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Role for Epithelial Dysregulation in Early-onset Colitis-associated Colon Cancer in *Gi2-alpha*^{-/-} Mice

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Abstract

Background—Inflammatory bowel disease (IBD) is a risk factor for developing colorectal cancer but the mechanisms are poorly characterized. Mice lacking the G-protein alpha subunit *Gi2-alpha* spontaneously develop colitis and colon cancer with high penetrance. Compared to canonical Wnt/APC signaling-based animal models of colon cancer, the tumors in *Gi2-alpha*^{-/-} mice more closely recapitulate the features of IBD-associated cancers seen in humans. They are predominantly right-sided, multifocal, mucinous, and arise from areas of flat dysplasia.

Methods—In evaluating the potential contribution of epithelial *Gi2-alpha* signaling to this phenotype, we found that *Gi2-alpha*^{-/-} colonic epithelium is hyperproliferative even before the onset of colitis, and resistant to the induction of apoptosis. We generated colon cancer cell lines overexpressing dominant-negative *Gi2-alpha*.

Results—Like other cells lacking *Gi2-alpha*, these cells release less arachidonic acid, an important antiinflammatory and epithelial growth regulator. They are also hyperproliferative and resistant to camptothecin-induced apoptosis and caspase-3 activation.

Conclusions—The colitis-associated cancers in *Gi2-alpha*^{-/-} mice appear very similar to those seen in human IBD patients, and *Gi2-alpha* is a direct negative regulator of colonic epithelial cell growth.

Keywords

colitis associated colon cancer; inflammatory bowel disease; animal models of colon cancer; heterotrimeric G-protein signaling

Colorectal cancer (CRC) is a feared complication of chronic inflammatory bowel disease (IBD), a condition that affects nearly half a million patients in the U.S. Although IBD-associated CRC shares some of the same genetic alterations seen in sporadic CRCs, there are differences in the timing and nature of these changes, including earlier loss of heterozygosity at the p53 locus and a lower incidence of ras mutations (reviewed in Ref. 1). There are important clinical and histopathological differences between sporadic and IBD-associated CRC as well (reviewed in Ref. 2). In the latter there is a greater incidence of right-sided/proximal bowel cancers, particularly in Crohn's disease (CD).³ The incidence of tumors with mucinous

differentiation is also higher in IBD patients, as is the likelihood of multiple synchronous malignancies⁴ and the presence of areas of flat dysplasia. On these grounds, IBD-associated colon cancer appears to be a distinct entity from sporadic colon cancer.

Of the mediators that have been shown to be important in the regulation of mucosal immunity as well as carcinogenesis, the arachidonic acid (AA)-COX-PGE₂ axis is particularly well studied. The role of PGE₂ is controversial in IBD; PGE₂ levels are commonly upregulated in diseased colonic mucosa, yet COX inhibition (and thus PGE₂ production) following nonsteroidal antiinflammatory (NSAID) use is strongly associated with relapse and worsening of symptoms.⁵ While there is a great deal of evidence that COX inhibitors inhibit polyp growth and decrease the relative risk of developing sporadic colon cancers,⁶ the data are considerably less clear in IBD-associated carcinogenesis. Some studies have shown a benefit in ulcerative colitis (UC) patients,⁷ while other studies show no preventative benefit of NSAID use by patients.^{8–10} We have previously shown that Gi2-alpha^{-/-} mice develop cancer despite lower colonic mucosal PGE₂ levels, even in the setting of inflammation.¹¹ IBD-associated carcinogenesis therefore appears to be less dependent on the COX-PGE₂ pathway.

