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Bacteria-Induced Intestinal Cancer in Mice with Disrupted *Gpx1* and *Gpx2* Genes

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ABSTRACT

Two glutathione peroxidase (GPX) isozymes, GPX-1 and GPX-2 (GPX-GI), are the major enzymes that reduce hydroperoxides in intestinal epithelium. We have previously demonstrated that targeted disruption of both the *Gpx1* and *Gpx2* genes (GPX-DKO) results in a high incidence of ileocolitis in mice raised under conventional conditions, which include the harboring of *Helicobacter* species [non-specific-pathogen-free (non-SPF) conditions]. In this study, we have characterized GPX-DKO mice that have microflora-associated intestinal cancers, which are correlated with increased intestinal pathology/inflammation. We found that GPX-DKO mice raised under germ-free conditions have virtually no pathology or tumors. After colonizing germ-free mice with commensal microflora without any known pathogens (SPF), <9% of GPX-DKO mice develop tumors in the ileum or the colon. However, about one-fourth of GPX-DKO mice raised under non-SPF conditions from birth or transferred from SPF conditions at weaning have predominantly ileal tumors. Nearly 30% of tumors are cancerous; most are invasive adenocarcinomas and a few signet-ring cell carcinomas. On the basis of these results, we conclude that GPX-DKO mice are highly susceptible to bacteria-associated inflammation and cancer. The sensitivity exhibited in these mice suggests that peroxidative stress plays an important role in ileal and colonic pathology and inflammation, which can lead to tumorigenesis.

INTRODUCTION

Enteric microflora begin to colonize the gut at birth and affect development and maintenance of the mucosal immune response and epithelial cell functions (1). Recently, commensal bacteria have emerged as cofactors in the development of ileocolitis and intestinal malignancies. Human inflammatory bowel disease (IBD), which includes Crohn's disease and ulcerative colitis, increases cancer risk by 20–30-fold in patients with prolonged IBD histories (2). Many mouse models have been generated to facilitate studying the etiology, prevention, and treatment of IBD and gastrointestinal cancer associated with IBD. The essential role of enteric microflora in ileocolitis-associated cancer has been established in several of these mouse idiopathic IBD models with defects in the immune response. Interleukin (IL)-2 KO, IL-10 KO, T-cell receptor β/p53 DKO, or tumor growth factor β1/Rag-2 DKO mice, when maintained under germ-free (GF) conditions, fail to develop severe inflammation, as well as small intestinal or colonic cancers that often occur when those animals are raised under conventional housing conditions (3–6). Because microflora can modulate epithelial cell signaling for immune reactions (7–9), this may explain the susceptibility of these immune-compromised mice to the development of IBD. Because GPX-DKO mice have an intact immune system at the outset of these studies, the role of microflora on the subsequent development of any pathological condition in these mice was unclear.

Glutathione peroxidases (GPX) are a family of four selenium-dependent antioxidant enzymes in mice (five in humans; Ref. 10) that reduce H_2O_2 and organic hydroperoxides by oxidizing glutathione. Taken together, the ubiquitous GPX-1 and epithelium-specific GPX-2 contribute nearly all glutathione-dependent H_2O_2-reducing activity in the intestinal epithelium (11). We have previously reported that GPX-DKO mice, with targeted disruption of *Gpx1* and *Gpx2* genes, exhibit ileocolitis between 2 and 7 weeks of age, which is accompanied by accumulation of lipid hydroperoxides, weight loss, and proctitis (12). Because some colitis models such as *Mdr1*-KO mice do not appear to develop cancer (13, 14), we set out to determine whether GPX-DKO mice would develop intestinal cancer. Because most mouse IBD and ileocolitis-associated cancer models are either immunodeficient or defective in membrane proteins that affect epithelial barrier integrity (15, 16), showing that intracellular GPX activity could prevent microflora-induced ileocolitis and cancer would strengthen the notion that peroxidative stress is one basis for the pathogenesis of inflammation-associated cancer. Although elevated reactive oxygen and nitrogen species are recognized as an integral part of the pathophysiology of IBD, there is little evidence to specify the precise role of hydroperoxides in IBD pathology (17, 18). Demonstrating that epithelial GPX activity could inhibit both IBD and IBD-associated cancers might set the stage for the prevention of IBD-related cancers with inhibitors of the major hydroperoxide-generating enzymes that reside in the mucosal epithelium or inflammatory cells.

Our original non-specific-pathogen-free (non-SPF) GPX-DKO mouse colony harbors several enterohepatic *Helicobacter* species such as *H. hepaticus*, which is widely spread in rodents (19). Although *H. hepaticus* was originally identified as causing hepatitis and hepatocellular tumors in A/JCr mice, its primary site of colonization is in the intestine. Colonization of *H. hepaticus* to SPF mice causes ileocolitis and colon cancer in immune-deficient animals such as nude mice, IL-10 KO, and T-cell deficient mice (20–22). However, *H. hepaticus* only induces mild or no colitis in SPF and immune competent wild-type C57BL/6 and *Mdr1*-KO mice (19, 23). In this article, we address whether specific microflora are essential for ileocolitis and its associated cancer in GPX-DKO mice by comparing the extent of ileocolitis and cancer incidences in mice harboring non-SPF, SPF, or no enteric microflora.

