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Influence of myeloperoxidase on colon tumor occurrence in inflamed versus non-inflamed colons of $Apc^{Min/+}$ mice ☆

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ABSTRACT

Control of colorectal cancer needs to be tailored to its etiology. Tumor promotion mechanisms in colitis-associated colon cancer differ somewhat from the mechanisms involved in hereditary and sporadic colorectal cancer. Unlike sporadic or inherited tumors, some experimental models show that colitis-associated colon tumors do not require cyclooxygenase (COX) expression for progression, and non-steroidal anti-inflammatory drugs (NSAIDs) which prevent sporadic or inherited colon cancer do not prevent colitis-associated colon cancer. We report that myeloperoxidase (MPO), an ancestor of the COX isoenzymes, is a determinant of colitis-associated colon tumors in $Apc^{Min/+}$ mice. During experimentally induced colitis, inhibition of MPO by resorcinol dampened colon tumor development. Conversely, in the bowels of $Apc^{Min/+}$ mice without colitis, resorcinol administration or 'knockout' of MPO gene coincided with a slight, but discernible increase in colon tumor incidence. Acrolein, a by-product of MPO catalysis, formed a covalent adduct with the phosphatase tensin homolog (PTEN) tumor suppressor and enhanced the activity of the Akt kinase proto-oncogene in vitro and in vivo. Thus, MPO may be an important determinant of diet and inflammation on colon cancer risk via its effect on endogenous exposure to oxidants and acrolein. We propose a hypothetical model to explain an apparent dichotomy between colon tumor occurrence and MPO inhibition in inflamed versus non-inflamed colons.

1. Introduction

Chronic ulcerative colitis is an independent risk factor for colorectal cancer [1,2]. Consistent with epidemiological observations in humans, experimentally-induced colitis increases colon tumor progression in the $Apc^{Min/+}$ mouse, a model of intestinal cancer [3–7]. While the molecular pathways of inherited, sporadic and colitis-associated colon tumor progression overlap, they are not identical [8,9]. Tumor formation in some mouse models of colitis-associated colon cancer does not require cyclooxygenase (COX) expression [10]. In a study using COX-1 and COX-2 'knockout' mice, investigators concluded that the mechanism of colorectal tumor promotion in colitis-associated cancer differs from the mechanism of tumor promotion for hereditary and sporadic colorectal cancer [10]. However, the exact role of prostaglandins in colitis associated colorectal cancer models varies with the model [11]. Additionally, the pharmacological rationale for colon cancer prevention with non-steroidal anti-inflammatory drugs (NSAIDs) does not apply to colitis-associated tumors [12,13]. In fact, NSAIDs aggravate inflammation and malignant progression in rodent models of colitis-associated tumors [14–16], albeit with some exceptions [17]. One NSAID, 5-aminosalicylic acid, can maintain remission of ulcerative colitis, which ought to prevent colitis-associated colon cancer. However, that hypothesis is unproven despite many attempts at validation [12,18].

Myeloperoxidase (MPO), an ancestor of COX enzymes [19], helps gut associated lymphoid tissue defend against harmful enteric microbes, while tolerating harmless commensal bacteria and dietary antigens. Because MPO activity correlates with the severity of experimentally induced colitis [20] and its expression is an

Abbreviations: Apc, adenomatous polyposis coli; BME, β-mercaptoethanol; COX, cyclooxygenase; DTT, dithiothreitol; DSS, dextran sodium sulfate; ECL, enhanced chemiluminescence; EPO, eosinophil peroxidase; FBS, fetal bovine serum; HRP, horse radish peroxidase; MBTH, 3-methyl-2-benzothiazolinone hydrazone hydrochloride; MEM, modified Eagle's medium; MPO, myeloperoxidase; NSAIDs, non-steroidal anti-inflammatory drugs; PBS, phosphate buffered saline; PI3, phosphatidylinositol; PTEN, phosphatase and tensin homolog on chromosome 10; PVDF, polyvinylidene difluoride; WT, wild type

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indicator of colon cancer risk [21] we investigated its influence on colon tumor development in $Apc^{Min/+}$ mice. We report that elevated MPO activity in the inflamed bowels of $Apc^{Min/+}$ mice correlated with greater colon tumor occurrence; inhibition of MPO activity in inflamed colons of $Apc^{Min/+}$ mice partly suppressed colon tumor occurrence. Conversely, tumors were absent or rare in non-inflamed colons with low, basal MPO activity in $Apc^{Min/+}$ mice. Unexpectedly, either pharmacological or genetic suppression of basal MPO activity correlated with a small, but discernible rise in colon tumors in $Apc^{Min/+}$ without colitis. Thus, the relationship between MPO activity and colon tumor occurrence in $Apc^{Min/+}$ mice varies with the status of inflammation in the gut. Our mechanistic experiments found that a carcinogenic by-product of MPO catalysis, acrolein, formed a protein adduct with phosphatase tensin homolog (PTEN) in colonocytes isolated from the inflamed bowel of $Apc^{Min/+}$ mice. Modification of the PTEN tumor suppressor coincided with activation of the Akt kinase proto-oncogene, which favors cell growth and survival. Since acrolein can originate endogenously from MPO mediated oxidation of threonine or serine [22] this mechanism may contribute to complex effects of diet on inflammation and colon cancer risk.

2. Materials and methods

2.1. Reagents

The following were used: resorcinol, acrolein, L-threonine, hydrogen peroxide (H_2O_2) and 3-methyl-2-benzothiazolinone hydrazone hydrochloride monohydrate (MBTH) (Sigma Aldrich, St.