Although there is great interest in the mechanisms underlying IBD-associated colon cancer, efforts have been impeded by a relative lack of suitable animal models. Mice on a 129Sv/C57 background that lack the G-protein alpha subunit Gi2-alpha spontaneously develop colitis starting at 6–8 weeks of age. During the initial characterization of Gi2-alpha^{-/-} mice,¹² it was noted that a subset of animals (8/26, or 31%) developed adenocarcinomas of the colon. However, the natural history and histopathologic features of cancer in Gi2-alpha^{-/-} mice have not been completely enumerated, and it is not clear whether loss of Gi2-alpha signaling has a direct or indirect effect on the epithelium that may contribute to carcinogenesis. We tested the hypothesis that the loss of Gi2-alpha signaling would directly affect the balance of growth and apoptosis in the colonic epithelium, independent of the influence of other cell types or mediators. In this study we evaluated the natural history of colitis and colon cancer and studied whether known defects in arachidonic acid metabolism in the absence of Gi2-alpha are conserved in colonic epithelia, and whether the lack of Gi2-alpha signaling directly affects the balance of growth and apoptosis in colon cancer cells.

MATERIALS AND METHODS

Mice

Wildtype (WT) and Gi2-alpha^{-/-} mice on a crossbred 129Sv/C57BL6 background were housed in a pathogen-free facility at the University of California, Irvine, vivarium. Male Gi2-alpha^{-/-} mice were bred with heterozygous females in order to maximize fertility and the yield of Gi2-alpha-knockout animals. Genotyping was performed on genomic tail DNA using a multiplex polymerase chain reaction (PCR) reaction that generates an 805 bp product for the WT allele and a 509 bp product for the mutant allele.

Ethical Considerations

All experiments were carried out in accordance with protocols approved by the Institutional Animal Care and Use Committee at the University of California, Irvine (IACUC Protocol #2002-2357-1) and were consistent with Federal guidelines.

Histopathologic Assessment of Colitis and Colon Cancer

In all, 123 Gi2-alpha^{-/-} mice ranging in age from 4–53 weeks of age were sacrificed and their colons removed. The lumens were rinsed thoroughly with phosphate-buffered saline (PBS) and the serosal surfaces inked with 4 colors of surgical marking ink to denote cecal, proximal, mid, and distal colonic segments. The tissues were submitted for fixation either as intact “jelly

rolls” or as linear segments on edge. The degree of colitis (none, minimal, mild, moderate, or severe) was assessed based on the following criteria: 1) extent and severity of inflammatory infiltrates, 2) presence of mucosal ulceration, 3) depletion of goblet cells, 4) presence of transmural inflammation and/or serositis, and 5) presence of crypt ulcers. Colon cancers were defined as the presence of glands that had invaded into (at least) the submucosa and were scored for their location and presence or absence of mucinous differentiation.

Anti-BrdU Staining

For immunofluorescent staining of BrdU-labeled sections, age-matched, colitis-free 6-week-old ($n = 8$ each) WT and Gi2-alpha^{-/-} mice were treated with 50 mg/kg BrdU 2 hours before sacrifice. Following formalin fixation / paraffin embedding, deparaffinized sections were stained using a Zymed anti-BrdU staining kit (Invitrogen, Carlsbad, CA) and counterstained with DAPI following the manufacturer’s instructions. For each age group the number of BrdU⁺ and DAPI⁺ epithelial nuclei from 10 well-oriented crypts per mouse were counted and the mean \pm SEM percentage of BrdU⁺ cells was calculated. At least 5000 DAPI⁺ cells were counted in each cohort of young mice. The data presented are from 3 separate iterations of the same experimental procedure.

In Vivo Assessment of Apoptosis Induction

Groups of age-matched WT and Gi2-alpha^{-/-} mice ($n = 7$ each) were injected i.p. with 15 mg/kg of azoxymethane (AOM) in sterile saline. Four hours later (2 hours before sacrifice) the animals were injected i.p. with 200 μ L of 5 mg/mL BrdU in sterile saline. Following sacrifice, whole colons were removed, the tip of the cecum was snipped, and the fecal contents washed out via a 4” gavage needle. Colonic tissues were pinned out and fixed overnight in 10% neutral buffered formalin and then submitted on edge for paraffin embedding. Then 5- μ m-thick sections were stained for apoptotic cells using an anti-digoxigenin antibody-based Apoptag staining kit (Chemicon, Temecula, CA) and counter-stained with DAPI per the manufacturer’s instructions. For the evaluation of basal colonic epithelial apoptosis in vivo, untreated tissue sections were stained with 1:200 rabbit anti-cleaved caspase 3 (Cell Signaling #9661, Danvers, MA) and developed with DAB. Ten medium-power fields from cleaved caspase-3- and TUNEL-stained sections each colon were photographed and counted. Cleaved caspase-3⁺ and TUNEL⁺ cells were counted and expressed as a percentage of hematoxylin-counterstained or DAPI⁺ (respectively) epithelial cells. The data are expressed as the mean \pm SEM of the normalized ratio of TUNEL⁺/PI⁺ cells and represent the results of 2 identical experiments.