MATERIALS AND METHODS

Animals. GPX-DKO mice were generated by mating *Gpx1*$^{tm1Ysh/tm1Ysh}$ (*Gpx1*-KO) and *Gpx2*$^{tm2Coh/tm2Coh}$ (*Gpx2*-KO) mice. Both lines were on a mixed C57BL/6J and 129Sv/J or 129S3 mixed background, as we have reported previously (12). The original colony harbors *Helicobacter* species, including *H. hepaticus*, evaluated by PCR on fecal samples (Missouri University Research Animal Diagnostic Laboratory). A GF GPX-DKO colony was established by neonatal transfer at the Gnotobiotic Laboratory of the University of Wisconsin-Madison. Upon the closure of the University of Wisconsin-Madison gnotobiotic facility, the GF colony was transferred aseptically to the Gnotobiotic Laboratory kindly provided by Kathryn A. Eaton, DVM, Ph.D., at Ohio State University-Columbus for an additional 5 months. To establish a SPF colony, we have transferred GF mice from University of Wisconsin-

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Madison to the City of Hope Animal Resources Center and naturally colonized the mice with bacteria by handling and feeding a nonirradiated diet (catalogue no. 5001 Laboratory Rodent Diet; Purina Mills, Inc., Richmond, IN) for 1 month, then switching to an irradiated diet. The non-SPF colony was fed with nonirradiated Laboratory Rodent Diet with 6% fat (catalogue no. 5001). Breeders in SPF and non-SPF colonies were fed, respectively, with irradiated and nonirradiated Mouse Diet 9F containing 9% fat (catalogue no. 5020) to maintain health. The original non-SPF colony and the SPF colony were kept in separate rooms and maintained in polycarbonate microisolator cages. The GF colony was fed with Autoclavable Rodent Diet with 6% fat (catalogue no. 5010). All mice had free access to food and water. The City of Hope Research Animal Care Committee approved the housing and care for our mouse colonies.

Tumor Analysis. Six non-SPF mice $>$ 5 months of age were found dead or appeared moribund and were thus euthanized for cause. Four of these mice had tumors. The remaining asymptomatic mice not warranting euthanasia were thus examined after 5 months of age to evaluate tumor prevalence. We visually examined the entire length of the small and large intestine of every animal sacrificed for any abnormal growth or tumor formation. In most cases, we excised tumors and made Swiss rolls on the remaining ileum and colon to avoid missing small lesions (24). All tissue samples were fixed in 10% formalin in phosphate buffer and then processed for sectioning. Most sections were stained with H&E for histological analysis. Only those lesions that had dysplastic histology (as defined below) examined by our staff pathologists were scored as tumors. Goblet cells were stained with Alcian Blue and counterstained with Nuclear Fast Red.

The tumors were analyzed for the stages of cancer progression described by Riddel *et al.* (2). Because of the nature of the GPX-DKO intestinal epithelium, which always had focal inflammation or pathology in the presence of luminal microflora, we did not include hyperplastic epithelium, which only showed crypt distortion or enlarged and branched glands as seen in polyps, as tumors. We scored tumors with low-grade dysplasia and high-grade dysplasia as precancerous lesions; cancers included both invasive carcinomas and signet-ring cell malignancies. The criteria for low-grade dysplasia were hyperplasia with cytological abnormalities, primarily loss of nuclear polarity, marked stratification of nuclei, nuclear hyperchromatism, and cellular and nuclear pleomorphism. High-grade dysplasia was scored when dysplastic epithelium extended to the apical surface or involved a large area. High-grade dysplasia also included carcinoma *in situ*, which has neoplastic epithelium forming a complex cribriform pattern (back-to-back glands with no intervening stroma) yet without evidence of invasion into the muscularis mucosa. Invasive carcinoma was scored when the atypical glands were seen in the muscularis mucosa, serosal adipose tissue, or muscle layers.