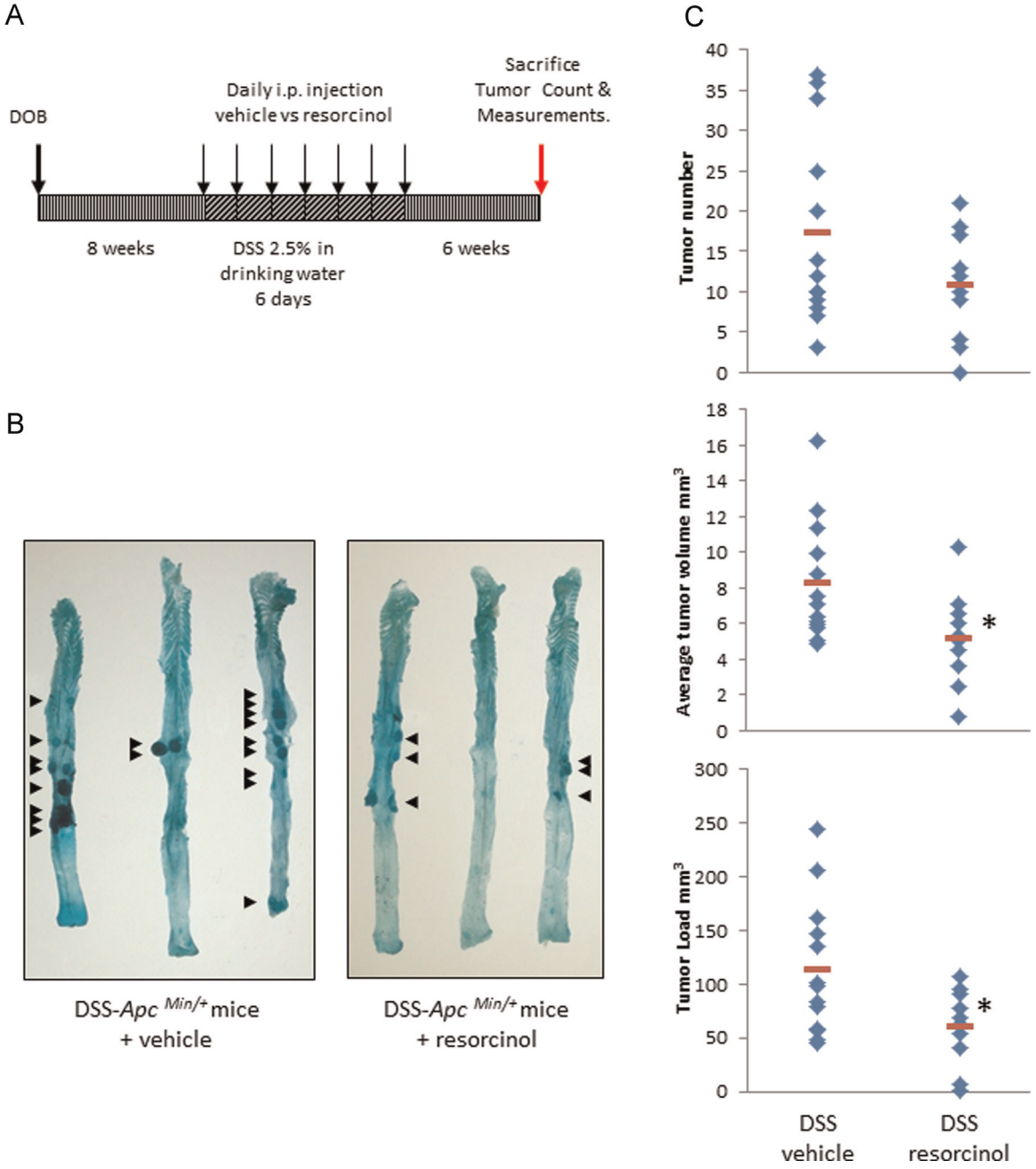

Fig. 1. Myeloperoxidase and colitis-associated colon tumors in $Apc^{Min/+}$ Mice. Panel A: protocol for induction of colitis with dextran sodium sulfate (DSS) and modulation of MPO activity with resorcinol in $Apc^{Min/+}$ mice. Panel B: colitis-associated tumors (arrows) in representative colons from DSS-$Apc^{Min/+}$ mice treated with vehicle or resorcinol. Panel C: graphs of colon tumor numbers, volumes and loads in colons from DSS-$Apc^{Min/+}$ mice treated with vehicle ($n=13$) or resorcinol ($n=10$).

Louis, MO); recombinant MPO (Athens Research & Technology, Athens, GA); myeloperoxidase activity EnzChek® assay kits; complete protease inhibitor mixture (Roche Molecular Biochemicals, Indianapolis, IN); lysis buffer A, 0.6% Igepal CA-630 in PBS (Promega, Madison, WI); wortmannin, primary antibodies against PTEN, Akt, phospho-(Ser^{473})Akt (Cell Signaling Technologies, Danvers, MA); rabbit polyclonal antibodies to PTEN (Upstate, Lake Placid, NY); anti-acrolein adduct antibody (Cosmo Bio Co. Ltd., Tokyo, Japan); horseradish peroxidase-conjugated (HRP) secondary antibodies and protein A/G PLUS-Agarose (Santa Cruz Biotechnology; Santa Cruz, CA); polyvinylidene difluoride (PVDF) membranes and Western Lightning chemiluminescence reagents (Perkin-Elmer, Waltham, MA); and dextran sodium sulfate, MW 36,000–50,000 (MP Biomedicals, Solon, OH). Lysis buffer B was 50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.2% deoxycholic acid sodium salt, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 5 μg/ml leupeptin, and 5 μg/ml pepstatin A. Authenticated HCT116 colon cancer cells and MCF-7 breast cancer cells were from ATCC (Manassas, VA).