Generation of Human Colon Cancer Cell Lines Overexpressing G203T-Gi2-alpha

The human colon cancer cell lines T84 (ATCC #CCL-248) and RKO (ATCC #CRL-2577) were grown in DMEM/F12/5% FCS or RPMI/10% FCS, respectively. Cells were stably transfected with either a control “Neo” plasmid (pcDNA-Neo3.1(+)) (Invitrogen, Carlsbad, CA), or a plasmid encoding the dominant-negative human Gi2-alpha cDNA,13 G203T-Gi2-alpha (from the University of Missouri, Rolla cDNA resource center). The transfections were performed using 4 μ g of cDNA and an Amaxa nucleofector (Amaxa USA, Gaithersburg, MD) with their primary mammalian epithelial nucleofector kit per the manufacturer’s instructions. Forty-eight hours after nucleofection, G418 selection with 200 μ g/mL (for T84 cells) or 700 μ g/mL (for RKO cells) was initiated. These concentrations were determined by kill curves on untransfected cells. Following the establishment of resistant colonies, pooled clones were evaluated for the overexpression of human Gi2-alpha by semiquantitative real-time PCR (using primers hGi2-alpha-forward: 5'-GAACGACCTGGAGCGTATTG-3'; hGi2-alpha-reverse: 5'-GAGGATGATGGACGTGTCTG-3'), normalized to beta actin (actin-forward: 5'-CATGTACGTTGCTATCCAGGC-3'; actin-reverse: 5'-CTCCTTAATGTACGCACGAT-3'). Overexpression of Gi2-alpha protein was evaluated by

Western blotting using a 1:500 dilution of mouse monoclonal anti-Gi2-alpha antibody, clone L5 (Santa Cruz Biotechnology, Santa Cruz, CA).

Human Colon Cancer Cell Line ³H-Arachidonic Acid Labeling and Release

For experiments involving arachidonate release, 2×10^5 cells/well were plated in 12-well dishes, allowed to adhere in RPMI-5 for 8 hours, then loaded with 0.3 μ Ci/well ³H-arachidonate (208 Ci/mmol, 1 mCi/mL; Amersham Biosciences, Buckinghamshire, UK) in RPMI-0.2% bovine serum albumin (BSA) overnight. Typical cellular uptake was >90% and was similar in WT and Gi2-alpha^{-/-} cells. Each well was washed 3 times with RPMI-0.2% BSA, then treated with 500 μ M adenosine triphosphate (ATP) or 0.1 U/mL thrombin (Sigma, St. Louis, Mo) in 500 μ M for 60 minutes with or without 5 minutes of pretreatment with 200 nmol/L phorbol 12-myristate 12-acetate (PMA). Two hundred out of the 500 μ L of supernatants was removed and the incorporated cellular arachidonate collected by lysing adherent cells in 10% sodium dodecylsulfate. Fractions were assayed for ³H-arachidonate release by scintillation counting and the percentage of ligand-stimulated arachidonate release calculated as $[(2.5 \times \text{counts per minute in supernatant}) / (2.5(\text{counts per minute in supernatant}) + \text{counts per minute in lysate})] * 100$.