Logistic regression was used to test the association of cancer prevalence

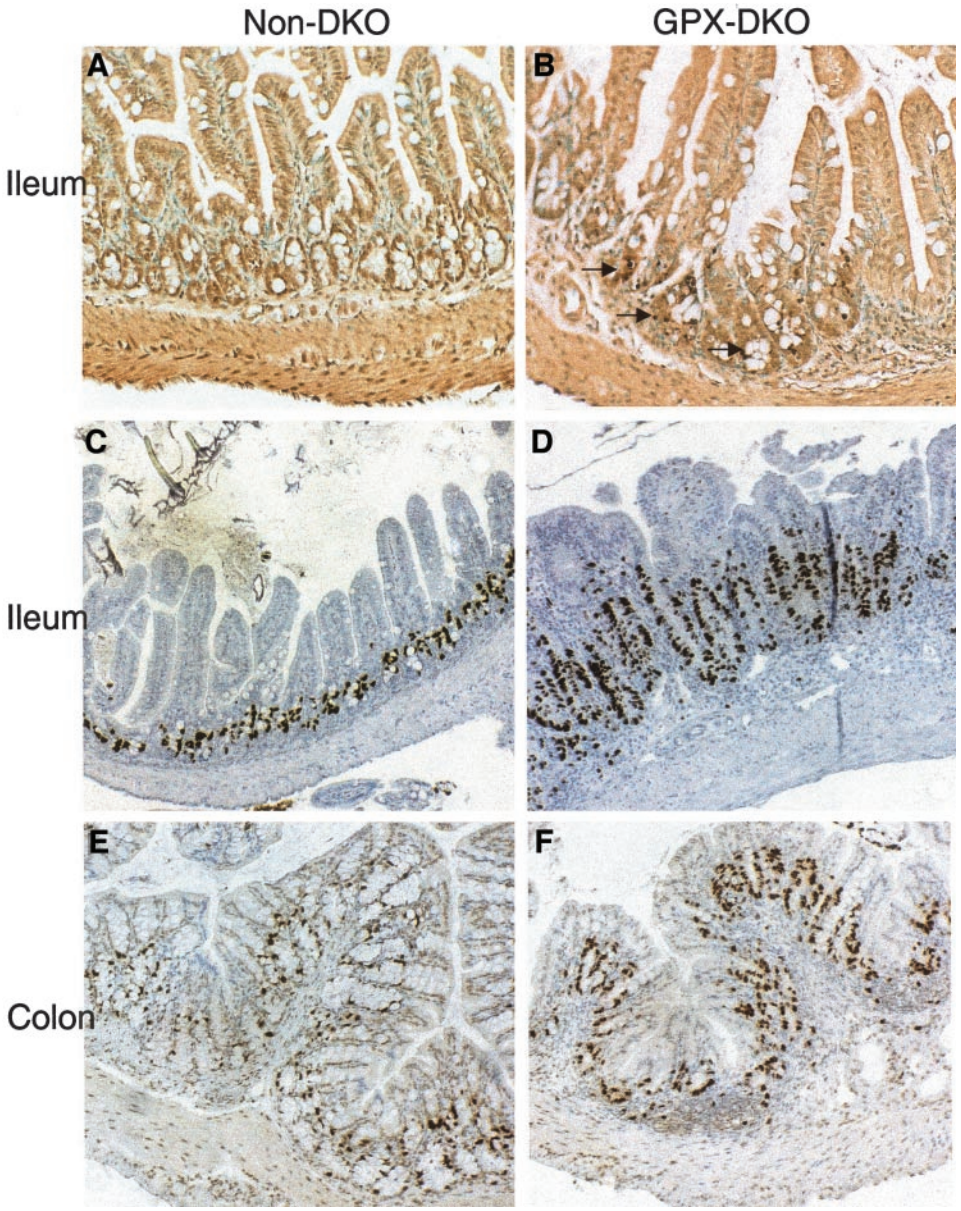

Fig. 1. Immunohistochemical staining of apoptotic cells in the ileum of 10-week-old germ-free mice and proliferating cells in the ileum and colon of 10-week-old specific pathogen-free mice. *A* and *B* demonstrate the apoptotic cells detected by terminal deoxynucleotidyl transferase assay, and the incorporated biotinylated deoxynucleotides were visualized with peroxidase staining. The tissues were counterstained with methyl green. *A* is an ileum of a GPX-DH mouse (*Gpx1*+/−*Gpx2*+/−) with no positive staining, and *B* is an ileum of a GPX-DKO mouse with dark brown positive staining (*arrows*). Both mice were euthanized on the day of arrival from the University of Wisconsin-Madison by air in germ-free isolators. *C–F* demonstrate mitotic cells in the ileum and colon of specific pathogen-free mice. Mice were injected with BrdUrd 2 h before euthanasia. BrdUrd was detected using an anti-BrdUrd antibody and visualized with streptavidin-peroxidase and 3,3′-diaminobenzidine, then cells were counterstained with hematoxylin. *C* and *E* are a GPX-DH and a 3/4-DKO mouse with (*Gpx1*−/−*Gpx2*+/−), and *D* and *F* are two GPX-DKO mice. The original magnification of *A* and *B* is ×200, and *C–F* is ×100. The apparently larger size of the GPX-DKO mouse ileum is due to underlying pathology.

with age at sacrifice, sex, parity, and colony conditions, each factor being adjusted for the others. Significant ($P < 0.05$) likelihood-ratio tests with multiple degrees of freedom were followed by specific contrasts (Wald tests).

Immunohistochemistry. Mouse monoclonal anti-β-catenin (1 μg/ml) antibody (BD Transduction Labs) was used to determine cellular localization of β-catenin, which was detected with horseradish peroxidase and 3,3′-diaminobenzidine (Ultravision Detection System; Lab Vision Co., Fremont, CA). Cell proliferation was detected with a BrdUrd immunohistochemistry kit (Oncogene Research Products, San Diego, CA). Mice were injected i.p. with 5′-bromo-2′-deoxyuridine (BrdUrd, 120 mg/kg) and 5′-fluoro-2′-deoxyuridine (12 mg/kg) dissolved in sterile Ringer's solution. Mice were euthanized 2 h after injection, and the small and large intestines were processed for routine paraffin embedding. Apoptotic cells were detected *in situ* by a TdT-FragEL DNA Fragmentation Detection kit (Oncogene) on the paraffin-embedded tissue sections. Rabbit polyclonal anti-myeloperoxidase antibody (7 μg/ml; DakoCytomation, Carpinteria, CA) was used to detect polymorphonuclear neutrophils and monocytes (25). The proliferative index (stained cells/crypt) was determined by counting the number of stained cells in at least seven crypts of the most distorted region of the intestine for each mouse.

