection of the anastomotic sites on POD6 as the hard end point of the study given that it is a common time at which an intestinal anastomosis leaks clinically.

Rat model of colorectal anastomosis
Adult, male Wistar rats (250 to 300 g) (Charles River Laboratories) were used for all experiments. Rats underwent general anesthesia and laparotomy with segmental colon resection at the peritoneal reflection and primary colorectal anastomosis. Devascularization of the anastomotic segment was carried out by dividing the feeding blood vessel 1 cm above and below the anastomotic suture line (55). Full details of the anastomotic surgery and other related procedures such as involving treatment with antibiotics and MMP9 inhibitors, anastomotic leak evaluation, blood vessel visualization, and intestinal collagen content are outlined in the Supplementary Materials.

Bacterial strains
Bacterial strains isolated in this study were used in defined experiments for collagen-degrading activity and MMP9 activation assays. Among them, E. faecalis E1 and E. faecalis E2 were additionally used in rat model. E. faecalis V583 and its derivative mutants ΔgelE, ΔsprE, ΔgelEΔsprE, and complemented mutants ΔgelE/gelE, ΔsprE/sprE, and ΔgelEΔsprE/gelE+sprE were provided by L.E.H. (56). All strains were stored in 10% glycerol stock at ~80°C. Only cells freshly plated from stock were used in experiments. Cells from stock were plated onto tryptic soy broth (TSB) plates, grown overnight at 37°C, and were further used as designed. For complemented mutants, spectinomycin (500 μg/ml) was added to TSB.

Microbial culture and anastomotic tissue analysis
In a separate experimental run, four groups of 10 rats each were randomly assigned to normal unoperated controls, devascularization alone (Dvasc), resection + anastomosis only (Anast), and resection, anastomosis, and devascularization (Dvasc + Anast). Colorectal anastomotic tissues were then inspected for leak, opened, and swabbed for microbial analysis. Swabs were cultured on medium specific for Gram-negative and Gram-positive bacteria, and bacterial species were identified as previously described (57, 58). Tissues were collected and analyzed for MMP9 and inflammatory markers.

Enterococcal inoculation of colorectal anastomosis
To test the hypothesis that E. faecalis strains can cause anastomotic leak, we altered the animal model described above. As previously described, 1 hour before laparotomy, rats were given two 5-ml rectal enemas of atropine (0.2 mg/ml; Sigma-Aldrich) and N-acetyl-l-cysteine (20 mg/ml; Sigma-Aldrich), followed by two 5-ml rectal enemas of 0.9% NaCl to remove the protective mucus layer (59). Additionally, to rid the intestine of normal flora, cefoxitin (50 mg/kg) was given via intramuscular injection 30 min before incision. After completion of the anastomosis, E. faecalis strains were intestinally inoculated via a 5-ml rectal enema of 1:100 diluted overnight culture in Todd-Hewitt broth (THB). As described above, the animals were sacrificed on POD6 to evaluate for anastomosis integrity.

Human studies
Under approval from the IRB at the University of Chicago and North Shore University Hospital IRB11-0481, 11 consecutive patients undergoing colon surgery were consented to participate in the study. When the colon sample was removed by the operating surgeon, the distal and proximal ends were immediately swabbed for 16S rRNA analysis and aerobic culture. In each case, whether the patient recovered and was discharged uneventfully or whether the patient
developed an anastomotic leak was determined by communicating with the operating surgery.

**Collagen-degrading activity**
Collagen-degrading activity was assessed as previously described (5) using an EnzChek Gelatinase/Collegenase Assay Kits (Molecular Probes). Details are outlined in Supplementary Materials.

**MMP9 activation assays**
We have used human recombinant MMP9, murine macrophage MMP9, and intestinal tissue MMP9 to test for the MMP9 activation. Detailed methods are displayed below.

**Recombinant MMP9**. Recombinant human proenzyme MMP9 (r-MMP9) (Calbiochem, catalog #PF038) was used as a substrate. The r-MMP9 was diluted to a final concentration of 1 µg/ml in the assay buffer [50 mM tris-HCl (pH 7.5), 10 mM CaCl2, and 0.05% Triton X-100]. Detection of MMP9-cleaving activity was performed as previously described (60). Briefly, 30 µl of bacterial culture grown in THB for 6 hours [~OD 600 nm = 1.0, which corresponded about 5 × 10^8 cells/ml as measured by plating of 10-fold dilutions] was incubated with 20 µl of r-MMP9 (1 µg/ml) for 2 hours. After centrifugation, 5 µl of the supernatant was mixed with an equal volume of 2x SDS loading buffers and subjected to zymography. The positive control included 4-aminophenylmercuric acetate (Sigma) known to induce autocleavage of MMP9 (61). Fresh 100 mM stock of APMA in DMSO was prepared, and MMP9 was activated by adding APMA to a final concentration of 1 mM, followed by incubation at 37°C for 3 hours.

**Murine macrophage MMP9**. The murine macrophage cell line (J774) was cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum. The macrophage cells were harvested and resuspended in DMEM to a concentration of 2 × 10^6 cells/ml. The cells were seeded on a 12-well plate, 2 ml for each well, incubated for 1 hour, and allowed to adhere to plate surface. E. faecalis strains were grown in THB to log phase, and density of bacterial cell suspensions (30 µl) were added to each well. After 4-hour co-incubation, supernatants were collected, clarified by centrifugation for 10 min at 10,000 g, and analyzed by zymography and Western blot.