2.2. Mice experiments and husbandry

All mice experiment protocols (Fig. 1A) were approved by the University of Utah Institutional Animal Care and Use Committee. Equal numbers of male and female mice were used throughout the study. Apc$^{Min/+}$ mice (C57BL/6J-Apc^{Min}/J), and their corresponding C57BL/6J wild type littermates were obtained from Jackson Laboratories, Bar Harbor, ME. MPO 'knockout' mice (B6.129 × 1-Mpo^{tm1Lus}/J) procured from Jackson Laboratory were bred on a C57BL/6J background through at least 10 generations. A strain is considered fully congenic after ten generations of backcrossing (N10). Transgenic mice (Apc$^{Min/+}$/MPO$^{-/-}$) on a C57Bl/6J background were identified by PCR amplification of genomic DNA isolated from mouse tails using salt precipitation. All mice, kept in standard housing conditions, and fed 8656 Teklad sterilizable rodent diet (Harlan Laboratories, Denver, CO). Mice were sacrificed by CO_2 asphyxiation.

2.3. DSS induced colitis

At 8 weeks of age Apc$^{Min/+}$ or wild type mice were given drinking water containing 2.5% (*w*/*v*) DSS for 7 days. The occurrence and severity of colitis was measured by quantifying the MPO activity, a proxy for colitis, in the intestinal mucosa of mice [20]. Both peroxidase and chlorination activity were quantified, with chlorination being a more specific index of MPO activity. We used EnzChek® MPO activity assay kits, following the manufacturer's protocol. Briefly, the assay measures selective cleavage of 3′-(*p*-aminophenyl) by hypochlorite ($^{-}$OCl) to yield fluorescein. Stock reagents include 3′-(*p*-aminophenyl) fluorescein (5 mM in dimethyformamide) and 5 mM H_2O_2 prepared fresh daily in phosphate-buffered saline (PBS), pH 7.2. The substrate-cofactor working solution is a combination of 20 μM 3′-(*p*-aminophenyl) fluorescein and 20 μM H_2O_2 in PBS, pH 7.2. Experimental samples (50 μL of tissue lysate) or MPO standards were added to 96-well microplates suitable for measuring fluorescence. MPO catalysis was initiated by adding substrate-cofactor working solution (50 μL) at 25 °C. Plates were covered with tin foil to protect from ambient light. The fluorescence intensity of fluorescein formed by MPO activity in each sample was measured continuously using excitation at 485 nm and emission at 530 nm. MPO activity is proportional to the rate of change of fluorescence in the linear portion of the curve, typically around 30 min after starting the reaction. Reaction rates were calculated after correction using a negative control (boiled samples plus MPO inhibitor). MPO levels were calibrated using serially diluted (1:2) MPO standard solutions ranging from 0 to 200 ng/ml. When resorcinol was used as an inhibitor it was added simultaneously with the substrate co-factor solution.

2.4. Tumor studies

Apc$^{Min/+}$ mice were divided into four groups. Starting at 8 weeks of age two groups of Apc$^{Min/+}$ mice were treated with 2.5% (*w*/*v*) DSS for 7 days, while the remaining two groups were given water. The MPO inhibitor resorcinol (1.25 mg/kg) or saline vehicles (0.20 ml) were injected once daily, i.p., for the duration of the DSS treatment. Mice were allowed to mature a further 6 weeks before being sacrificed. In addition, two separate groups of Apc$^{Min/+}$ and Apc$^{Min/+}$ *x* MPO$^{-/-}$ double mutant mice were matured to the same age as above and sacrificed to compare the effect of pharmacological inhibition of MPO by resorcinol [23] versus a genetic 'knockout' of MPO in a non-colitis background.

2.5. Tissue harvesting and tumor assessment

The small intestine and colon were dissected longitudinally and washed with ice-cold PBS. They were then fixed for 4 h in 10% formalin in PBS, and stored in 70% ethanol at 4 °C. The intestine and colons were stained with methylene blue, and then polyps and colon tumors were counted with the aid of a dissecting microscope. The counting studies were independently performed by two individuals who had no knowledge of the genotype and/or type of treatment used for each mouse. Colon tumors were photographed, the largest and smallest diameters were recorded, and tumor volumes were calculated according to the equation (volume (mm^3) = $\pi/6 \times$ largest diameter × smallest diameter^2). Tumor load was calculated by summing all measured tumor volumes in a mouse.

2.6. Generation of acrolein in situ by MPO

2-Hydroxy-propenal and acrolein (2-propenal) were generated by incubating 1.5 μg MPO (10 nM), 150 mM chloride and 200 μM L-threonine in 1.0 ml of 0.05 M phosphate buffer, pH 7.0, at 37 °C [22]. The reaction was initiated by the addition of 200 μM H_2O_2. For some experiments incubation times were varied, MPO was omitted, or the MPO inhibitor resorcinol (0–100 μM) was added. Formation of 2-hydroxy-propenal and acrolein were quantified spectrophotometrically by measuring the absorbance of their MBTH derivative at 598 nm. The assay was calibrated with acrolein standards. Briefly, a sample of the MPO reaction mixture (200 μl) was incubated for 20 min, 25 °C with 568 μl of sodium phosphate buffer (5 mM, pH 7.0), 25 μl HCl (6 N), and 132 μl of MBTH (155 mM). The derivative was then reacted with 75 μl $FeCl_3$ (370 mM) and incubated for 10 min at 25 °C. The MBTH derivative was stable for at least 5 h.

