Enterococcus faecalis Induces Aneuploidy and Tetraploidy in Colonic Epithelial Cells through a Bystander Effect

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Abstract

Intestinal commensals are potential important contributors to the etiology of sporadic colorectal cancer, but mechanisms by which bacteria can initiate tumors remain uncertain. Herein, we describe mechanisms that link Enterococcus faecalis, a bacterium known to produce extracellular superoxide, to the acute induction of chromosomal instability. Immortalized human and nontransformed murine colonic epithelial cells, along with a mouse colonic ligation model, were used to assess the effect of E. faecalis on genomic DNA stability and damage. We found that this human intestinal commensal generated aneuploidy, tetraploidy, and γH2AX foci in HCT116, RKO, and YAMC cells. In addition, direct exposure of E. faecalis to these cells induced a G2 cell cycle arrest. Similar observations were noted by exposing cells to E. faecalis–infected macrophages in a dual-chamber coculture system for detecting bystander effects. Manganese superoxide dismutase, catalase, and tocopherols attenuated, and caffeine and inhibitors of glutathione synthase exacerbated, the aneugenic effects and linked the redox-active phenotype of this intestinal commensal to potentially transforming events. These findings provide novel insights into mechanisms by which E. faecalis and intestinal commensals can contribute to cellular transformation and tumorigenesis. [Cancer Res 2008;68(23):9909–17]

Introduction

Intestinal commensals are potential, important contributors to the development of sporadic human colorectal cancer (CRC), but the identification of relevant microorganisms has been impeded by the complex ecology of the large intestine (1, 2). Several bacteria are definitively linked to gastrointestinal tumorigenesis; for example, Helicobacter pylori is considered a class I carcinogen for gastric cancers (3). However, in contrast to the stomach, wherein the microbiota only consists of Helicobacter spp., the human colon contains >400 species with >1011 colony-forming units (cfu) per gram of feces (4). At least 75% of these microorganisms cannot be cultivated using current techniques and, therefore, remain largely uncharacterized. These difficulties have made the epidemiologic approach to identifying CRC-associated microorganisms problematic. An alternate approach involves focusing on commensals that can confer long-term exposure to the host and are potentially mutagenic.

Enterococcus faecalis is a minority constituent of the intestinal microbiota that uniquely produces reactive oxygen species (ROS), including extracellular superoxide (5). This phenotype is rare among bacteria and is known to damage colonic epithelial DNA (6). E. faecalis also causes inflammation and CRC in interleukin-10 knockout mice (7, 8) and promotes chromosomal instability (CIN) in mammalian cells (9). CIN is the most common form of genomic instability in solid tumors. It consists of gains and losses of chromosome segments or whole chromosomes, along with inversions, translocations, and complex rearrangements in the genome sequence and structure (10). The dramatic and unstable changes in genomic content for cells with CIN are typified by aneuploidy. Recent mathematical modeling and experimental evidence suggest that CIN is an early event in tumorigenesis (11, 12). The origin of CIN, however, remains uncertain. We recently showed that E. faecalis promotes CIN through free radical mechanisms initiated by superoxide (9). This effect was linked to the expression of cyclooxygenase-2 (COX-2) in macrophages and production of clastogens (or chromosome-breaking factors) that diffuse into neighboring cells to damage DNA.

The production of clastogens by E. faecalis–infected macrophages is analogous to the induction of CIN through the radiation-induced bystander effect (13). This effect is generated when cells are activated by radiation to produce diffusible mediators that damage DNA in neighboring, unirradiated cells. The bystander effect has been shown to occur in vivo in congenic sex-mismatched bone marrow transplantation experiments (14). Although causative mediators are not characterized, the bystander effect is associated with COX-2 and DNA-damaging products from this inflammatory pathway may be important (9, 15, 16).

A macrophage-induced bystander effect is consistent with the potential contribution of innate immune cells to tumorigenesis through prostaglandins, cytokines, and chemokines that have transforming, proliferative, and metastatic consequences (17). In this study, we found that E. faecalis induced aneuploidy, tetraploidy, and DNA damage in colonic epithelial cells. Superoxide production was associated with DNA double-strand breaks (DSB), G2 arrest, and changes in chromosome number. These findings occurred through a bystander effect and provide a mechanism by which redox-active intestinal commensals may contribute to cellular transformation and tumorigenesis.

