Induction of DNA Damage in Mouse Colorectum by Administration of Colibactin-producing *Escherichia coli*, Isolated from a Patient With Colorectal Cancer

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Abstract. *Background/Aim:* Among colorectal cancer-associated intestinal microbiota, colibactin-producing (*clb*⁺) bacteria are attracting attention. We aimed to clarify the interaction between *clb*⁺ *Escherichia coli* and normal colorectal epithelial cells in vivo and in vitro. *Materials and Methods:* Five-week-old female Balb/c mice were divided in an untreated group, a group treated with *clb*⁺ *E. coli* isolated from a Japanese patient with colorectal cancer (*E. coli*-50), and a group treated with non colibactin-producing *E. coli* (*E. coli*-50/ Δ *clbP*). Mice were sacrificed at 18 weeks of treatment. *Results:* Treatment with *clb*⁺ *E. coli* increased positivity for H2A histone family member X phosphorylated

at Ser-139 (γ H2AX) in epithelial cells of the luminal surface of the mouse rectum but this did not occur in the *E. coli*-50/ Δ *clbP* and untreated groups. In an in vitro setting, the ratio of apoptotic cells was increased and cell counts were reduced by treatment with *clb*⁺ *E. coli* more than in untreated cells and normal rat colorectal epithelial cells. *Conclusion:* *E. coli*-50 induced DNA damage in the mouse rectum, possibly by direct interaction between *clb*⁺ *E. coli* and normal colorectal epithelial cells. Our findings imply that regulation of *clb*⁺ *E. coli* infection may be a useful strategy for colorectal cancer control.

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It is well known that diet strongly affects the incidence of colorectal cancer (1). As suggested from fecal and colonic mucosa of healthy individuals, a poor diet results in potentially adverse intestinal microbiota profiles (2, 3). Recently, it was found that intestinal microbiota are involved in changes in physical condition and the development of diseases. It has been clarified that the intestinal microbiota may change at a very early step of colorectal carcinogenesis (4). *Atopobium parvulum* and *Actinomyces odontolyticus* have been identified as bacteria that are elevated only in

patients with multiple polyps (adenoma) and intramucosal cancer (4). Thus, it is strongly suggested that these bacteria are related to the early stage of colorectal cancer. *Fusobacterium nucleatum* and *Peptostreptococcus stomatis* are bacteria that increase from the stage of intramucosal cancer and increase as the disease progresses (4). These bacteria have already been reported to be elevated in advanced colorectal cancer. It is interesting to clarify the relation between diet and such a specific bacterial increase in detail. Moreover, it is interesting to clarify the mechanism of how such bacteria might induce colorectal cancer.

Recently, colibactin-producing bacteria are attracting attention because they may contribute to colorectal carcinogenesis (5, 6). This bacterium is interesting because it may not be transmitted through food intake but rather from mother to infant by intimate contact, such as natural childbirth and breastfeeding (7). Colibactin is a gut bacterial genotoxin that is suggested to be linked to the development of colorectal cancer. Colibactin is produced by several bacteria, especially by *Enterobacteriaceae*, including certain *Escherichia coli* strains, those harbored in the human intestine. Colibactin is a hybrid non-ribosomal peptide-polyketide, synthesized by multi-enzyme complexes encoded by genes carried on the 54-kb genomic region (*pks* island) of *E. coli* (8). Exposure to *E. coli* containing the *pks* island causes DNA double-strand breaks in human cells and animals, and accelerates colonic tumor growth under conditions of host inflammation (9-11). However, the underlying molecular mechanisms that induce mutagenesis/carcinogenesis have not fully revealed yet. This is largely due to a lack of research clarifying the structure of colibactin. Of note, we recently successfully identified colibactin metabolites with the rearranged form of an intact colibactin (12).