Proliferation Assays of Human Colon Cancer Cell Lines

In all, 2.5×10^4 WT, Neo, or G203T-Gi2-alpha expressing RKO or T84 clones were plated in triplicate in 96-well plates and allowed to attach overnight. Four hours after the addition of fresh complete medium, 10 μ L/well of WST-1 reagent (Roche Applied Science, Nutley, NJ) was added to each well, and the conversion to formazan monitored by measuring the OD₄₅₀ at varying times up to 2 hours. The slope of the resulting curves is proportional to cell proliferation.

In Vitro Assessment of Apoptosis Induction and Caspase 3 Activity Assays

Basal and camptothecin-induced caspase 3 activities were determined in cultures of control and G203T-Gi2-alpha-expressing T84 and RKO cells. Briefly, subconfluent 100 mm dishes of cells were preincubated with 20 μ M arachidonic acid overnight and then treated with 5 μ M camptothecin for 6 hours. Trypsinized cells were resuspended at 5×10^7 cells/mL and lysed in 50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 10 mM DTT, 1 mM EDTA, and 10% glycerol. Cleared lysates were assayed in triplicate for caspase 3 activity in 100 μ L reactions containing 80 μ L assay buffer, 10 μ L lysate, and 10 μ L of 500 μ M DEVD-pNA (Biomol, Plymouth Meeting, PA), monitoring the OD₄₀₅ of the reaction over time. Samples containing buffer and DEVD-pNA substrate alone were used as a negative control. The activity of caspase 3 in pmol/min/mg protein was calculated according to the manufacturer's instructions.

For Western blot analysis of cleaved caspase-3 activity, RKO cells were treated with 5 μ M camptothecin for 0, 3, 6, and 24 hours, lysed in basal lysis buffer (20 mM Tris pH 7.9, 137 mM NaCl, 5 mM EDTA, 10% glycerol, 1% Triton X-100, 1 mM EGTA 1 Roche complete-mini protease inhibitor tablet, 1 mM NaPPi, and 10 mM NaF), and analyzed for cleaved caspase-3 by Western blot using a 1:1000 dilution of anti-cleaved caspase-3 antibody.

Statistical Analyses

Statistical significance was assessed using Student's paired *t*-test for linear data (epithelial cell proliferation in vivo and in vitro, arachidonic acid release, caspase-3 activity), and the Mann-Whitney *U*-test for nonlinear data (normalized TUNEL-staining data).

RESULTS

Spectrum of Colitis and Colon Cancer in Gi2-alpha^{-/-} Mice

We summarize the prevalence of increasingly severe colitis and colon cancer in Gi2-alpha^{-/-} mice, stratified into 4 age groups, in Figure 1. These age groupings correspond to the age where colitis begins (4–6 weeks), colitis but not cancer is present (7–10 weeks), cancer incidence is increasing (11–18 weeks), and cancer incidence is prevalent (19–52 weeks). In our breeding colony, nearly all Gi2-alpha^{-/-} mice develop colitis by the seventh week of life (Fig. 1), consisting of an expansion of the lamina propria by mononuclear infiltrates. However, 2 mice sacrificed at 7.9 and 11.9 weeks were still considered to have a “normal” number of lamina propria mononuclear cells.

In these mice the inflammatory infiltrates are often lymphoplasmacytic, although some animals with mild colitis have a predominance of neutrophils. As the degree of colitis progresses, the infiltrates expand into the submucosa and eventually through the muscularis propria and serosal layer (Fig. 2A). Some animals with severe colitis have significant numbers of mast cells, scattered within the muscularis propria (Fig. 2B). An additional curious feature in some cases is the presence of dilated serosal lymphatic vessels which have a large cuff of surrounding mononuclear cells around them (Fig. 2C). Importantly, the distribution of colitis along the length of the colon is patchy, with areas of involvement interspersed with “skip” areas. In our experience, large areas of ulcerated mucosa, crypt ulcers, strictures, and adhesions between adjacent loops of bowel are not prominent, nor have we identified any granulomas in any colitic tissues.