Materials and Methods

Cell lines and bacterial strains, HCT116 and RKO cells are near-diploid human colonic epithelial cell lines (43–45 and 46–49 chromosomes per cell, respectively) with defective mismatch repair (American Type Culture Collection [ATCC]; ref. 18). These cell lines were maintained in McCoy’s 5A

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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tetraploidy. Among healthy adults, *E. faecalis* varies in concentration in feces from $10^9$ to $10^7$ cfu g$^{-1}$ (22). This strain was grown in brain-heart infusion (Difco) at $37^\circ$C and washed in PBS with the multiplicity of infection (MOI) defined as bacterial cfu per cultured cell. Superoxide production was verified using ferricytochrome c reduction (5). MnSOD was purchased from Roche, and additional reagents were from Sigma, unless otherwise specified. Tocopherols (Tama Chemicals) and γ-carboxyethyl hydroxychroman (Encore Pharmaceuticals, Inc.) were provided by Kenneth Hensley.

For cells directly exposed to *E. faecalis*, a 2-h treatment was carried out in a medium without serum or antibiotics and restored to a complete medium after treatment. The pH of the medium during experiments was maintained in a physiologic range during coculture with *E. faecalis*. Antioxidants and inhibitors were added with *E. faecalis* in experiments, as specified. Experiments with the dual-chamber coculture system were carried out, as previously described (9).

Cytogenetics. Anaphase bridging was analyzed using cells grown on Cell Signaling Technology (BD Biosciences). After 24 h incubation, cells were fixed with 2% paraformaldehyde and stained with propidium iodide. Anaphases were scored by one of us (X.W.) using fluorescent microscopy according to previously described criteria (23).

Fluorescence-activated cell sorting analysis for aneuploidy and tetraploidy. Ploidy was determined by fluorescence-activated cell sorting (FACS) analysis, as previously described (24). In brief, cells were treated with $1 \times 10^5$ cfu mL$^{-1}$ *E. faecalis* for 2 h. After 48 h, cells were arrested in mitosis with colcemid, fixed (70% ethanol), and stained with phosphorlated (p-) histone H3 (Ser10) mouse monoclonal antibody (1:400 dilution, Cell Signaling Technology). Phosphorylation of histone H3 is a marker of chromosome condensation and mitosis. Cells were secondarily stained with the Alexa Fluor 488 (Invitrogen) fragment of goat anti-mouse IgG (H+L; 1:1,000 dilution, Invitrogen) and propidium iodide. Cell sorting was performed on FACS Calibur (BD Bioscience) using CellQuest software.

To investigate the induction of aneuploidy, *E. faecalis*-exposed HCT116 cells were double-stained with Hoechst 33342 and propidium iodide. Cells with DNA content of $>5N$ were sorted as single cells into wells onto 96-well plates (in Flux Cell Sorter, Cytotopia) and incubated for 2 wk. Colonies were enumerated and compared with untreated controls.

Cell cycle assay. Cells were synchronized in G, using 2 mM/L thymidine, followed by thymidine-free medium, and then thymidine-supplemented medium (25). Cells were treated with $1 \times 10^5$ cfu mL$^{-1}$ *E. faecalis* for 2 h (MOI = $1,000$) and fixed. For the dual-chamber system, uninfected and *E. faecalis*-infected macrophages were placed in the upper compartment (9). *E. faecalis* is readily phagocytosed by macrophages and survives as an intracellular pathogen (26). Synchronized cells in the lower compartment were used as targets, fixed, stained with propidium iodide, and sorted by FACS. Data were analyzed using ModFit LT (Verity Software House).

Western blotting. Protein extraction and blotting were carried out using enhanced chemiluminescence (GE Healthcare). Phosphorylated ataxia telangiectasia–mutated (ATM; Ser$^{1981}$), p-ATM and Rad53-related (ATR; Ser$^{296}$), p-Chk1 (Ser$^{332}$), p-Chk2 (Ser$^{345}$), and p-Cdkl (Thr$^{13}$) monoclonal antibodies and polyclonal anti–cyclin B1 antibody were purchased from Cell Signaling Technology. Murine anti–β-actin loading control was purchased from BioVision. Donkey anti-mouse IgG–horseradish peroxidase (HRP) conjugate (Santa Cruz Biotechnology) and goat anti-rabbit IgG–HRP conjugate (Zymed) were used as secondary antibodies.

