Pro-oxidant environment of the colon compared to the small intestine may contribute to greater cancer susceptibility

Lisa M. Sandersa, Cara E. Hendersona, Mee Young Honga, Rola Barhoumib, Robert C. Burghardtb, Raymond J. Carrolla,b,c, Nancy D. Turnera,b, Robert S. Chapkina,b, Joanne R. Luptona,b,*

aFaculty of Nutrition, Texas A & M University, 2471 TAMU, College Station, TX 77843-2471, USA
bCenter for Environmental and Rural Health, Texas A & M University, College Station, TX, USA
cDepartment of Statistics, Texas A & M University, College Station, TX 77843-3143, USA

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Abstract

The colon and small intestine have inherent differences (e.g. redox status) that may explain the variation in cancer occurrence at these two sites. This study examined basal and induced (oxidative challenge) reactive oxygen species (ROS) generation, antioxidant enzyme activity and oxidative DNA damage. Basal ROS and antioxidant enzyme activities in the colon were greater than in the small intestine. During oxidative stress, 8-oxo-deoxyguanosine (8-oxodG) DNA adducts in the colon exceeded levels in the small intestine concomitant with increased ROS. Thus the colon responds to oxidative stress less effectively than the small intestine, possibly contributing to increased cancer incidence at this site.

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1. Introduction

It is predicted that the cases of colorectal cancer this year will outnumber small intestine cancer cases 28 to 1 [1], despite the fact that the small intestine has over three times the surface area of the colon. While the variation in cancer occurrence between these two intestinal sites is not completely understood, environmental differences such as the presence or absence of bacterial microflora, and intrinsic differences such as the rate of cell turnover have been examined [2,3]. Yet, these differences provide an incomplete explanation for the variation in cancer susceptibility. For example, cell turnover involves the proliferation of cells as well as cell death. The more rapid rate of cell death in the small intestine as compared to the colon should infer protection against cancer development as potentially tumorigenic cells could be quickly eliminated. However, the small intestine also has a greater rate of cell proliferation which may promote tumorigenesis as transformed
cells must propagate for tumors to develop [4]. One inherent difference which may play a critical role in the tumorigenic process that has not been well investigated is the oxidative environment of these two tissues.

Damage to DNA from ROS is a consequence of oxidative stress, and several oxidative DNA adducts, including 8-oxodG, have been implicated in the tumorigenic process [5–8]. Oxidative stress exists when pro-oxidants such as ROS exceed antioxidant capabilities. This environment can result from increased generation of ROS as well as impaired removal of ROS by antioxidant defenses such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) enzyme systems (Fig. 1). Differences in ROS generation or antioxidant enzyme activities between the small intestine and colon may alter the levels of oxidative DNA damage, thus contributing to the variation in cancer susceptibility at these two intestinal sites.

The current study evaluated the differences in ROS generation, antioxidant enzyme activities and oxidative DNA adducts in the small intestine and colon in an effort to explain, in part, the dramatic difference in cancer occurrence at these two similar organ sites.

2. Materials and methods

2.1. Materials

Reagents for cell isolation were obtained from Sigma (St. Louis, MO) with the exception of Ca\(^{2+}\)- and Mg\(^{2+}\)-free phosphate buffered saline (PBS) that was obtained from GibcoBRL (Grand Island, NY) and glutamine obtained from Life Technologies (Rockville, MD). Chloromethyl-2,7-dichlorodihydrofluorescein diacetate (CM-H\(_2\)DCFDA) and ethidium homodimer-1 were purchased from Molecular Probes (Eugene, OR). Fragment Length Analysis using Repair Enzymes (FLARE) kits were purchased from Trevigen (Gaithersburg, MD). Antioxidant enzyme kits (CAT, GPx and SOD) and the SOD standard were from Calbiochem (San Diego, CA). Coomassie Plus protein kits were obtained from Pierce Biotechnology (Rockford, IL).

2.2. Animals and diets

Animal protocols were approved by the University Animal Care Committee of Texas A & M University and conformed to the National Institutes of Health guidelines (NRC 1985). Sixty male weanling (28-day old) and forty 9-month old Sprague-Dawley rats (Harlan Sprague Dawley, Houston, Texas) were housed individually in raised wire cages and maintained in a temperature- and humidity-controlled animal facility with a daily photoperiod of 12 h light and 12 h dark. The rats were stratified by body weight and assigned to one of four defined diets (25 rats/diet), which were consumed for 2 weeks.