2.7. Cell culture

HCT116 cells were grown in modified Eagle's medium (MEM) with 10% fetal bovine serum (FBS), 100 units of penicillin/streptomycin, 2 mM L-glutamine, and 1 mM pyruvate. MCF-7 cells were grown in MEM with 10% v/v FBS, 2 mM L-glutamine, 1.5 g/l $NaHCO_3$, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 0.01 mg/ml bovine insulin, and 0.01 mg/ml gentamicin.

2.8. MPO dependent modulation of PTEN–Akt signaling

HCT 116 and MCF-7 cells were grown to ~80% confluency; culture media was removed and replaced with 2 ml of 0.05 M phosphate buffer, pH 7.0, MPO, chloride and threonine as detailed

above. Acrolein formation was initiated with 200 μM H_2O_2 before addition to cells. Cells were exposed to the reaction products at 37 °C for intervals from 0 to 30 min. For some experiments MPO was omitted, or resorcinol (0–100 μM) was added to modulate MPO dependent generation of reactive aldehydes. A phosphatidylinositol-3-kinase (PI3-K) inhibitor, wortmannin, was used as a control to modulate Akt activation. Treated cells were lysed in 250 mM sucrose, 50 mM Tris pH 7.4, 5 mM $MgCl_2$, 1 mM EGTA, 1 × complete protease inhibitor, 2 mM NaF and 2 mM sodium orthovanadate. Lysates from treated cells (15 μg protein) were fractionated on SDS-10% PAGE and proteins were transferred to polyvinyldifluoride membranes for immunoblot analysis of Akt and phospho-(S473) Akt as described [24,25].

2.9. Detection of acrolein-PTEN adducts in colonocytes from inflamed and non-inflamed colons of mice

After treatment of $Apc^{Min/+}$ mice with either vehicle or DSS, ± resorcinol, (Fig. 1A) colons were removed. Colon crypts, and the colonocytes within, were isolated as described [26]. Longitudinally dissected colons were incubated in PBS containing 3 mM EDTA and 50 μM DTT for 90 min at 4 °C. The colons were then washed and vigorously shaken in ice-cold PBS 3–4 times to release intact colon crypts and wash away any leukocytes. Colon crypts and their colonocytes were enriched via centrifugation at 40*g* for 10 min at 4 °C, and then reconstituted in ice-cold cell lysis buffer.

Colon crypts were lysed in buffer B and their PTEN protein was isolated by immunoprecipitation. Briefly, lysates were pre-cleared by incubation with 2 μg of mouse IgG and 20 μl of protein G PLUS agarose at 4 °C for 30 min and centrifuged at 1000*g* at 4 °C for 5 min. Aliquots containing equivalent amounts of total cellular protein were immunoprecipitated using 1 μg of mouse monoclonal anti-PTEN by incubation at 4 °C for 2 h. Immune complexes were precipitated by incubation with 20 μl of protein G PLUS agarose at 4 °C overnight and collected by centrifugation. Immunoprecipitates were washed four times with lysis buffer B, resuspended in Laemmli buffer, separated on a 10% SDS-polyacrylamide gel, and transferred to PVDF membranes. After blocking with 5% powered milk in 10 mM Tris–HCl, pH 7.5, 100 mM NaCl, and 0.1% Tween 20, acrolein-modified PTEN was detected with the monoclonal antibody mAb5F6 (1:5000 dilution) [27] followed by horseradish peroxidase-conjugated mouse anti-goat IgG (1:10,000 dilution) followed by detection with ECL Plus®. Blots were stripped using buffer containing 62.5 mM Tris–HCl, pH 6.7, 100 mM 2-mercaptoethanol, 2% SDS, at 55 °C for 30 min, and reprobed with the rabbit polyclonal antibodies to PTEN and visualized with HRP-conjugated secondary antibody followed by ECL Plus®.

2.10. Western immunoblotting

Samples were dissolved in 50 μl of Laemmli loading buffer, 0.5% BME and heated at 95 °C for 10 min. Samples (15–30 μg protein) were fractionated by SDS-PAGE and transferred to polyvinyldifluoride membranes. Membranes were blocked with 5% *w*/*v* nonfat dry milk in tris buffered saline with Tween 20, and then incubated for 16 h at 4 °C with primary antibodies, followed by HRP-conjugated secondary antibody (1:5000). Antigen–antibody complexes were detected with Western Lightning ECL reagents. The intensity of chemiluminescent protein-antibody complexes was quantified with a Kodak Image Station 440. Bar graphs depict the mean ± standard error from densitometric analyses of separate experiments.

2.11. Statistical analysis

Data was analyzed using the Microsoft Excel statistical package. A two-tail homoscedastic or heteroscedastic unpaired Student's *t*-test was used. A p-value of less than 0.05 was considered statistically significant.

3. Results

3.1. Leukocyte MPO activity in DSS-induced colitis correlates with colon tumor occurrence in $Apc^{Min/+}$ mice

At 10–12 weeks after birth, $Apc^{Min/+}$ mice fed a standard diet and normal drinking water have intestinal polyps, but few or no colon tumors [28]. In our experiments 42% of $Apc^{Min/+}$ control mice were free of any colon tumors and the others had only 1.3 colon tumors per mouse (range 1–3 colon tumors/mouse).