In vivo DSBs caused by *E. faecalis*. To assess the DNA-damaging effect of *E. faecalis in vivo*, we developed a colonic ligation model (27). Colonies of wild-type BALB/c mice were surgically filled with 1.0 mL of PBS as a control (n = 3) or *E. faecalis* at $1 \times 10^5$ cfu mL$^{-1}$ in PBS (n = 3), and 5 mM/L α-glucose were added to initiate superoxide production (5). Colon contents were retained using a rectal suture and ligature at the proximal colon. Care was taken to preserve blood flow with no ischemia observed in the colons of 24 mice previously studied using this protocol (27). At 6 h postinoculation, colons were removed for analysis.

Wild-type C57BL/6 mice were subjected to 13 Gy whole body radiation in a $^{60}$Co irradiator. Animals were euthanized 4.5 h after irradiation, and colons were removed for analysis. Protocols were approved by animal study committees of the University of Oklahoma Health Sciences Center and Oklahoma City VA Medical Center.

Immunofluorescence and immunohistochemistry. HCT116 and RKO cells treated with *E. faecalis* were fixed with 2% paraformaldehyde and stained using p-histone H2AX (Ser$^{139}$) rabbit monoclonal antibody conjugated to Alexa Fluor 488 (Cell Signaling). DNA was counterstained with 4′-6-diamidino-2-phenylindole. γH2AX-positive cells were identified by laser scanning confocal microscopy (Leica Microsystems) or enumerated by FACS Calibur and analyzed using Summit software (v.4.3, DAKO).

Epitope retrieval of paraffin-embedded colon sections was done using a pressurized decloaking chamber (Biocare Medical, LLC). Sections were blocked using avidin/biotin blocking kit (Vector Lab), and peroxidase activity was quenched using 3% H$_2$O$_2$. After washing, slides were incubated in horse serum and bovine albumin and exposed to rabbit–anti-phospho-H2AX antibody (Cell Signaling). Slides were incubated in biotinylated donkey anti-rabbit secondary antibody (Jackson Immuno Research Lab) and SA-HRP (Dako), and chromogenic development was performed using 3′,3′-diaminobenzidine substrate (brown) with hematoxylin as the counterstain (Biocare Medical). Slides were scored for γH2AX in a blinded fashion by one of us (S.L.), counting only nuclear staining as positive.

Statistical analysis. Data were expressed as means with SDs. Experimental and control groups in ploidy assays were compared by $t$-test and the number of positive nuclei in colon biopsies by the Student's $t$ test. $P$ values of <0.05 were considered statistically significant.

Results

*E. faecalis* promotes anaphase bridging, aneuploidy, and tetraploidy. To assess mechanisms by which *E. faecalis* damages DNA and causes CIN, we initially evaluated the diploid and chromosomally stable HCT116 cell line for anaphase bridging after exposure to *E. faecalis*. Anaphase bridges appear as extended chromosomes between spindle poles during the separation of sister chromatids (23, 28). HCT116 cells showed numerous abnormal mitotic patterns after exposure to *E. faecalis*, including lagging chromosomes, anaphase bridges, and tripolar anaphases (Fig. 1A). We found a significant increase in the frequency of anaphase bridging after *E. faecalis* exposure (86 of 151 anaphases, 57%) compared with untreated control cells (35 of 194 anaphases, 18%; $P < 0.001$). The frequency of anaphase bridging was significantly reduced when MnSOD was added (48 of 158, 30%; $P < 0.001$), suggesting that superoxide promoted abnormal mitoses in these cells.