2.3. Cell isolation

Enterocytes and colonocytes were isolated based on a procedure by Zoran et al. [9]. After rats were euthanized the colon and small intestine were removed and flushed with warm Ca\(^{2+}\)- and Mg\(^{2+}\)-free PBS. The first 10 cm of small intestine and last half of the colon were taken as duodenum and distal colon, respectively. Each segment was cut
longitudinally to expose the lumen and placed in warm (37 °C) Ca and Mg free Hank’s Balanced Salt Solution (HBSS), 30 mM EDTA, 5 mM dithiothreitol (DTT), 0.1% fatty acid free BSA, 1 mM glutamine and 1 mM butyrate (pH 7.4). Following a 15 min shaking incubation, the mucosal side of each segment was gently scraped with a rubber policeman to remove surface epithelial cells as well as intact intestinal crypts [10]. Removal of crypts and surface cells was confirmed by histological examination of remaining intestinal tissue following the scraping procedure. The isolated epithelial cells were washed in warm HBSS containing Ca²⁺ and Mg²⁺, 0.1% BSA, 1 mM glutamine, and 1 mM butyrate. Two aliquots of cells from the duodenum and distal colon were taken for antioxidant enzyme analysis and FLARE analysis [11]. The remaining cell suspensions were used for ROS analysis as described below.

2.4. Detection of reactive oxygen species

Isolated enterocytes and colonocytes (maintained at 37 °C) were divided into two treatment groups, which received either an oxidative challenge of 50 μM H₂O₂ for 5 min or no treatment (basal). This concentration of H₂O₂ was chosen as it was sufficient to create an oxidative stress without being toxic to the cells. Exposure time was necessarily limited due to the short-term viability of cells ex vivo. Samples were prepared in duplicate and incubated for 15 min with CM-H₂DCFDA, a fluorescence probe sensitive to such cellular oxidants as hydrogen peroxide (H₂O₂), hydroxyl radicals (OH·), and peroxyl radicals (OOH·). This probe passively diffuses into cells and upon oxidation by ROS forms a fluorescent adduct which remains trapped in the cell. Fluorescence was monitored on a Meridian Ultima confocal microscope (Meridian Instruments, Okemos, MI) with a 530 nm barrier filter and laser excitation at 488 nm, as previously described [12]. Intensity of fluorescence is used as an indirect measure of prevalence of ROS. Data for each sample were collected from 15 fields/treatment/rat. Viability of the cells used for analysis was determined after each treatment by staining with ethidium homodimer-1. Mean viability was 81 ± 4.5%.

2.5. Measurement of oxidative DNA damage using the FLARE assay

This assay is a modification of the single cell gel electrophoresis (comet) assay, which uses fpg to introduce DNA strand breaks at 8-oxo-deoxyguanosine (8-oxoG), a prevalent and potentially mutagenic oxidative adduct. This process allows measurement of 8-oxoG on a single cell level in intact nuclei thus reducing the confounding factor of artifactual adducts [11]. Isolated surface epithelial cells and crypts from the duodenum and distal colon were divided into two treatment groups receiving either an oxidative challenge of 50 μM H₂O₂ for 5 min or no treatment (basal). Intact crypts were broken into single cells by aspiration through 27 gauge needle and plated with agarose in duplicate on comet slides. Slides were then exposed to a lysis buffer, followed by immersion in 1 × FLARE buffer according to the kit protocol. Slides were treated with fpg enzyme diluted 1:50 with reaction buffer and a control slide received reaction buffer without fpg enzyme. Following treatment with alkali solution, the slides were exposed to electrophoresis (1 V/cm, 20 min), immersed briefly in 70% ethanol and stored horizontally. Nuclei were viewed by epifluorescence microscopy using SYBR green staining. Quantitation of the relative tail moment (tail moment/(tail moment + head moment) × 100) [13] was measured using the Metamorph Imaging System (v.4.6r3, Universal Imaging Corp., Downington, PA) and a macro designed by Nikon. One hundred randomly selected cells were analyzed per treatment group for each rat.