**Intestinal tissue MMP9**. E. faecalis strains E1 and E2 were grown overnight in THB, followed by centrifugation (5000 rpm, 6 min) to separate bacterial cells and secreted fraction (supernatant). Bacterial pellet was then diluted in fresh THB to OD 600 nm = 0.2, and 500 µl of culture suspension was added to 100 µg of colon tissues. Similarly, 500 µl of filter-sterilized supernatant was added to 100 µg of colon tissues. After 4 hours of incubation at 37°C, colon tissues and their conditioned media were collected and subjected to zymography and Western blot.

**Western blot**
The resected colon tissues were weighted, immediately placed in liquid nitrogen, and kept at ~80°C. The ice-cold lysis buffer [50 mM tris-HCl (pH 7.4), 150 mM NaCl, 0.5% Triton X-100, and phenylmethylsulfonyl fluoride] was added to ~80 µg of grinded tissues (1:10; 80 µg of tissues/800 µl of buffer). After vortexing and 15-min incubation on ice, the lysates were centrifuged at 10,000 rpm for 15 min to remove the debris. The supernatants (15 µl that contained ~40 µg of protein) were boiled for 5 min with Laemmli sample buffer, electrophoresed through 10% SDS–polyacrylamide gels, and then transferred onto polyvinylidene difluoride membranes (Immobilon-P, Millipore). Ponceau staining was used as a loading control. The membranes were blocked in tris-buffered saline containing Tween 20 (pH 7.4) added with 4% nonfat dry milk (LabScientific) for 1 hour and incubated with primary anti-MMP9, iNOS (Abcam), HIF-1α, and MPO (Novus Biologicals) antibodies, followed by the corresponding horseradish peroxidase–coupled secondary antibody. The dilution of antibodies was performed as recommended by the vendors. The membranes were developed using ECL Western Blotting Detection Reagents (GE Healthcare). Densitometry analyses were performed using ImageJ software.

**Collagen-degrading activity in anastomotic tissues**
Tissue extracts from the above-described preparation were used to measure total collagen-degrading activity in anastomotic tissues. Tissue extracts containing 60 µg of total protein were suspended in buffer containing 50 mM tris-HCl, 0.15 M NaCl, 5 mM CaCl2, 1 µM ZnCl2 (pH 7.6), supplemented with 10 µl of fluorescent gelatin (1 mg/ml) in total volume of 200 µl in 96-well plates. Incubation was performed at 37°C under static conditions, and fluorescence was measured at 480/520 nm. All experiments were performed in triplicate. Zymography assay is described in details in the Supplementary Materials.

**C. elegans killing assay**
E. faecalis E1 and E2 strains were tested for virulence/killing capacity by assessing mortality of C. elegans fed on E. faecalis lawns (62). Full details are outlined in the Supplementary Materials.

**16S rRNA analysis of bacterial community structure**
Composition of the bacterial microflora was analyzed by 16S rRNA V4 ITAG amplicon sequencing analysis (63, 64) using DNA recovered from tissue swabs. Full details are outlined in the Supplementary Materials.

**Statistical analysis**
Statistical analysis was performed using SigmaPlot software. Student’s t tests were used when analyzing the differences between two means, whereas analysis of variance (ANOVA) with Bonferroni correction was used when more than two means were compared. Kaplan-Meier survival plot was analyzed using SPSS software. Significance was determined as a P value <0.05.

**SUPPLEMENTARY MATERIALS**
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Materials and Methods
Fig. S1. Images of selected rat colon segments after colon resection with and without segmental devascularization.
Fig. S2. Collagenolytic activity of tissue extracts from leaking anastomosis compared to nonleaking anastomosis.
Fig. S3. Time course–dependent degradation of gelatin by E. faecalis isolates E1 and E2.
Fig. S4. Kaplan-Meier survival curves of C. elegans feeding on E1 and E2 strains of E. faecalis.
Fig. S5. Gelatin degradation by complemented mutants.
Fig. S6. Effect of zinc-chelating compound 1,10-phenanthroline on collagen-degrading activity in E. faecalis E2 and V583 strains.
Fig. S7. Zymography of tissue extracts in leaking tissues.
Fig. S8. Zymography analysis of macrophage activation of MMP9 by wild-type V583, its mutants lacking GelE and SprE, and its complemented mutants ΔgelEΔsprE and ΔgelEΔsprE/pETV583.
Fig. S9. Western blot analyses to identify MMP9 in tissues of rats subjected to devascularization (Dvasc), anastomosis (Anast), or Anast + Dvasc, in the presence or absence of direct topical application of ciprofloxacin, metronidazole, and neomycin via enema.
Fig. S10. Effect of topical antibiotics on the activation of MMP9.
Fig. S11. Gelatin degradation by individual strains recovered from anastomotic tissues after intra-muscular injection of cefoxitin or topical application of ciprofloxacin, metronidazole, and neomycin.
REFERENCES AND NOTES


RESEARCH ARTICLE


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Collagen degradation and MMP9 activation by *Enterococcus faecalis* contribute to intestinal anastomotic leak
Benjamin D. Shogan *et al.*
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**Editor’s Summary**

**Can our gut microbes prevent wound healing?**

In a new study, Shogan *et al.* examined whether the bacterium *Enterococcus faecalis*, normally present in the intestine, contributes to anastomotic leak, the most feared complication after intestinal surgery. They demonstrated that intestinal *E. faecalis* can produce a tissue-destroying enzyme that affects the normal healing process by breaking down collagen, a protein that is critical to fully seal the intestine after its removal and reconnection. *E. faecalis* also activates a host gut enzyme, MMP9, further contributing to anastomotic leak. Finally, the authors demonstrated that the most common antibiotic used in intestinal surgery does not eliminate *E. faecalis* and thus does not prevent anastomotic leak.

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