Because anaphase bridging can lead to aneuploidy (23), we next determined whether exposure to *E. faecalis* resulted in aneuploidy. Mitotic HCT116 cells were sorted using a monoclonal antibody to p-histone H3 and DNA content determined by FACS. Approximately, 10% of untreated mitotic HCT116 cells were nondiploid with a DNA content greater or less than 4N (Fig. 1B, left). After a 2-hour exposure to *E. faecalis*, the average frequency of nondiploid cells was significantly increased at 24, 48, 72, and 96 hours postexposure (Fig. 1B, middle, and C). This correlated with significant increases in both aneuploid and tetraploid cells (i.e., 8N DNA content for cells in G$_2$ or mitosis; 8.3% compared with 0.6% for untreated control; $P < 0.001$). Similarly, the percentage of cells in G$_2$ (i.e., nonmitotic) with 8N DNA content increased after
E. faecalis treatment compared with untreated controls (7.8% versus 0.0%; \( P < 0.001 \)). MnSOD was associated with a reduction in the number of mitotic aneuploid cells at 24, 48, and 72 hours (Fig. 1B, right, and C). Reductions in mitotic cells and cells in G2 with 8N DNA content were also observed (8.2 to 3.5% and 7.8 to 2.0%, respectively, at 48 hours). We next assessed whether tocopherols, scavengers of lipid radicals, protected against aneuploidy and tetraploidy. Addition of \( \alpha \)-tocopherol or \( \gamma \)-tocopherol significantly decreased the percentage of aneuploid cells compared with treatment with E. faecalis alone (Fig. 1D). Similar reductions were seen in the percentage of tetraploid cells. Notably, the tocopherol metabolite, \( \gamma \)-carboxyethyl hydroxychroman (\( \gamma \)-CEHC) decrease the frequency of aneuploidy and tetraploidy at 48 h compared with E. faecalis alone. *, \( P < 0.05 \); **, \( P < 0.01 \); ***, \( P < 0.001 \).

To determine whether E. faecalis generated long-term changes in chromosome number, we exposed HCT116 cells to E. faecalis and selected highly aneuploid or tetraploid cells by FACS (i.e., DNA content of >5N). Single cells were sorted, and after 2 weeks, only 20 of 288 cells (6.9%) grew compared with 51 of 288 untreated cells (17.7%; \( P < 0.001 \)). Growth of untreated nondiploid cells was similar to untreated diploid cells with 60 of 288 cells (20.8%) viable (\( P = 0.40 \)). E. faecalis–exposed clones were then somatically selected over 20 generations for heritable changes in DNA content. Reexamination by FACS detected only one tetraploid clone (Supplementary Fig. S2, right), with the remainder being diploid (Supplementary Fig. S2, middle). This implied that the acute induction of aneuploidy and tetraploidy by E. faecalis was unstable (or lethal) with few clones able to propagate only by reverting back to diploidy or, on occasion, maintaining tetraploidy.

E. faecalis activates DNA damage responses and G2 arrest. We noted that HCT116 and RKO cells developed megalocytosis after exposure to E. faecalis (data not shown). This effect was abrogated by catalase, but not MnSOD, and seemed similar to cytotoxic effects produced by bacterial cyclomodulin known to interfere with the eukaryotic cell cycle. Cyclomodulins, such as the cytolethal distending toxin, delay entry into mitosis by damaging...
host cell DNA (25). We, therefore, analyzed by FACS HCT116 and RKO cells after exposure to E. faecalis and found that both developed arrest at the G2-M transition (Fig. 2A).

We hypothesized that E. faecalis-induced G2-M arrest was due to DNA damage from ROS and, therefore, investigated phosphorylations involved in checkpoint activation. ATM and ATR are kinases that initiate DNA damage response signal transduction cascades (31). After DNA damage, ATM and/or ATR are activated by autophosphorylation and, in turn, phosphorylate Chk1 and/or Chk2 (32–34). Chk1/Chk2 further phosphorylates Cdc25C leading to sequestration in the cytoplasm and failure to activate Cdk1 (cyclin B–dependent kinase 1), a key regulator of mitotic progression. Phosphorylation of Cdk1 at Thr161 and dephosphorylation at Tyr15/Thr14 activates Cdk1, leading to progression from G2 to M (35). Western blots for p-ATM/p-Chk2 and p-ATR/p-Chk1 showed activation in HCT116 and RKO cells after exposure to E. faecalis (Fig. 2B and C). However, unlike HCT116 cells, RKO cells expressed p-ATR at baseline, suggesting constitutive activation, as has been reported for many colon cancers (36). Despite the G2-M arrest after exposure to E. faecalis (Fig. 2A), phosphorylation of Cdk1 at Tyr15 failed to increase (Fig. 2B and C), although doxorubicin, the positive control, produced this effect. Cyclin B1 levels, in contrast, were markedly increased in treated cells compared with controls.