RESULTS

GPX-DKO mouse intestinal epithelium is highly susceptible to bacteria-induced pathology and inflammation. GF GPX-DKO mice have no pathology or symptoms except after shipping, which induces a temporary increase in crypt apoptosis and proliferation in the ileum (Fig. 1, *A* and *B*). When bacterial colonization occurs at birth, the ileal and colonic epithelium always exhibits focal pathology or inflammation throughout the life of the GPX-DKO mice (Fig. 2). The pathology and inflammation was scored in nontumorous areas based on a 17-point system, which includes inflammation as shown by lymphocyte or neutrophil infiltration (0–3 points), mucin depletion (0–2 points), reactive epithelium such as crypt distortion (0–3 points), number of intraepithelial lymphocytes (0–3 points), inflammatory foci (0–3 points), and apoptotic figures (0–3 points; Ref. 12). The pathology/inflammation scores in GPX-DKO ileal epithelium vary in degree based on exposure to microflora, with scores greatest in non-SPF > SPF > GF = non-DKO mice. In colonic epithelium, the order of pathology scores was non-SPF = SPF > GF = non-DKO (Fig. 2). SPF mouse ilea have significantly lower inflammation scores than non-SPF ilea when compared by age ($P < 0.04$, t test), whereas the colon scores remain the same (with Ps ranging between 0.06 and 0.37, t test). We have previously reported that colonization with SPF microflora of adult GF GPX-DKO mice caused acute ileocolitis (26). Similarly, colonization with non-SPF microflora at weaning (4 weeks old) of the SPF GPX-DKO mice also caused acute ileocolitis, which resulted in one death in a group of 15 at the 53rd day after transfer. These transferred (SPF→non-SPF) GPX-DKO mice had similar inflammation scores as non-SPF GPX-DKO mice when analyzed 4–6 months later. On the basis of these observations, we conclude that non-SPF microflora provoked a more severe and persistent pathology than SPF microflora in the ilea of GPX-DKO mice.

Table 1 *Tumor incidence in mouse ileum and colon*

	DKO			Non-DKO[a]		
Age (months)	Male	NB[b]-Female	Breeder[b]	Male	NB-Female	Breeder
Non-SPF colony[a]						
4.0–5.9	8/20[c] (40%)[c]	1/8 (13%)	—[c]	0/13	0/7	0/1
6.0–8.9	10/64 (16%)	3/19 (16%)	3/9 (33%)	0/38	0/16	0/1
9.0–13.0	6/15 (40%)	0/4 (0%)	5/9 (56%)	0/16	0/4	0/2
All ages	24/99 (24%)	4/31 (13%)[c]	8/18 (44%)	0/67	0/27	0/4
SPF colony[a]						
<4–5.9	2/12 (17%)	0/4	0/2	0/5	0/1	0/1
6.0–8.9	4/46 (9%)	0/13	—	0/20	0/6	—
9.0–13.0	0/11 (0%)	—	—	0/14	—	—
All ages	6/69 (9%)	0/17	0/2	0/39	0/7	0/1
SPF→ non-SPF at weaning[a]						
<4–5.9	0/1	—	—	0/2	—	—
6.0–7.9	4/13 (31%)	—	—	0/8	—	—
All ages	4/14 (27%)			0/10		
GF colony[a]						
4.0–5.9	0/13	0/4	—	0/6	—	—
6.0–8.9	0/2	0/3	0/9	0/1	0/2	—
9.0–12.0	0/6	—	1/8[d]	0/2	—	0/5
All ages	0/21	0/7	1/17 (6%)	0/9	0/2	0/5

[a] The conventionally reared specific pathogen-free (SPF) mice did not have detectable pathogens, and non-SPF mice harbored *Helicobacter* species, including *H. hepaticus*. Germ-free (GF) mice were derived from non-SPF mice and housed under GF conditions, and SPF mice were established from GF mice. The SPF→ non-SPF colony contains mice transferred from SPF containment at 4 weeks of age to cages with soiled bedding from non-SPF mice. Non-DKO mice were littermates of the GPX-DKO mice with one wild-type *Gpx1* or *Gpx2* allele or double heterozygous KO.

[b] NB stands for non-breeder. Breeder consists of female mouse with one or more parity.

[c] The numerator is the number of mice with dysplasia and adenocarcinoma in the ileum or colon, and the denominator is the number of mice analyzed. Number in the parenthesis is the percentage of mice bearing tumors. Dash means no mice were analyzed in the category.

[d] A 9.1-month-old GF female breeder had an invasive ileal carcinoma.