To our knowledge, data on the effects of colibactin exposure in *in vitro* and *in vivo* settings are limited. A previous study showed that colibactin alkylated DNA in germ-free C57BL/6J mice (13). DNA alkylation is considered one of the pivotal factors in carcinogenesis. Recently, we isolated various colibactin-producing (*clb*⁺) *E. coli* strains, and found that *E. coli*-50, from a Japanese patient with colorectal cancer, producing a high amount of colibactin, showed strong genotoxicity in *in vitro* assays (14-17). Thus, we used this strain for an experiment to determine the effects of *clb*⁺ *E. coli* on rodent colorectal mucosa epithelial cells *in vivo* and *in vitro*, revealing that *clb*⁺ *E. coli*-50 induces DNA damage on the surface mucosa of mouse epithelial cells, possibly by direct interaction between *clb*⁺ *E. coli* and normal colorectal epithelial cells.

Materials and Methods

***E. coli* strains.** Isolation of *E. coli*-50 from the colorectal cancer specimen was reported elsewhere (14). An *E. coli*-50/ Δ *clbP* strain

that was deficient in producing colibactin owing to a disruption of the *clbP* gene was established as described elsewhere (12).

Animals and animal experiment. Four-week-old female Balb/c mice were purchased from Japan SLC (Hamamatsu, Japan). The mice were housed in plastic cages with sterilized softwood chips as bedding in a barrier-sustained animal room maintained at 24±2°C and 55% humidity under a 12 h light/dark cycle. After 1 week adaptation, mice were randomized by body weight into control (n=5), *E. coli*-50 (n=5) or *E. coli*-50/ Δ *clbP* (n=5) groups. Each *E. coli* strain was cultured overnight in LB medium (0.5 ml) and suspended in drinking water (100 ml) at a concentration of 5×10⁸ cells/ml, which was prepared every 2-3 days, and given to mice for 18 weeks. Standard chow (AIN-93M; Oriental Yeast, Tokyo, Japan) and water were available *ad libitum*. The animals were observed daily for clinical symptoms and mortality. Body weight and food consumption were measured every 2-3 days.

At the time of sacrifice, the mice were anesthetized, and blood released from their abdominal veins. Their colorectum was removed, opened longitudinally and fixed flat between sheets of filter paper in 10% buffered formalin. The colorectum was divided into the rectum (1 cm in length), and the remaining segments divided into proximal, middle and distal parts, and used for later immunochemical staining. All experiments were performed according to the Guidelines for Animal Experiments in the University of Shizuoka and were approved by the Institutional Ethics Review Committee for Animal Experimentation of the University of Shizuoka (#175175). The animal protocol was designed to minimize pain and discomfort to the animals. All animals were euthanized for tissue collection by isoflurane overdose.

Cell culture. Rat primary colorectal epithelial cells (PriA/MUTO2 cells) were derived from a 4-week-old F344 rat. In brief, the colorectum was removed and treated with Dispase I (Merck KGaA, Darmstadt, Germany) for 30 min, 37°C and the luminal side of the colorectum scraped to obtain intact crypts. Crypts were cultured in a collagen-coated dish. Cells showing a cobblestone pattern of growth were further cultured. PriA/MUTO2 cells were maintained in RPMI 1640 (Nacalai Tesque Inc., Kyoto, Japan) supplemented with 10% heat-inactivated fetal bovine serum and antibiotics (100 µg/ml streptomycin and 100 U/ml penicillin; Nacalai Tesque Inc.) at 37°C in 5% CO₂.

Immunohistochemical staining. Mouse intestines were fixed, embedded, and sectioned for further immunohistochemical examination using the avidin-biotin complex immunoperoxidase technique after heating with 10 mM citrate buffer (pH 6.0). The primary antibody was anti-H2A histone family member X with phosphorylated Ser-139 (γH2AX) (Cell Signaling Technology, Danvers, MA, USA) at 100× dilution. As the secondary antibody, biotinylated horse anti-mouse IgG (Vector Laboratories, Burlingame, CA, USA) was used at 200× dilution. Staining was undertaken using avidin-biotin reagents (Vectastain ABC reagents; Vector Laboratories), 3,3'-diaminobenzidine, and hydrogen peroxide, and the sections were counterstained with hematoxylin to facilitate orientation. As a negative control, consecutive sections were immunostained without exposure to the primary antibody. The ratio of γH2AX-positive cells was calculated by the formula: 100× number of γH2AX-positive cells/total number of cells in the intestine (at 100× magnification).