2.6. Measurement of antioxidant enzyme activity

Activities of CAT, GPx, and SOD in isolated enterocytes and colonocytes during basal condition (no H₂O₂ treatment) were measured spectrophotometrically using assay kits from Calbiochem. Cell lysates were prepared by homogenization of cells in 50 mM potassium phosphate buffer (250 mM sucrose/1 mM EDTA/1 mM DTT/0.1% Triton X-100) followed by centrifugation for 3 min at 10,000 × g. Supernatant was used for enzyme assays following protocols provided in the kit. Briefly, SOD activity was determined by measuring the rate of generation of a chromophore at 525 nm. CAT activity was
determined by measuring absorbance of quinone-imine dye at 520 nm. GPx was determined indirectly by oxidation of NADPH to NADP⁺ measured at 340 nm. (Due to use of cell lysates H₂O₂ was the preferred substrate over tert-butyl hydroperoxide as this compound is also a substrate for some glutathione transferases [14]. Sodium azide (NaN₃) was used to inhibit CAT competition for H₂O₂). Samples were assayed in triplicate in 96 well microplates with standards provided in kits or purchased separately. Microplates were read on a Spectra Max 250 microtiter plate reader using SoftMax Pro v.1.2 software (Molecular Devices, Sunnyvale, CA). Activity was normalized to protein concentration.

2.7. Statistical analysis

Analysis of ROS, antioxidant enzyme activity and oxidative DNA damage data was performed by analysis of variance (ANOVA) using SAS 8.0 (SAS Institute, Inc). Results were considered significant at \( P < 0.05 \). The results presented here were not affected by the diet or age of the animal.

3. Results

3.1. Generation of reactive oxygen species

The basal levels of ROS, as determined by indirect fluorescence microscopy with the probe CMH₂DCFDA, in the epithelial cells of the colon were significantly greater than the ROS levels found in the small intestine \( (P < 0.0001) \) (Fig. 2). As shown in the same figure, when exposed to an oxidative challenge of 50 \( \mu \text{M} \) H₂O₂, the colon continued to maintain greater levels of ROS as compared to the small intestine \( (P < 0.0001) \). Both tissues experienced a significant increase in ROS over basal levels with the addition of an exogenous oxidant stress \( (P < 0.0001) \).

3.2. Oxidative DNA damage

Levels of 8-oxodG as measured by the FLARE assay were used as a marker of oxidative DNA damage. During basal conditions (without H₂O₂ treatment), the level of oxidative DNA damage did not differ between the small intestine and colon (Fig. 3). However, in an oxidatively stressful environment, the difference in damage between the colon and small intestine was enhanced. During exogenous stress with 50 \( \mu \text{M} \) H₂O₂, the colon exhibited significantly greater oxidative DNA damage compared to the small intestine \( (P < 0.038) \). Both tissues exhibited a significant increase in oxidative DNA damage over basal levels with the addition of an exogenous oxidant stress \( (P < 0.0001) \).

3.3. Antioxidant enzyme activity

Antioxidant enzyme activities for SOD, CAT and GPx were greater in the colon as compared to the small intestine \( (P < 0.008, P < 0.006, P < 0.0001 \) respectively) (Fig. 4). CAT and SOD displayed 20–35% greater activity in the colon than the small intestine, while GPx activity was 72% greater in the colon.

4. Discussion

Limited research is available on small intestine cancer due to the rarity of this disease. However, there
are abundant investigations into the etiology and cause of colon cancer, the second leading cause of cancer death in the US. While these anatomical sites share structural and functional similarities, there also exist inherent differences, which may contribute to the variation in cancer incidence. In this investigation, we propose that intrinsic differences in redox status and response to oxidative stress contribute to differences in cancer occurrence in the small intestine and colon.

ROS are frequently implicated as key players in tumorigenesis primarily by their potentially mutagenic oxidation of DNA. Our current data show the colon to have significantly greater basal levels of ROS than the small intestine, thereby creating an environment for more extensive oxidative DNA damage. While many exogenous sources of ROS in the intestine have been determined, endogenous sources have not been well investigated. There is considerable evidence that a key producer of endogenous ROS is mitochondrial oxidative phosphorylation [15–17]. When this process operates inefficiently, superoxide radicals ($O_2^-$) are produced that, if not quenched by antioxidant defenses, can lead to the formation of other damaging oxidants including hydrogen peroxide ($H_2O_2$) and hydroxyl radicals (OH$^-$). There is compelling evidence that oxidative metabolism in the colon is less efficient than in the small intestine. Wu [18] has shown the oxidation of butyrate (the primary energy substrate for colonocytes) yields 4.4 ATP/O$_2$ while the oxidation of glutamine (the primary energy substrate for enterocytes) yields 5.3 ATP/O$_2$. This strongly suggests less efficient mitochondrial respiration exists in the colon, which could lead to increased production of ROS.