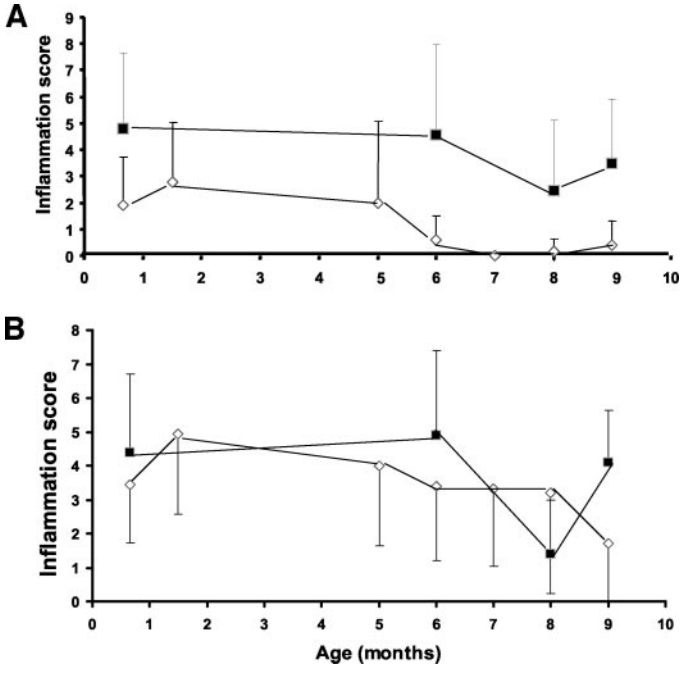

Fig. 2. Comparison of the mean inflammation/pathology scores (±SD) between the non-specific-pathogen-free (non-SPF; ■) and the SPF (◇) colonies in the ileum (*A*) and in the colon (*B*) by age. Only *one-sided error bars* are shown for clarity. Between 5 and 15 mice were analyzed at each time point. The ilea of the mice in the non-SPF colony had significantly higher inflammation/pathology scores than those of the SPF colony at matched ages ($P < 0.04$ for each time point, t test). Colonic inflammation/pathology scores were similar for the non-SPF and SPF colonies.

Most of the pathology/inflammation scores are reflective of high numbers of focally appearing mitotic and apoptotic cells in the ileum and colon, with a small number of infiltrating neutrophils. We compared the extent of cell proliferation in the ileal and colonic crypts of SPF GPX-DKO and non-DKO littermates using a mitotic index (MI) scale 2 h after bromodeoxyuridine injection (Fig. 1 *C*, *D*, *E*, and *F*). At least seven crypts were counted in the most distorted regions. The ilea of GPX-DKO mice had 2.2-fold higher numbers of proliferating cells than non-DKO control mice. The ileal MI for GPX-DKO mice was 17 ± 5 (mean ± SD, $n = 5$) labeled cells/crypt and that for non-DKO (with one or one each wild-type *Gpx1* and *Gpx2* allele) 8 ± 4 ($n = 6$; $P = 0.004$, Wilcoxon test). Similarly, colonic crypts of GPX-DKO mice also had significantly higher numbers of proliferating cells than in non-DKO mice. The colonic MI for GPX-DKO was 11 ± 2 ($n = 4$) and for non-DKO 4 ± 2 ($n = 4$; $P < 0.03$, Wilcoxon

test). This higher MI in GPX-DKO mice may be necessary to avoid crypt atrophy caused by high levels of apoptosis. We found a lower number of ileal crypts (68 ± 8/cross-section, $n = 5$) in GPX-DKO mice compared with non-DKO mice (95 ± 19/cross section, $n = 5$, $P = 0.03$) at 7–10 weeks of age. This suggests that the crypt loss due to apoptosis may not be fully compensated by a higher MI.

To determine tumor incidence, we euthanized mice primarily between 5 and 9 months of age in non-SPF, SPF, SPF→non-SPF, and GF colonies (Table 1). Because age variation at sacrifice could confound comparisons among the colonies, we adjusted for age variation by using a logistic regression test. We found age at sacrifice was not significantly associated with tumor prevalence ($P = 0.3$). However, tumor prevalence varied significantly across colony conditions after adjustments for age and sex ($P < 0.0001$, 3 degrees of freedom, logistic regression test; Table 1). The effect was primarily due to the difference between "the clean conditions" (the GF and SPF) as opposed to "the dirty conditions" (non-SPF and SPF→non-SPF conditions; $P = 0.0003$). Comparisons within the clean and dirty conditions were not statistically significant ($P = 0.15$ between GF and SPF, and $P = 0.7$ between non-SPF and SPF→non-SPF). However, the data are consistent with the hypothesis that the risk of developing a tumor to GPX-DKO mice in SPF conditions was intermediate between that in GF and non-SPF conditions. The tumor rate among females varied significantly with parity adjusted for age and conditions, with nonbreeder females having significantly fewer tumors ($P = 0.02$). Male mice were not statistically distinguishable from either group of females ($P > 0.07$). No tumors were found under any conditions in mice that had at least one wild-type *Gpx1* or *Gpx2* allele. These non-DKO