$Apc^{Min/+}$ mice exposed to 2.5% *w*/*v* DSS in drinking water have increased colon tumor occurrence [29]. In our protocol all mice developed colon tumors, secondary to their colitis (100% incidence). The colon tumor number rose 13-fold to a mean = 17.3 tumors per colon, range 3–37; with a median tumor volume = 8.3 mm^3, range 4.9–16.2 mm^3 and an average tumor load = 112.8 mm^3, range 44.9–245 mm^3. Leukocyte MPO activity in the colon, a proxy for colitis [22], rose 2.5-fold in DSS-treated mice compared to controls given normal drinking water.

Resorcinol, administered once daily (1.25 mg/kg, i.p.) for 7 days [Fig. 1A], inhibited MPO activity by ~50% on day 1 and 3, and 75% on day 6 in $Apc^{Min/+}$ mice with DSS colitis [Table 1]. On day 6, leukocyte MPO activity in the mucosa of mice treated with DSS plus resorcinol was comparable to basal MPO activity in the mucosa from $Apc^{Min/+}$ mice given normal drinking water. Corresponding with its suppression of MPO activity in the inflamed gut, resorcinol lessened colitis-associated colon tumor development [Fig. 1B and C]. Resorcinol reduced incidence by 10% and tumor multiplicity and tumor volume by 38%. Resorcinol-treated mice had a mean tumor number = 10.7, range 0–21, and a median tumor volume = 5.2 mm^3, range 0.8–10.3 mm^3. As mentioned above, the vehicle control mice had a comparative mean tumor number = 17.3, range 3–37, and a median tumor volume = 8.3 mm^3, range 4.9–16.2 mm^3. Resorcinol also reduced tumor load by 46%. Resorcinol-treated mice had an average tumor load = 60.7 mm^3, range 0.8–106.6 mm^3; while the control mice had an average tumor load = 112.8 mm^3, range 44.9–245 mm^3. In these same mice, resorcinol reduced the number of polyps in the small intestine, by 20%, but this was not statistically significant.

3.2. MPO activity in the non-inflamed gut of $Apc^{Min/+}$ mice correlates inversely with colon tumors

$Apc^{Min/+}$ mice fed a standard diet and normal drinking water-with no DSS-had few or no colon tumors [Fig. 2, upper panel]. Administration of resorcinol (1.25 mg/kg) to these mice was associated with a small but discernible increase in colon tumor incidence from 58% during vehicle treatment, to 85% during

Table 1
MPO activity* in colon crypts from Apc $^{Min/+}$ mice treated with DSS ± resorcinol.

	DSS control	DSS + resorcinol	% MPO inhibition
Day 1	53.7 ± 8.1 (*n* = 3)	29.0 ± 4.3 (*n* = 3)	46.0 ± 8.0
Day 3	59.9 ± 7.5 (*n* = 3)	33.1 ± 2.2 (*n* = 3)	44.7 ± 3.6
Day 6	36.8 ± 8.4 (*n* = 6)	9.0 ± 2.1 (*n* = 6)	75.5 ± 5.7

* Mean ± std. dev. (pg MPO/μg protein).

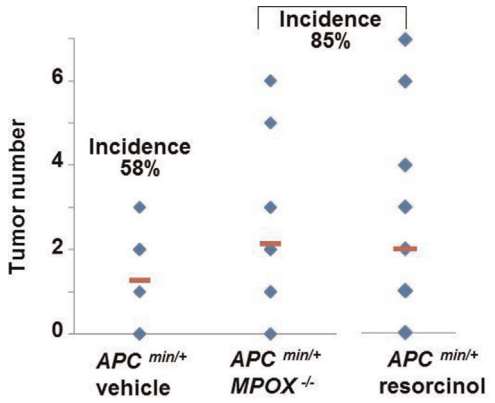

Fig. 2. Effects of resorcinol and genetic 'knockout' of myeloperoxidase on inherited colon tumors in $Apc^{Min/+}$ mice without DSS-colitis. Tumor numbers in colons from $Apc^{Min/+}$ mice treated with vehicle ($n=15$); $Apc^{Min/+}/MPO^{-/-}$ treated with vehicle ($n=16$) or $Apc^{Min/+}$ mice treated with resorcinol ($n=14$) administered at times and doses shown in Fig. 1A. Incidence of tumor occurrence was 58%, 85% and 85%, respectively.

resorcinol treatment. The number of tumors rose from a mean=1.3 (range 0–3) in mice treated with normal drinking water, to a mean=2 (range 0–7) in corresponding $Apc^{Min/+}$ mice treated with resorcinol [Fig. 2, upper panel]. Exposure to resorcinol did not alter the tumor volume or tumor load in these mice under our protocol (not statistically different, $p>0.05$) [Fig. 2, middle and lower panels]. We draw attention to the fact that fewer tumors occur in non-inflamed colons (Fig. 2), compared to inflamed colons (Fig. 1).