To investigate whether the spindle-assembly checkpoint contributed to E. faecalis-induced cell cycle arrest, we determined the proportion of cells containing p-histone H3 as a marker of chromosome condensation and mitosis. There was, however, no change in the proportion of cells with p-histone H3 after exposure to E. faecalis (Supplementary Fig. S3A and B). Nocodazole-treated cells, in contrast, showed a 5-fold increase by 24 hours. This provided evidence that the spindle-assembly checkpoint was not activated by E. faecalis. Finally, caffeine, an ATM/ATR inhibitor (37), significantly increased the percentage of aneuploid and tetraploid cells after exposure to E. faecalis (Fig. 2D), and partially rescued cells from E. faecalis–induced G2 arrest (Supplementary Fig. S3C). In sum, these findings indicated that E. faecalis–induced G2 arrest did not occur through the canonical Cdk1 pathway, and the elucidation of underlying mechanisms are under investigation.

E. faecalis forms γH2AX foci. Cellular responses to DNA damage include DNA repair cascades and, for severe injury, apoptosis. In mammalian cells, γH2AX is rapidly formed by ATM at sites of DSBs. We identified γH2AX foci in the nuclei of cells after treatment with 1 μmol/L doxorubicin, an inducer of DSBs, and

Figure 2. E. faecalis activates DNA damage responses. A, cell cycle analysis shows E. faecalis induced a G2-M cell cycle arrest in HCT116 cells (left) and RKO cells (right). Top, untreated control; bottom, cells treated with 1 × 10^9 cfu mL−1 E. faecalis for 2 h. Syn, cells synchronized to G1 by double thymidine block. B and C, Western blots of p-ATM (Ser1981), p-ATR (Ser428), p-Chk1 (Ser345), p-Chk2 (Ser356), p-Cdk1 (Tyr15), and cyclin B1. ATM/ATR is activated by transient infection with E. faecalis in HCT116 cells (B) and RKO cells (C). Dephosphorylation of Cdk1 is evident at 48 h in both cell lines. Phosphorylation of Cdk1 at Tyr15 was seen in untreated cells for both cell lines (bottom). D, caffeine blocks ATM/ATR leading to increased frequency of aneuploidy and tetraploidy in E. faecalis–exposed HCT116 and RKO cells. Black columns, E. faecalis; white columns, E. faecalis with 2.5 mmol/L caffeine. ***, P < 0.001 compared with E. faecalis alone.
after exposure to *E. faecalis* (Fig. 3A and Supplementary Fig. S4). The proportion of γH2AX-positive cells was bimodal with peaks appearing immediately after exposure to *E. faecalis* and 48 hours later (Fig. 3B). The proportion of γH2AX-positive cells was also noted to increase with greater MOIs (Fig. 3C). Finally, caffeine decreased the percentage (±SD) of cells positive for γH2AX (*E. faecalis* versus *E. faecalis* with 2.5 mmol/L caffeine: 43.4 ± 0.5 versus 20.2 ± 1.8 for HCT116 cells and 79.2 ± 0.2 versus 67.4 ± 0.4 for RKO cells; *P* < 0.01 for both comparisons). These observations show that *E. faecalis* generates γH2AX foci and inhibition of ATM/ATR promotes *E. faecalis*-induced aneugenesis.

**ROS promote γH2AX foci.** The temporal pattern of γH2AX formation (Fig. 3B) suggested rapid DNA damage (38). To confirm that ROS generated γH2AX foci and, presumably, DSBs, MnSOD and catalase were added to cells exposed to *E. faecalis*. Compared with *E. faecalis* alone, MnSOD partially protected against γH2AX formation in HCT116 cells at 0 h (top left), but not 24 h (top right) after exposure to *E. faecalis*. In contrast, MnSOD has no effect on the proportion of γH2AX-positive RKO cells at 0 h (bottom left) and 24 h (bottom right). Catalase eliminates DSBs for HCT116 and RKO cells at all time points: gray trace, untreated control; red trace, MOI of 1,000; green trace, addition of MnSOD (1,200 units mL⁻¹); blue trace, addition of catalase (1,200 units mL⁻¹).
E. faecalis induces DNA damage and G2 arrest in nontransformed cells. To determine whether induction of aneuploidy, tetraploidy, and G2 arrest by E. faecalis in HCT116 and RKO cells extended to nontransformed cells, we exposed YAMC cells to E. faecalis. A similar set of responses was found with increased aneuploidy and tetraploidy, γH2AX formation, decreased γH2AX foci after treatment with MmsOD and catalase, and G2 arrest (Supplementary Fig. S5). Caffeine decreased γH2AX foci (data not shown) and partially rescued YAMC cells from E. faecalis–induced G2 arrest (Supplementary Fig. S5). These findings confirmed that the DNA-damaging and cyclomodulatory effects of E. faecalis were not unique to transformed cells.