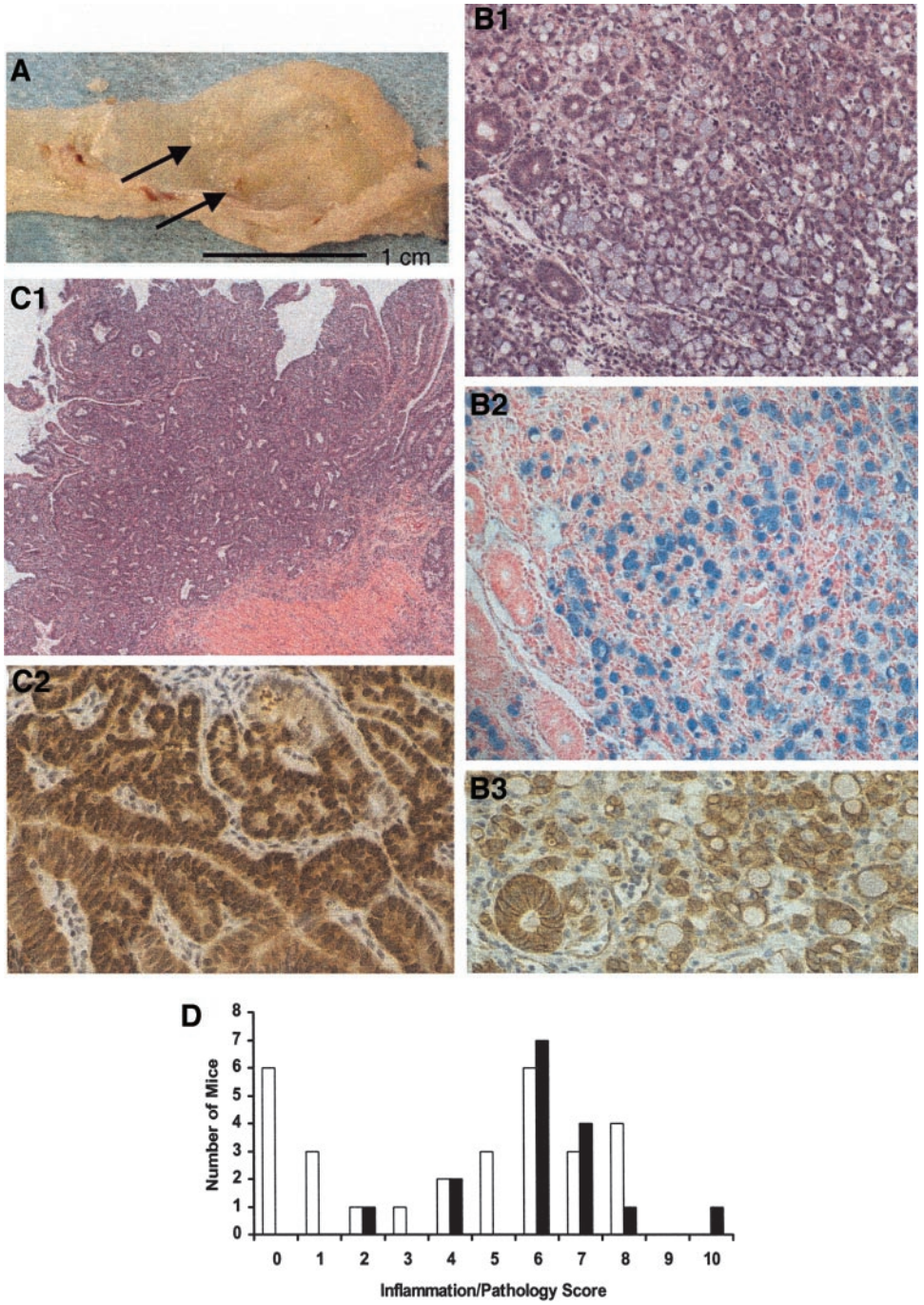

Fig. 3. Histopathology of tumors from GPX-DKO mice. *A* demonstrates a nonpolypoid tumor mass in the ileum with arrows pointing to the periphery of the 1-cm tumor. *B1* and *C1* are a signet-ring cell carcinoma (×100) and an invasive adenocarcinoma (×40) stained with H&E. The signet-ring cell carcinoma has abundant Alcian blue-stained mucin with a Nuclear Fast Red counterstain (B2). *B3* shows membranous β-catenin localization, and *C2* illustrates nuclear accumulation of β-catenin in this invasive cancer (both panels are originally amplified ×200). *D* correlates the ileal epithelial inflammation/pathology scores with tumor incidence in male nonspecific pathogen-free GPX-DKO mice. Ileal histology is shown for mice free of tumors (□) and with ileal tumors (■). Scoring criteria included lymphocytic or neutrophilic infiltration (0–3 points), mucin depletion (0–2 points), reactive epithelium such as crypt distortion (0–3 points), number of intraepithelial lymphocytes (0–3 points), inflammatory foci (0–3 points), and apoptotic figures (0–3 points). A significant difference was noted in scores for tumor-bearing (mean ± SD; 6.1 ± 1.8, $n = 16$) and nontumor bearing (4.1 ± 3.0, $n = 29$) mice ($P = 0.04$, Wilcoxon rank-sum test with continuity correction).