Results in Fig. 2 imply that resorcinol may have impeded any role that MPO has in anti-tumor host-defense processes under basal conditions in the colon of $Apc^{Min/+}$ mice. Alternatively, resorcinol, or its metabolites, in $Apc^{Min/+}$ mice may have been carcinogenic. To address this issue we examined colon tumor formation in $Apc^{Min/+}/MPO^{-/-}$ mice fed a standard diet and normal drinking water. In these mice genetic 'knockout' of the MPO gene heightened colon tumor incidence and number to the same extent (85%) as pharmacological suppression of MPO by resorcinol [Fig. 2]. Like resorcinol, 'knockout' of the MPO gene only altered tumor incidence and number, but not the tumor volume or tumor load in $Apc^{Min/+}$ mice in our protocol ($p>0.05$).

3.3. Acrolein, a by-product of threonine and MPO catalysis, forms an adduct with PTEN, thereby enhancing Akt kinase proto-oncogene signaling

Reactive lipid-enals and -enones generated by COX or lipoxygenase enzymes during inflammation can irreversibly modify the PTEN tumor suppressor, inactivate its inositol phosphatase activity, and thereby nullify its restraint of Akt proto-oncogene signaling [24,25]. MPO can generate acrolein, a prototypical-enal carcinogen [30], from hydroxyl-amino acids at sites of inflammation [22] [Fig. 3A and B]. When HCT 116 or MCF 7 cells were exposed in situ to acrolein generated by MPO, threonine, hydrogen peroxide, and chloride ions, the cells accumulated higher amounts of phospho-Ser^{473}Akt, an index of Akt kinase activation. The MPO inhibitor, resorcinol, suppressed the formation of cellular phospho-Ser^{473}-Akt [Fig. 3C] to about the same extent as 0.5 μM wortmannin, an inhibitor of phosphatidylinositol-3-kinase.

During DSS-induced colitis, inflammation may expose colonocytes of $Apc^{Min/+}$ mice to the processes depicted in Fig. 3. Consistent with this mechanistic hypothesis, colon crypts isolated from inflamed bowels of $Apc^{Min/+}$ mice contained PTEN protein that was post-translationally modified by acrolein [Fig. 4A]. Formation of acrolein-PTEN adducts in colonocytes also corresponded with Akt kinase activation (increased phospho-Ser^{473}-Akt levels) and with the MPO activity in the samples [Fig. 4B].

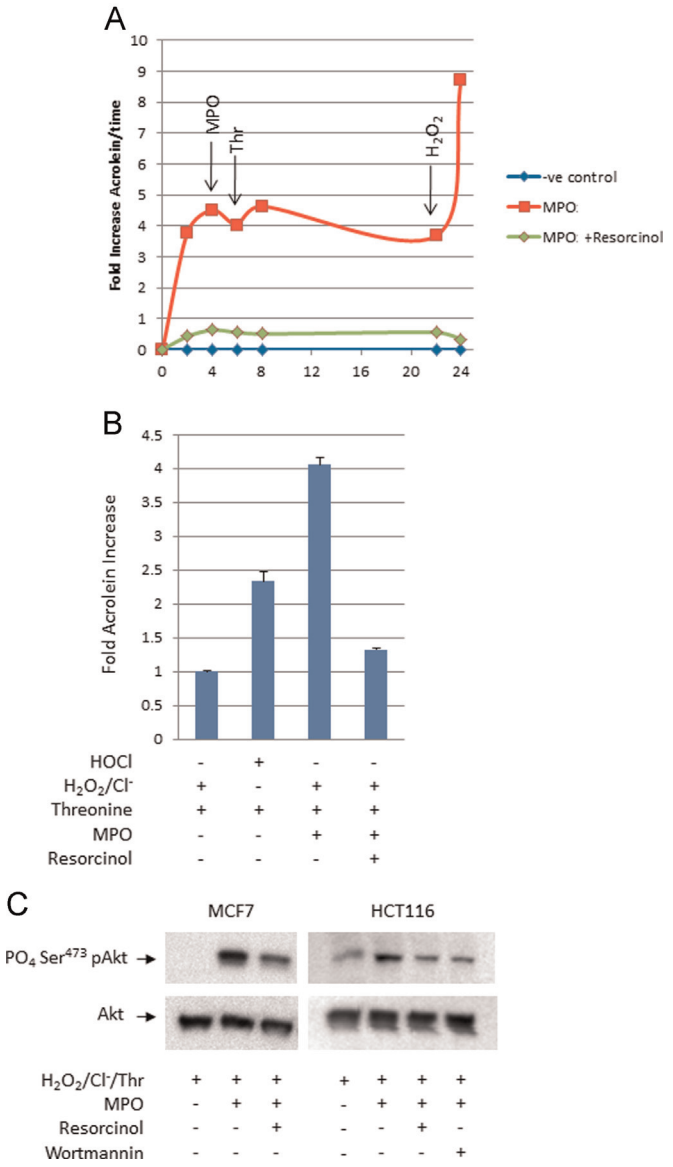

Fig. 3. Myeloperoxidase enhances cellular Akt kinase activation via acrolein generation in vitro. Panel A. Time-course of acrolein generation via MPO-mediated formation of threonine chloramine. Active MPO+substrate —■—; active MPO+substrate+resorcinol —◆—; substrate alone —◆—. Arrows indicate supplemental addition of MPO, threonine, or H_2O_2 to determine the relationship between their consumption and acrolein production: depletion of H_2O_2 limited acrolein generation. Panel B. Relative levels of acrolein generated via chemical (HOCl) versus MPO mediated formation of threonine chloramine. These data correspond with experiments shown in panel C. Panel C. Immunochemical analysis of phospho-Ser473 Akt, a biomarker of cellular Akt activation in HCT 116 and MCF 7 cells exposed to acrolein via MPO-mediated formation of threonine chloramine±resorcinol and wortmannin.