Figure 1. Effects of colibactin on DNA damage of the intestines in mice. A: Balb/c mice were treated with *clb*⁺ *Escherichia coli* isolated from a Japanese patient with colorectal cancer (*E. coli*-50) or with non colibactin-producing *E. coli* (*E. coli*-50/ $\Delta clbP$) at 5×10^8 cells/ml in drinking water for 18 weeks. Body weights of the groups are shown. B: Representative data of immunohistochemical staining for H2A histone family member X with phosphorylated Ser-139 (γ H2AX) from each group are shown. Bars are 200 μ m. C: Data are presented as the ratio of positive- γ H2AX cells relative to all intestinal cells of the luminal surface in each group. Statistical analyses were performed using Tukey's test.

Figure 2. Effects of apoptosis and cell death induced by colibactin. Rat primary colorectal epithelial cells (PriA/MUTO2 cells) were plated in 12-well plates (1×10^5 cells/well) and treated with or without *clb*⁺ *Escherichia coli* isolated from a Japanese patient with colorectal cancer (*E. coli-50*) ($OD_{600}=0.5$). After 1 h (A) or 2 h (B) treatment, cells were harvested and then stained using annexin-V Alexa Fluor® 488/propidium iodide (PI) to evaluate cell status and the ratio of apoptosis (left panel) or counted using a Tali® Image-based cytometer (Right panel). Data are presented as the mean±standard deviation. Statistical analyses were performed using Student's *t*-test. Live: Live cells, annexin-V negative and PI-negative; early: early apoptotic cells, annexin-V positive and PI-negative; late: late apoptotic cells, annexin-V positive and PI-positive.

Apoptosis assay. PriA/MUTO2 cells were plated in 12-well plates (1×10^5 cells/well) and treated with *E. coli-50* ($OD_{600}=0.5$). Cells were treated with the *E. coli* strains for 1 or 2 h, harvested and stained using annexin-V Alexa Fluor® 488 propidium iodide (PI), as described by the Tali® Apoptosis Kit – Annexin V Alexa Fluor® 488 and PI (Life Technologies Corporation, Carlsbad, CA, USA). Cell counts, death and apoptosis were evaluated using a Tali® Image-based Cytometer (Life Technologies Corporation). Annexin-V positive/PI-negative cells were recognized as early apoptotic cells

by cytometry, whereas the annexin V-positive/PI-positive cells were identified as late apoptotic cells; similarly, annexin V-negative/PI-negative cells were identified as viable cells and annexin V-negative/PI-positive cells were identified as dead cells.

Statistical analysis. The results are expressed as means±SD and statistical analyses were performed using Tukey's test or Student's *t*-test. Differences were considered to be statistically significant at $p < 0.05$.

Results

DNA damage by *clb*⁺ *E. coli*-50 in the colorectum of mice. Administration of *E. coli*-50 or *E. coli*-50/ Δ *clbP* in the drinking water for 18 weeks did not affect body weight (Figure 1A), food intake, daily drinking water intake or clinical symptoms throughout the experimental period in the mice.

We assessed colibactin-induced DNA damage using immunohistochemical staining of γ H2AX in mice colorectal epithelial cells (Figure 1B). In the luminal surface of the rectum, the ratio of γ H2AX-positive cells was increased by treatment with *E. coli*-50 compared to the untreated control mice (Figure 1C). Moreover, the ratio of γ H2AX-positive cells in the rectum in the group treated with *E. coli*-50/ Δ *clbP* was not increased compared with the controls. In the distal, middle and proximal part of the colon, the ratio of γ H2AX-positive cells in the luminal surface did not significantly differ between the groups (Figure 1C).

Cell death induced by colibactin. *In vivo* experiments showed that DNA damage at the luminal surface of the rectum was increased with *clb*⁺ *E. coli*-50 treatment. Thus, we next confirmed the direct interaction of *clb*⁺ *E. coli* with normal epithelial cells *in vitro*. The ratio of apoptosis did not significantly change between *E. coli*-50 cells and untreated cells (Figure 2A, left panel) after the 1-h treatment, whereas there was a slight reduction of the cell count compared to untreated cells (Figure 2A, right panel). *E. coli*-50 treatment for 2 h resulted in an increase of apoptotic cell ratio and reduction of the cell count (Figure 2B).