Table 2 *Histological analysis of intestinal tumors in GPX-DKO mice*

Colony	Age (months)	n^a	LG dysplasia	HG dysplasia	Cancer[b]
Non-SPF	5–12	36	10 (28%)[c]	16 (44%)	10 (28%)
SPF	2–8	6	4 (66%)	2 (33%)	0
SPF→non-SPF	7	4	0	2 (50%)	2 (50%)
GF	10	1	0	0	1 (100%)
Total	All ages	47	14 (30%)	20 (43%)	13 (28%)
Nuclear β-catenin		47	7/14 (50%)[d]	19/20[e] (95%)	9/13[f] (69%)

[a] *n*, number of tumor-bearing mice in the colony; LG, low grade; HG, high grade; SPF, specific pathogen-free.

[b] Thirteen cancers include 10 invasive adenomas and 3 signet-ring cell carcinomas (SRCC). Three ileal SRCC were found in a 5-month-old and a 6-month-old non-SPF male mouse, as well as a 7-month-old male mouse transferred from SPF to non-SPF at weaning.

[c] The number in the parenthesis is the percentage of tumors exhibiting the specified histology in the colony.

[d] The number in the parenthesis is the percentage of tumors with nuclear β-catenin accumulation within the characterized group.

[e] One HG-dysplastic tumor from a 10-month-old breeder non-SPF female did not have nuclear accumulation of β-catenin.

[f] Three SRCC and an invasive tumor from a 7-month-old male mouse transferred from SPF to non-SPF at weaning did not have nuclear accumulation of β-catenin.

mice were littermates of GPX-DKO mice and shared identical housing conditions. These observations exclude genetic background, non-intestinal environmental and dietary factors as etiologies for cancer susceptibility in this model.

All tumors were nonpolypoid masses > 3 mm in diameter on inspection (Fig. 3*A*). Most mice had single tumors, except for a few mice that had 2 tumors each. Ten of 36 (28%) tumors analyzed in the non-SPF colony (from birth) were adenocarcinomas with 8 invasive carcinomas and two signet-ring cell carcinoma (SRCC; Table 2). Two of 4 tumors in the SPF→non-SPF colony were cancerous with 1 invasive and 1 SRCC. Most of the tumors were located in the distal ileum, except for 1 non-SPF mouse that had a tumor in the distal jejunum. Six mice had colonic tumors; this includes 1 SPF female, 1 SPF→non-SPF male, and 4 SPF male GPX-DKO mice.

Multiple gene mutations have been reported during colorectal carcinogenesis, and mutations in the *adenomatous polyposis coli* (*Apc*) gene occur often in both sporadic tumors and with colitis-associated cancers (27–32). The tumor suppressor activity of Apc sequesters the bifunctional β-catenin for degradation. Cells with mutant *Apc* genes have detectable accumulations of cytoplasmic and nuclear β-catenin (33–35). We stained for β-catenin and used nuclear accumulation, only, as indirect evidence for *Apc* inactivation because it was difficult to distinguish cytoplasmic staining from background staining. We found that 7 of 14 (50%) low-grade dysplastic adenomas, 19 of 20 (95%) high-grade dysplastic adenomas, and 9 of 13 (69%) adenocarcinomas had nuclear accumulation of β-catenin, including an invasive ileal adenocarcinoma in a GF breeder (Table 2 and Fig. 3). Two SRCCs from the non-SPF colony and 1 from the SPF→non-SPF colony did not show nuclear accumulation of β-catenin (Fig. 3). Signet-ring morphology appeared to be caused by the large amounts of mucin (stained by Alcian Blue) pressing nuclei against the plasma membrane. The only invasive adenocarcinoma that did not have nuclear accumulation of β-catenin was from the SPF→non-SPF colony.

The higher ileal tumor rates in non-SPF mice than SPF and GF mice correlated with higher pathology scores. Among the male non-SPF GPX-DKO mice, we also found that tumor-bearing mice had significantly higher ileal pathology scores than those that were tumor free (Fig. 3). Also, similar ileal scores were found between male (6.0 ± 2.4, $n = 29$) and female (6.6 ± 2.6, $n = 12$) GPX-DKO mice regardless of breeding status. However, although similar scores were found in the ileum and colon in the SPF colony, colon tumors were most prevalent.

DISCUSSION

In this article, we provide the first direct evidence that GPX prevents ileocolitis and intestinal cancer. Elevation of reactive metabolites of oxygen and nitrogen during the inflammatory response are believed to cause some of the intestinal and colonic injury and dysfunction observed in IBD (17). However, most studies have focused on the damaging effect of superoxide and nitric oxide; few studies have examined the effect of hydroperoxides. Some indirect evidence suggests a protective role for *Gpx1* and *Gpx2* genes against oxidative stress. These data include induction of *Gpx1* gene expression in gastric mucosa by *H. pylori* infection (36) and induction of *Gpx2* gene expression in intestinal mucosa by commensal bacteria or by γ-irradiation (26, 37). However, elevated GPX gene expression has also been associated with tumorigenesis, presumably because of its antiapoptotic activity (38). Elevated *Gpx2* gene expression is observed in squamous cell carcinomas, Barrett's mucosa, and colorectal adenomas compared with normal tissues (39–41), and overexpression of the *Gpx1* gene increases skin cancer risk (42). Therefore, it has been a quandary whether increased GPX activity is anti-inflammatory or procarcinogenic. Our results suggest that GPX can prevent tumorigenesis in intestinal epithelium by its antioxidant activity.