4. Discussion

Colon tumor occurrence correlates with high MPO activity found in DSS-inflamed colons of $Apc^{Min/+}$ mice. MPO can generate oxidants that cause DNA damage, and mutagenesis [30]; it can also generate acrolein as an oxidative by-product from unsaturated fats, serine, or threonine [31]. Acrolein–protein adducts in colon

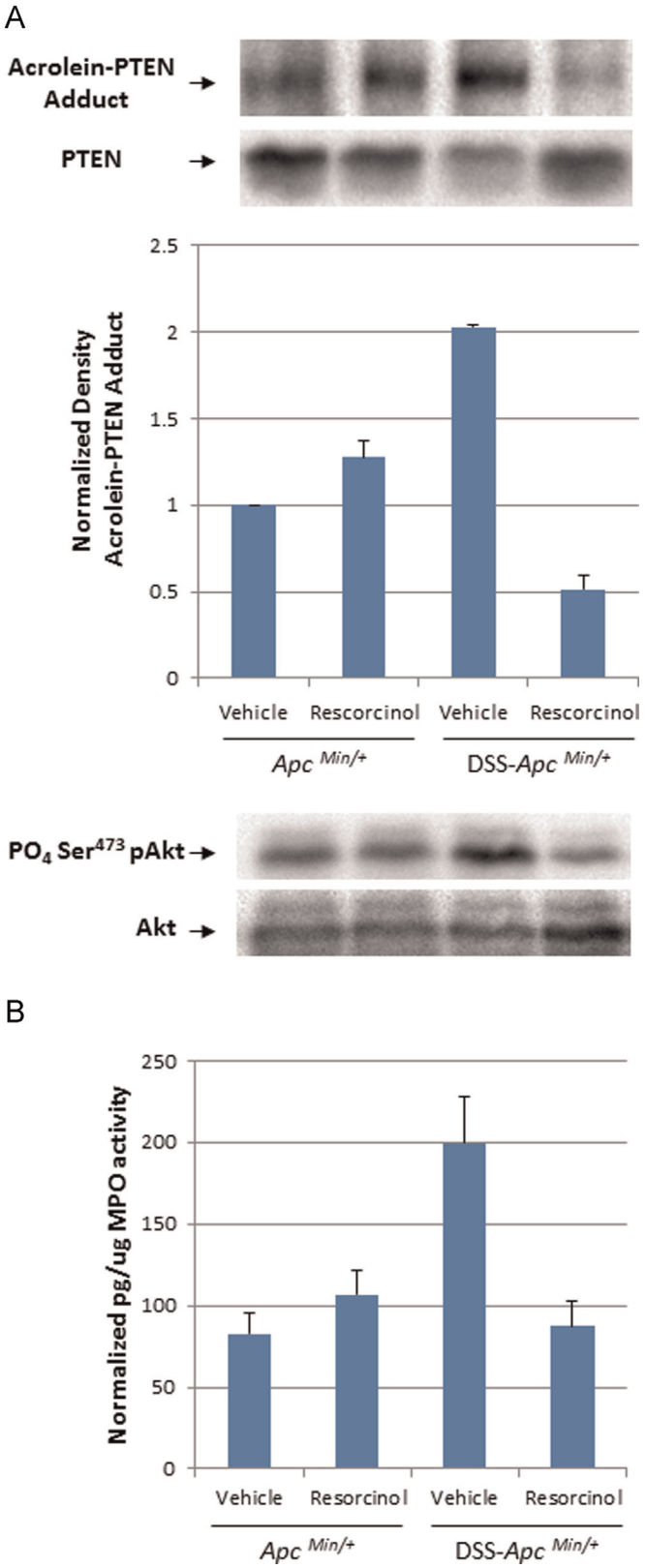

Fig. 4. Post-translational modification of the PTEN tumor suppressor and activation of cellular Akt kinase activation in colonocytes isolated from $Apc^{Min/+}$ mice. Panel A. Immunoblot and bar graph of acrolein-PTEN adducts in the total PTEN content isolated from colonocytes of $Apc^{Min/+}$ mice or DSS-$Apc^{Min/+}$ mice treated with vehicle or resorcinol, respectively. The lower immunoblot depicts phospho-Ser^{473} Akt, a biomarker of cellular Akt activation in the same samples of colonic mucosa. Panel B. MPO activity in the mucosa of $Apc^{Min/+}$ mice or DSS-$Apc^{Min/+}$ treated with vehicle or resorcinol, respectively.

tissue are associated with the transition from benign to malignant colon tumors in humans [32]; however, little is known about the identity of proteins that form acrolein adducts, or their role in the etiology of colon tumor progression. In colonocytes isolated from $Apc^{Min/+}$ with inflamed colons we found that PTEN, a prominent intestinal tumor suppressor, formed a protein adduct with acrolein. PTEN modification coincided with Akt proto-oncogene activation in these colonocytes. Reactive nitrogen species also reportedly oxidize PTEN and disable its restraint of Akt kinase signaling [33]. Thus, MPO metabolites or by-products may augment colitis-associated colon tumor occurrence via genetic and energetic mechanisms [34]. Genetically, oxidants and acrolein have mutagenic potential [30,35]. Energetically, oxidants and acrolein enhance anabolic signaling via the PI3-kinase–PTEN–Akt kinase axis. While speculative, this latter mechanism can account for the increased size of colon tumors reported in the DSS-$Apc^{Min/+}$ model [3,5].