Of 123 Gi2-alpha^{-/-} mice whose whole colons were available for study, 31 mice were found to have at least 1 carcinoma, defined as the presence of glands invasive through (at least) the muscularis mucosa into the submucosa. Table 1 summarizes the distribution and multiplicity of tumors, divided into 2 age groups. In the 9 mice aged 12–24 weeks, 80% of the tumors (16/20) were either in the cecum or proximal colon, and 90% consisted of smaller, nonmucinous tumors. The earliest invasive cancer occurred at 11.9 weeks of age. Cancers in the older age group (25–53 weeks of age) showed a similar (76%) predilection for the cecum and proximal colon, but there were a greater proportion of tumors containing mucinous differentiation (17/48, or 35%). With respect to the issue of multifocality, there was an average of 2.2 ± 1.3 (mean \pm SD) malignancies per colon, indicating a high degree of multifocality; nearly half (14/31) of the mice had 3 or more separate cancers.

We defined tumors as mucinous if they contained areas of mucin lakes lined at least in part by strips of epithelium; overall, 28% of the cancers showed mucinous differentiation (Fig. 2D). Other notable features of cancers in Gi2-alpha^{-/-} mice include very low-grade histologic features, the absence of obvious polypoid structures, and their tendency to invade downwards into the underlying submucosa and muscularis propria. While inflamed colonic mucosa and sporadic colon cancers often display a loss of goblet cells and/or cytoplasmic mucin vacuoles, many of the nonmucinous tumors in Gi2-alpha^{-/-} mice were composed of monotonous tubular glands containing some goblet cells and many secretory cells with prominent apical vacuoles. Notably, although there are areas of flat dysplasia overlying many of the invasive cancers, in many other examples the overlying epithelium is inflamed but appears to be otherwise uninvolved. Indeed, many of these cancers would not be identifiable at all if looked at endoscopically.

Colonic Epithelium in *Gi2-alpha*^{-/-} Mice Is Hyperproliferative Before the Onset of Colitis and Is Resistant to Apoptosis

The development of invasive cancers so soon after the onset of colitis led us to test whether the loss of *Gi2-alpha* signaling in the colonic epithelium might have direct effects on the balance of growth and apoptosis. To that end, we evaluated basal proliferative indices in the colonic epithelium of WT and *Gi2-alpha*^{-/-} before and after the onset of colitis. As shown in Figure 3, colitis-free 6-week *Gi2-alpha*^{-/-} mice exhibited a greater than 2-fold increase in the percentage of BrdU-labeled cells compared to WT littermates ($15.68 \pm 4.04\%$ versus $7.63 \pm 4.03\%$ [mean \pm SD], $n = 8$, $P < 0.0001$). A similar result was obtained from the inflamed epithelium of old *Gi2-alpha*^{-/-} mice (mean age 40 weeks), with an average proliferative index of $20.21 \pm 11.55\%$ versus WT ($7.03 \pm 2.68\%$, $n = 3$), $P < 0.0001$, data not shown due to small sample size). The BrdU⁺ cells in the *Gi2-alpha*^{-/-} crypts could be found extending as high as two-thirds of the way up the crypt base, in contrast to WT crypts, where the signal did not extend past the bottom 40% of the crypt.

We next tested whether the increased colonic epithelial proliferation observed in colitis-free *Gi2-alpha*^{-/-} mice might be accompanied by a decrease in apoptosis. We attempted to assess basal levels of apoptosis in WT and *Gi2-alpha*^{-/-} tissues by performing immunofluorescent TUNEL staining but TUNEL⁺ cells were identified too infrequently to perform meaningful analysis (fewer than 1 per crypt on average, data not shown). We then stained the same untreated tissues for cleaved caspase-3, which resulted in comparable levels of caspase-3 plus epithelial cells (40 medium power fields of crypt epithelium; WT: $0.19 \pm 0.03\%$ versus *Gi2-alpha*: $0.22 \pm 0.03\%$, $P =$ not significant), indicating no significant difference in basal apoptosis rates between WT and *Gi2-alpha*^{-/-} crypt epithelium.