Despite these elevations of endogenous ROS in the colon, levels of basal 8-oxodG did not differ significantly between the two intestinal sites. The resistance of the colon to oxidative DNA damage may be partially explained by enhanced protection against ROS via increases in the activity of antioxidant enzymes found at this site. Indeed, over expression of certain antioxidant enzymes has been shown to decrease urinary excretion of 8-oxodG adducts [19] while elimination of specific antioxidant enzymes in knockout mice have resulted in enhanced cellular oxidative damage [20,21]. While our findings reveal antioxidant enzyme activity to be greater in the colon, other investigations have shown varied results. One report found no difference in GPx and SOD in...
the mucosa of rat small intestine and colon [22], while another investigation found GPx activity to be slightly greater in the colon and rectum [23]. Yet another study found CAT and SOD to be greater in the small intestine than the colon [24]. This study included in its measurements the submucosal layer of the intestine, which contains immune cells and possibly vascular endothelial and blood cells [25] rich in antioxidant enzymes. The present study examined enzyme activity exclusively in the epithelial cells of the intestine, as these are the cells that undergo the process of malignant transformation as well as directly endure the exogenous stressors contained in the lumen.

Other mechanisms besides antioxidant enzymes may also be at work to control oxidative DNA damage. There are several DNA repair enzymes responsible for the removal of oxidative DNA adducts, including the excision repair enzyme oxoguanine glycosylase (OGG-1), specific for 8-oxodG repair in eukaryotes. However, the contribution of these repair enzymes in situations of elevated ROS may be minimal considering that even in basal conditions, the amount of DNA damage exceeds the repair capacity of these enzymes [26]. While comparisons of repair enzymes in the small intestine and colon has not been well characterized, findings in our laboratory indicate expression of the repair enzyme OGG-1 in the colon is not greater than in the small intestine despite enhanced generation of ROS in the colon [11]. Therefore, differences in DNA repair may not completely explain the resistance of the colon to oxidative DNA damage in the presence of elevated ROS.

It is demonstrated here that while antioxidant enzyme activity is elevated in the colon, there is no significant enhancement of protection against oxidative DNA damage when compared with the small intestine. Furthermore, basal ROS levels continue to be elevated despite enhanced enzyme activity. This suggests that under basal conditions in the colon, antioxidant enzymes are fully functional, which appears only sufficient to maintain, not reduce, oxidative DNA damage in the presence of elevated ROS. As a result, there exists a rather precarious pro-oxidant basal environment in the colon that when challenged with an exogenous oxidant, predisposes the colonocytes for more severe oxidative stress and increased oxidative DNA damage over the small intestine. This is evidenced by the dramatic rise in ROS and 8-oxodG adducts in the colon following brief exposure to 50 μM H2O2. Specifically, the colon experienced a 50% increase in ROS from the basal level and 8-oxodG adducts increased by 43%. Exposure of the small intestine to the same exogenous stress yielded only a 24% increase in ROS and subsequent 36% increase in oxidative DNA damage.

The inefficiency of the colon in managing exogenous oxidative stress is critical as the colon experiences greater oxidant exposure compared to the small intestine. Prolonged transit time in the colon allows for longer exposure to oxidized food particles, toxins and redox active minerals. In addition, bacterial microflora consistently generate reactive metabolites [27]. This poses a significant hazard to the colon since oxidative stress is involved in the development of inflammatory bowel diseases [28] and has been shown to induce malignant transformation [29,30]. The inability of the colon to manage oxidative stress especially in the presence of chronic oxidant exposure may partially explain the predisposition of this site for inflammatory bowel diseases and cancer over the small intestine.

Overall, these data indicate that the colon generates more endogenous ROS than the small intestine which persists despite enhanced antioxidant enzyme function. This seemingly pro-oxidant environment of the colon may lead to this tissue’s inability to handle oxidative stress as effectively as the small intestine. As a result, oxidative DNA damage in the colon is greater than in the small intestine during times of exogenous oxidant stress. As oxidative damage has been implicated in the carcinogenic process, these results may help to explain the difference in cancer incidence between the small intestine and colon. However, further functional studies of biological endpoints, such as tumor formation, are necessary to establish a definitive link between altered oxidative status and cancer susceptibility. This is the first study to report ROS and oxidative DNA damage differences in the small intestine and colon. Continued investigation of the inherent differences in these two similar tissues and their relation to cancer susceptibility can contribute to the understanding of the pathogenesis and possibly the future prevention of colon cancer.
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