Colon tumors are absent or rare in non-inflamed colons of $Apc^{Min/+}$ mice with physiologically basal MPO activity. Under these conditions, inhibition or genetic deletion of MPO coincided with a small but discernible rise in colon tumors. Thus, there is a dichotomy between MPO activity and colon tumor occurrence in $Apc^{Min/+}$ mice. During DSS-induced colitis, high MPO activity favors colon tumor development; inhibition of MPO during DSS-colitis partly prevents colon tumors. However, under basal conditions – without DSS-colitis – inhibition or deletion of MPO activity favors colon tumor occurrence, suggesting that $Apc^{Min/+}$ mice may rely on basal MPO activity for mucosal host defense against tumors [36]. The occurrence of colon tumors that coincided with inhibition of basal MPO activity may relate to the disposition of H_2O_2, its substrate. Ordinarily, consumption of H_2O_2 by MPO may help restrain peroxide 'tone' below the level needed for optimal COX activity [37]. Accordingly, inhibition or deletion of MPO activity in non-inflamed bowels may allow H_2O_2 to accumulate and reach levels supporting COX-mediated or H_2O_2-mediated colon tumor progression [38]. Fig. 5 depicts a speculative model to explain the apparent dichotomy between tumor occurrence and inhibition of MPO activity in inflamed versus non-inflamed colons. A similar dichotomy exists in humans. On one hand, MPO protein levels correlate directly with colorectal cancer progression [21], and colon tumors have elevated MPO activity compared to non-inflamed colonic mucosa [39,40]. On the other hand, malignancies have been associated with human MPO deficiency and MPO deficient neutrophils fail to destroy malignant cells [41,42].

Resorcinol causes irreversible, H_2O_2-dependent loss of peroxidase activity in various heme-containing peroxidases [43], including both MPO and EPO. Strictly speaking, we cannot exclude a contribution from other granulocytes, such as eosinophils, in DSS-induced colitis in Apc^{Min+} mice. A role for eosinophil peroxidase (EPO) in DSS- induced colitis has been established using EPO 'knockout' mice ($EPO^{-/-}$) [23]. Environmental and genetic variation can modify mouse models of disease [44–47], consequently results with ($EPO^{-/-}$) mice on a 129/Ola/Hsd × 129/SvJ background, do not extrapolate seamlessly to $Apc^{Min/+}$ mice on a C57Bl/6J background. Generally, the involvement of neutrophils in DSS colitis in C57/Bl6J mice including $Apc^{Min/+}$ mice is well established [3–7]. It should be stressed that the investigators studying the role of eosinophils in DSS colitis were, themselves, unable to exclude a contribution from neutrophils [23].

Consistent with gut's dual role as a digestive organ and a lymphoid organ, diet and inflammation can modify colon cancer risk in complex ways. To interpret these complexities Bruce et al. [34] have proposed an intriguing model that integrates two separate mechanisms: (1) disruption of cellular energetic signaling processes, leading to growth and proliferation; (2) focal loss of

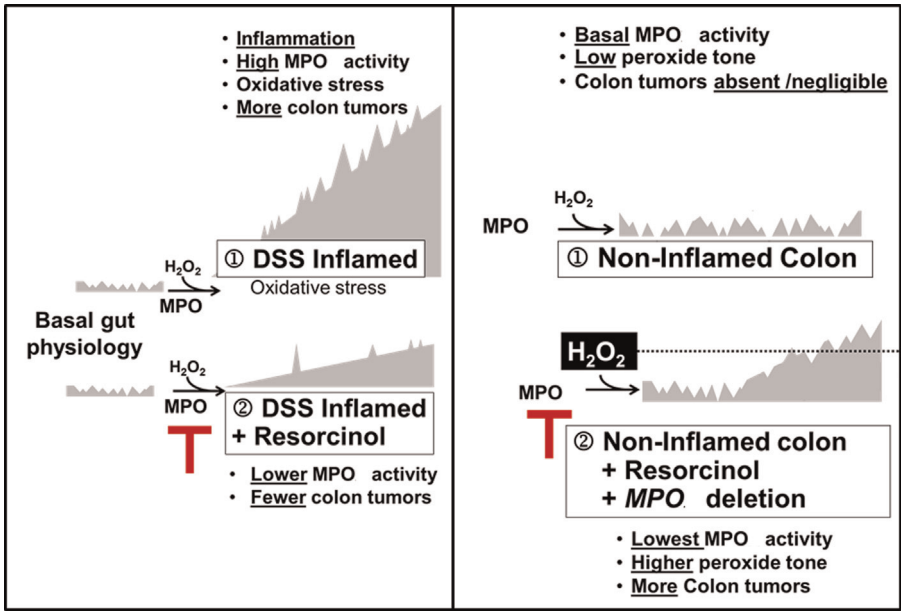

Fig. 5. Legend. Hypothetical model of the relationship between MPO activity and colon tumor occurrence in inflamed colon (left) and non-inflamed colon (right).

epithelial barriers, inflammation and oxidative stress, leading to proliferation and mutation. Our results lend support and refinement to this model. In evaluating cancer risk Swenberg et al. [48] and Wild [49] have championed a comprehensive approach which considers all exposure events – both exogenous and endogenous. This includes electrophilic molecules that are generated in living cells and organs through normal physiology, lifestyle, and nutrition. Our results suggest that MPO is an aspect of the 'exposome' [49] that deserves attention in the etiology and control of colorectal cancer [50–52].

Animal experiments

Approved and performed under the University of Utah IACUC Protocol number 09-02001.

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