The tumor incidence that we observed in the ilea of GPX-DKO mice is correlated with pathology/inflammation scores, with the highest cancer rate occurring in the non-SPF colony or SPF mice made non-SPF at weaning. However, the colon shows no correlation between the tumor incidence and pathology/inflammation scores. Both non-SPF and SPF colonies have similar pathology/inflammation scores in the colon, but SPF mice have more colonic tumors than non-SPF mice. Also, female non-SPF GPX-DKO mice had similar inflammation scores regardless of their breeding status. These results suggest that inflammatory changes are necessary but not sufficient for tumorigenesis.

Although male mice did not have a significantly higher tumor incidence than female mice, breeder females appeared to have a higher tumor incidence than nulliparous females. It has been noted that *H. hepaticus* causes more severe hepatic lesions in male mice, and *H. pylori* causes more severe intestinal-type gastritis and gastric cancer in men and male mice (19, 43, 44). Apparently, those microflora harbored in the non-SPF colony, from which most tumor-bearing mice were derived, did not have as strong an effect on ileal and colonic cancer incidence as *H. pylori* has on gastric cancer. In humans, parity is not recognized as a risk factor for colon cancer. However, it is unclear whether the pregnancy-associated risk in women with IBD can be obscured by decreased numbers of pregnancies due either to reduced fertility or choice and by more preterm births or smaller birth weights (45–47). Although we have found that multiparous female GPX-DKO mice had a significantly higher tumor incidence than nulliparous females because breeder females were on a diet with slightly higher fat content to promote their own and the litters' health and high-fat diet has been implicated in higher colorectal cancer risk (48), we cannot exclude the possibility that the different cancer rate is skewed by different diets. During normal pregnancy and preeclampsia, there is increased lipid peroxidation in multiple organs, which can increase oxidative DNA damage and gene mutations (49–52). Additional analysis will be needed to better evaluate whether parity increases cancer rate in GPX-DKO mice fed with the same 9% fat diet.

There are multiple sources for bacteria-induced oxidative stress. A few strains of commensal bacteria in *Enterococcus spp*. can generate reactive oxygen species in the lumen and cause inflammation-associated cancer in IL-10 KO mice (53–55). Epithelial cells also respond to bacterial colonization by modulating inflammatory responses (7, 9,

56). Colonization with *H. hepaticus* in A/JCr mice induces oxidative DNA damage in the liver and the expression of a subset of immune-related genes, including γ-IFN in cecal epithelium (57–59). *H. pylori* induces H_2O_2 production and cyclooxygenase-2 expression in gastric epithelial cells (60). Although our results are consistent with the notion that *H. hepaticus* is pathogenic in GPX-DKO mice, additional studies are needed to fulfill Koch's postulates and to elucidate the molecular mechanism for bacteria-induced ileocolitis.

The tumor pathology in these mice is unique. Although most of tumors were adenomas, among 13 adenocarcinomas, there were 3 SRCCs. In humans, the occurrence of SRCC in the small intestine or colon is a rare but distinctive event, typically found in younger patients; it carries an adverse prognosis (61, 62). Few murine intestinal cancer models demonstrate signet-ring histology. Among the exceptions are the azoxymethane-induced colorectal cancer model in rats, duodenal polyps of *Smad4*-heterozygous mice, and intestinal tumors in *Smad4/Apc*-double heterozygous mice (16, 63–65). All 3 SRCCs in our GPX-DKO mice still had membranous β-catenin localization, suggesting that the *Wnt* pathway remains intact. This is consistent with the hypothesis that a set of genes other than those in the *Apc*-β-catenin pathway may be mutated in SRCC (62, 64). This result also suggests that mutations in multiple pathways may occur during tumorigenesis in GPX-DKO mouse intestines.

Mutations in the *Apc* gene are a common early event in sporadic colon cancer. Whether *Apc* gene mutations occur as frequently in colitis-associated cancer is less certain (18). Opposing results have been found in mouse IBD models; IL-2/β_2-microglobulin-deficient mice have mutated *Apc* genes in all adenocarcinomas determined by DNA sequence analysis (32), but IL-10 deficient mice do not have *Apc* mutations in all tumors analyzed by immunohistochemistry of APC protein (66). Our results, showing most adenomas and non-SRCC adenocarcinomas have nuclear accumulation of β-catenin, support the notion that mutations in genes in the *Wnt* pathway occur in our inflammation-associated mouse model.

In summary, we have described a new mouse ileocolitis-associated cancer model. This is the first mouse ileal and colonic cancer model produced by a deficiency in antioxidant enzyme levels. Inflammation and cancer occurring in our GPX-DKO mice provide direct evidence, suggesting that intracellular hydroperoxides, which are reduced by GPX-1 and GPX-2, play an important role in cancer initiation, promotion, or progression. However, whether this goes beyond suppression of inflammation is not clear. Although some commensal microflora may be harmless or even beneficial to the host by preventing inflammation in intestinal mucosa (67, 68), certain luminal bacteria may drastically increase cancer incidence.

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Bacteria-Induced Intestinal Cancer in Mice with Disrupted *Gpx1* and *Gpx2* Genes

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