Discussion

In the present study, we found that treatment of *clb*⁺ *E. coli*-50, isolated from a Japanese patient with colorectal cancer, increased DNA damage in the epithelial cells of the luminal surface of mouse rectum. Moreover, in an *in vitro* setting, *clb*⁺ *E. coli*-50 increased the ratio of apoptotic cells and reduced the cell count compared to those of untreated cells.

Turnover of cell division is vigorous in intestinal epithelial cells. This is because intestinal epithelial cells are always exposed to substances that should be excreted, including toxins, and the resultant damaged epithelial cells should be removed from the intestine as fast as possible. Our finding that γ H2AX-positive cells were observed in the epithelial cells of the luminal surface of mouse rectum suggests that colibactin derived from *E. coli* damaged the DNA of epithelial cells by direct contact of *clb*⁺ *E. coli* with the epithelial cells. If increased DNA damage by colibactin from *E. coli* was observed in the growth zone of intestinal crypts (under 1/3 the area of crypts), it is assumed that organic substances, including colibactin produced by *E. coli*, play an important role in cell damage. However, we did not observe DNA damage by *clb*⁺

E. coli in the proliferative zone. Further investigation is needed to clarify the reason why *clb*⁺ *E. coli* was correlated with colorectal carcinogenesis. One possibility is that retained γ H2AX-positive cells in the epithelial cells affected structural maintenance and affected cell growth. In this case, experimental periods greater than we performed in the present study are needed to obtain change in the proliferative zone after treatment with *clb*⁺ *E. coli*. Another possibility is involvement of a pathological condition, such as ulcerative colitis, which induces crypt dilation *i.e.*, crypt abscess. In this condition, *clb*⁺ *E. coli* might reach the proliferative zone, and play an important role in the development of colorectal cancer.

To observe direct cell damage by *clb*⁺ *E. coli*, we performed *in vitro* assay in which *clb*⁺ *E. coli* was able to attach to colorectal epithelial cells. To this end, we derived normal epithelial cells from rat colorectum, and used them instead of cancer cells because the action of colibactin may be different between normal epithelial cells and cancer epithelial cells. As expected, the ratio of apoptosis was increased, and cell counts were reduced by 2 h-treatment with *clb*⁺ *E. coli* by more than that of untreated normal epithelial cells. Apoptosis is often observed after DNA damage. Thus, our results also suggest that DNA damage of cells occurs in a short period after *clb*⁺ *E. coli* meets epithelial cells.

Another report suggested that *clb*⁺ *E. coli* induces cell senescence when compared with colibactin-negative *E. coli* (18). Therefore, colibactin confers resistance to DNA damage-induced cell death in normal colorectal epithelial cells. This might be an additional reason why we observed more γ H2AX-positive cells in the *clb*⁺ *E. coli*-treated group. In a recent study, colorectal cancer cells were shown to have a mutation signature after long-term exposure to *pks*⁺ *E. coli* (19, 20). It is assumed that DNA mutation with resistance to apoptosis may play a pivotal role in colorectal carcinogenesis.

In conclusion, we showed that *clb*⁺ *E. coli*-50, derived from a patient with colorectal cancer, induced DNA damage in the rectum of mice, possibly by direct interaction between *clb*⁺ *E. coli* and normal colorectal epithelial cells. The findings of this study imply that regulation of *clb*⁺ *E. coli* infection may lead to the development of preventative methods against colorectal carcinogenesis. Further studies are needed to clarify the detailed mechanism by which colibactin induces colorectal cancer and to develop a useful strategy to prevent colorectal carcinogenesis.

Conflicts of Interest

The Authors declare no competing interests.

Authors' Contributions

TN and MM had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Moreover, K Watanabe, K Wakabayashi and MM

contributed to the concept, design and drafting of the article. K Watanabe obtained funding. TN, YT, NM, MK, TH, GF, YY, MS, MK, MT and YI were responsible for acquisition, analysis, or interpretation of data. HS and YT reviewed and supervised this work.

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