E. faecalis–infected macrophages induce aneuploidy through a bystander effect. Colonic mucus limits direct contact between intestinal commensals and epithelial cells. Thus, luminal bacteria would likely exert promutagenic effects through indirect mechanisms. For E. faecalis, this could occur via translocation of the intact colonic epithelium (39). We have shown in mice that this process can lead to acute changes in colonic mucosal gene expression and nuclear factor-κB activation in tissue macrophages (27). This finding is consistent with other work by us, showing activated macrophages produce clastogens that damage epithelial cells via a mechanism analogous to the radiation-induced bystander effect (9, 13). To investigate this hypothesis, we exposed HCT116 cells to murine macrophages infected with E. faecalis in a dual-chamber tissue culture system (9). We found that the percentage of aneuploid and tetraploid cells increased significantly at an MOI of 1,000 compared with uninfected controls and at lower MOIs (Fig. 4A).

Eukaryotic cells have numerous defenses against oxidative stress and maintenance of genomic integrity. Glutathione (GSH) is a cofactor for enzymes that protect cells against free radical toxicity and xenobiotics. To determine whether GSH-dependent antioxidant defenses protected against the bystander effect, we depleted HCT116 cells of intracellular GSH using buthionine sulfoximine (BSO), a specific inhibitor of GSH synthesis. Depleted cells were exposed to E. faecalis–infected macrophages in the dual-chamber culture system. As the MOI increased from 1 to 100, the proportion of aneuploid and tetraploid cells increased compared with uninfected macrophages (Fig. 4B). Cells treated with BSO alone showed no increase in the proportion of aneuploid and tetraploid cells. A slight increase was seen, however, in GSH-depleted cells cocultured with uninfected macrophages. This may represent basal production of clastogens by these cells.4 Thus, GSH-dependent cellular defenses protect against clastogen-mediated aneugenesis and support the notion that E. faecalis–infected macrophages induce CIN through a bystander effect.

Infected macrophages cause DSBs through a bystander effect. To assess the response of colon epithelial cells to E. faecalis–infected macrophages, we investigated DNA damage repair pathways using the dual-chamber coculture system. Cells were synchronized in G1 by double thymidine block and exposed to E. faecalis–infected macrophages at an MOI of 1,000. HCT116 cells developed a marked G2-M arrest (Fig. 5A, left). The percentage of cells in G2-M was significantly increased at 48 hours (8.0 ± 0.1) and 72 hours (9.6 ± 0.5) compared with cells exposed to uninfected macrophages. In comparison, the percentage of RKO cells in G2-M did not change significantly (<5%; Fig. 5A, right).

* M.M. Huycke, unpublished data.

Figure 4. E. faecalis–infected macrophages induce aneuploidy and tetraploidy in colonic epithelial cells through a bystander effect. A, macrophages infected with E. faecalis at an MOI of 1,000 increase the percentage of aneuploid and tetraploid HCT116 cells. ***, P < 0.001 compared with coculture with uninfected macrophages. NS, not significant. B, depletion of GSH in HCT116 cells by 50 μmol/L BSO increases the percentage of aneuploid and tetraploid cells when cocultured with E. faecalis–infected macrophages at MOIs from 1 to 1,000. *, P < 0.05; ***, P < 0.001, compared with exposure to uninfected macrophages (MOI = 0); ***, P < 0.001, compared with without macrophages.

Activation of the G2 checkpoint by DSBs can occur through protein kinase damage response pathways, p-ATM/p-Chk2 and p-ATR/p-Chk1 signaling pathways were activated in both cell lines exposed to uninfected macrophages (Fig. 5B and Supplementary Fig. S6). Activation was moderately stronger, however, after exposure to E. faecalis–infected macrophages. As previously shown for cells directly exposed to E. faecalis (Fig. 2B and C), phosphorylation of CdK1 at Tyr15 was evident at baseline and did not increase after exposure to E. faecalis–infected macrophages. A G2-M arrest, however, was only noted for HCT116 cells (Fig. 5A), suggesting differences in sensitivity to DNA damage or cell cycle checkpoints between these cell types.

We next identified γH2AX in cells exposed to supernatants from E. faecalis–infected macrophages (Fig. 5C, bottom, and Supplementary Fig. S7). By laser scanning confocal microscopy γH2AX foci appeared more frequently in cells exposed to supernatants from infected macrophages than uninfected macrophages. Similarly, the proportion of γH2AX-positive cells at 5 hours (data not shown) and 48 hours (Fig. 5D) after exposure to infected macrophages was significantly greater for cells exposed to uninfected macrophages. The proportion of γH2AX-positive RKO cells (Fig. 5D, right) was less than that of HCT116 cells (Fig. 5D, left), suggesting insufficient DNA damage as a potential explanation for the lack of arrest at G2. These data showed that the bystander effect can activate ATM and ATR, generate γH2AX foci, and, for HCT116 cells, induce a G2-M arrest.
E. faecalis induces γH2AX foci in vivo. To determine whether E. faecalis could generate γH2AX foci in vivo, we developed an intestinal ligation model that exposed the colonic mucosa to an inoculum of enterococci. In this system, wild-type mice were exposed to E. faecalis for 6 hours, PBS as sham, or irradiated with 13 Gy. There was no histologic evidence for inflammation or mucosal abnormality in control or E. faecalis–exposed mice. However, immunohistochemistry showed marked positivity for γH2AX in colonic epithelial and stromal cell nuclei for mice treated with E. faecalis or irradiated (Fig. 6A, C, and D). Comparison of E. faecalis–exposed colons to shams showed significant increases in γH2AX in the nuclei of epithelial and stromal cells (Fig. 6B–D). The average (±SD) number of positive nuclei per cross-section for surface epithelial, crypt epithelial, and stromal cells for E. faecalis–exposed compared with sham mice was 2.3 ± 1.7, 12.0 ± 7.2, and 3.1 ± 2.2 versus 0.3 ± 0.5, 0.7 ± 1.2, and 0.0 ± 0.0, respectively (P < 0.001 for all comparisons). These results show that E. faecalis can generate rapid induction of γH2AX and, presumably, DSBs in colonic epithelial and stromal cells—findings not dissimilar to radiation-induced intestinal injury.

Discussion

In this study, we found that the human intestinal commensal E. faecalis induced γH2AX foci in human colonic epithelial cells, activated DNA damage pathways, produced G2 arrest, and promoted missegregation of chromosomes, leading to aneuploidy and tetraploidy. The production of ROS by E. faecalis was associated with anaphase bridging, lagging chromosomes, and multipolar mitoses. MnSOD, catalase, and tocopherols attenuated aneugenic and cyclomodulatory effects, linking the redox-active phenotype of this commensal to these potentially transforming effects.
E. faecalis was associated with a significantly increased number that remained tetraploid. Indeed, the production of ROS by propagating, all reverted to near-diploid, except for one clone and found that few survived clonal expansion. Of those that $>$5N DNA content to determine if aneuploid cells could persist increased

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in nude mice (41). Cytochalasin, was rendered tetraploid and found to be malignant primary mouse mammary epithelial cell that, when exposed to homologous chromosomes can, in general, arise after cellular stress through a failure of cytokinesis by cell fusion or from haphazard chromosome segregation. The tumorigenic potential of tetraploid cells was recently confirmed using a p53-deficient

cells to anaphase bridging, chromosomal fragmentation, and chromosomal rearrangements. G2 arrest should prevent cell cycle progression until DNA damage is repaired. However, damage below threshold values of 10 to 20 DSBs needed for the activation of mammalian checkpoints, would lead to propagation of chromosome aberrations to daughter cells (43). Evidence for this has been noted in sporadic CRC with mutations arising via low-level, telomere-mediated anaphase bridging (23).

G2 arrest is typically induced by decreased dephosphorylation of Cdk1 at Tyr15/Thr14 (44). However, by Western blot, we found no evidence for Cdk1 inactivation in HCT116 or RKO cells exposed to E. faecalis. HCT116 and RKO cells are mismatch repair–deficient, and this trait can negatively affect G2 checkpoint activation (45). Cell cycle arrest during G2 was confirmed by the persistence of cyclin B1 and the lack of accumulation of cells in mitosis. However, classic activation of the G2 checkpoint through Cdk1 was not found, and the G2 arrest that was induced by E. faecalis may have occurred through potential alternate pathways, including p53/p21, GADD45, and 14-3-3, among others (45, 46). This is a subject of ongoing investigation.

We previously found that E. faecalis promoted CIN in colonic epithelial cells through a macrophage-induced bystander effect (9). As with radiation, superoxide is a trigger for this effect (47). COX-2 contributes to both the radiation-induced and macrophage-induced bystander effects (9, 15, 16), although mechanisms are unclear. The results reported here show that the macrophage-induced bystander effect can rapidly induce aneuploidy and tetraploidy in colonic epithelial cells. The bystander effect also generated γH2AX foci in target cells, a finding consistent with the eukaryotic cell cycle (42). One example, produced by extra-intestinal pathogenic and commensal strains of Escherichia coli, is the cytolethal distending toxin that induces DSBs, cell distension, G2 checkpoint activation, and, ultimately, cell death (25). Commensal bacteria with cyclomodulatory effects may initiate DSBs that lead to anaphase bridging, chromosomal fragmentation, and chromosomal rearrangements. G2 arrest should prevent cell cycle progression until DNA damage is repaired. However, damage below threshold values of 10 to 20 DSBs needed for the activation of mammalian checkpoints, would lead to propagation of chromosome aberrations to daughter cells (43). Evidence for this has been noted in sporadic CRC with mutations arising via low-level, telomere-mediated anaphase bridging (23).

G2 arrest was partially ameliorated by MnSOD and indicated that superoxide can act as a cyclomodulin. Cyclomodulins are a family of secreted bacterial toxins that interfere with events. E. faecalis infection of macrophages produced a bystander effect that resulted in γH2AX deposition, cell cycle arrest, and CIN. Finally, the colonic mucosa of mice exposed to E. faecalis showed increased γH2AX formation similar to that produced by irradiation. γH2AX formation is a marker of DSBs, and these findings support our previous work that E. faecalis can generate DNA damage in vivo (6). In general, DSBs are difficult to repair and can generate dicentric chromosomes through nonhomologous end-joining repair (28). Dicentric chromosomes can attach to both spindle poles and lead to anaphase bridging. If bridges fragment, chromosomes can enter breakage-fusion-bridge cycles that produce rearrangements and aneuploidy (28). These are hallmark features of CIN.

We selected E. faecalis–exposed colonic epithelial cells with $>$5N DNA content to determine if aneuploid cells could persist and found that few survived clonal expansion. Of those that propagated, all reverted to near-diploid, except for one clone that remained tetraploid. Indeed, the production of ROS by E. faecalis was associated with a significantly increased number of tetraploid cells. Polyploid cells with more than two sets of homologous chromosomes can, in general, arise after cellular stress through a failure of cytokinesis by cell fusion or from mitotic slippage (40). Tetraploid cells, in particular, are considered potential intermediates in the cellular progression from diploid to aneuploid. For example, supernumerary centrosomes in tetraploid cells can form multipolar mitoses and cause haphazard chromosome segregation. The tumorigenic potential of tetraploid cells was recently confirmed using a p53-deficient primary mouse mammary epithelial cell that, when exposed to cytochalasin, was rendered tetraploid and found to be malignant in nude mice (41). E. faecalis–induced G2 arrest was partially ameliorated by MnSOD and indicated that superoxide can act as a cyclomodulin. Cyclomodulins are a family of secreted bacterial toxins that interfere with
4-hydroxy-2-nonenal (47, 48), although no single agent has been shown to be causative. Multiple breakdown products of polyunsaturated fatty acids, such as 4-hydroxy-2-nonenal (47, 48), although no single agent has been definitively shown to be causative. 

In conclusion, this study shows that E. faecalis can induce h2AX foci, G2 arrest, anaphase bridging, and aneuploidy and tetraploidy in human colonic epithelial cells through direct contact or after exposure to E. faecalis−infected macrophages. The latter is analogous to the radiation-induced bystander effect (13). Blocking DNA repair with caffeine or depleting GSH-deficient defenses with BSO significantly increased the frequency of aneuploidy and tetraploidy. Finally, E. faecalis−induced h2AX formation in murine colons suggested that this commensal can generate DSBs in vivo. These findings provide insights into mechanisms by which commensal bacteria may contribute to the endogenous origin of CIN and cellular transformation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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