The natural history of colon cancer in IBD patients suggests that longstanding chronic inflammation predisposes to colon cancer via DNA damage due to oxidative stress.¹⁴ Because of the apparently extremely low basal levels of apoptosis in both WT and *Gi2-alpha*^{-/-} colonic epithelium, we decided to experimentally induce DNA damage in an attempt to provoke either a reparative or apoptotic response in vivo. For in vivo studies we chose the alkylating agent azoxymethane. Intestinal epithelial apoptosis occurs following injection of a single dose of azoxymethane (AOM) in rodents,¹⁵ and peak levels of apoptosis have been reported at 6 hours after AOM injection in mice.¹⁶ We therefore treated groups of 12–20-week-old mice with 15 mg/kg AOM for 6 hours, performed TUNEL staining on colonic sections, and counted the number of TUNEL⁺ cells. In 2 separate experiments we consistently observed a significant decrease in the percentage of TUNEL⁺ cells in *Gi2-alpha*^{-/-} epithelium compared to WT tissues (Experiment #1: 2-tailed $P = 0.0003$, Experiment #2: $P = 0.00002$) (Fig. 4B).

Generation of Stably Transfected Colon Cancer Cells Overexpressing Dominant-Negative (G203T)-Human *Gi2-alpha*

Taken together, these in vivo data suggest that the loss of *Gi2-alpha* signaling directly affects the balance of growth and apoptosis in the colonic epithelium. To rule out the possibility that the lack of *Gi2-alpha* indirectly affects epithelial growth via alterations in other adjoining cell types, we generated stably transfected lines of 2 separate human colon cancer cell lines (T84 and RKO) overexpressing the dominant-negative human *Gi2-alpha*2 point mutant, G203T-h*Gi2-alpha*.¹⁷ The pooled G418-resistant clones were found to overexpress *Gi2-alpha* protein compared to cells stably transfected with the neomycin-resistance plasmid alone (Fig. 5).

Increased In Vitro Proliferation of T84 and RKO Clones

To test the effect of overexpressing dominant-negative *Gi2-alpha*3 on colonic epithelial cell growth, we first measured basal proliferation rates of wildtype, vector-control, and G203T-*Gi2-alpha* expressing RKO and T84 cells using the WST-1 tetrazolium salt proliferation assay.

Both RKO (Fig. 6A) and T84 (Fig. 6B) cells overexpressing G203T-Gi2-alpha proliferated at a greater rate than either wildtype or vector-control cells. Overall, RKO clones proliferated at a much higher rate than the T84 cells. We corroborated the proliferation results by labeling RKO cells in culture with BrdU, counterstaining with DAPI, and counting BrdU⁺ cells. As shown in Figure 6C, G203T-Gi2-alpha-expressing RKO cells showed a significantly higher level of BrdU-labeled cells (G203T: $47.54 \pm 5.99\%$ versus Neo: $35.8 \pm 9.5\%$, $P = 0.004$). Attempts to perform BrdU labeling experiments on T84 cultures were hampered by an inability to accurately perform cell counts due to the tightly clustered growth pattern that T84 cells display.

Decreased AA Release from G203T-Gi2-alpha-Expressing RKO Cells

Defects in arachidonic acid metabolism have been implicated in colon carcinogenesis.^{18,19} Cells lacking Gi2-alpha harbor defects in cPLA₂ activation and arachidonate release,^{17,20} and we have previously shown that colonic stromal myofibroblasts release less AA and produce less PGE₂.¹¹ We therefore tested whether RKO cells expressing G203T-Gi2-alpha would also display the same general arachidonic acid release defect. As shown in Figure 7, while basal arachidonate release was comparable between Neo and G203T trans-fectants, the addition of either 1 μ M A23187 alone or in combination with 200 nM PMA resulted in significantly less arachidonate release from G203T-Gi2-alpha-expressing cells. The relative decrease in AA release was comparable to that seen previously in Gi2-alpha^{-/-} myofibroblasts. Therefore, loss of Gi2-alpha signaling either by congenital absence or by overexpression of the dominant-negative mutant results in decreased arachidonic acid mobilization both in epithelial cells and subepithelial myofibroblasts.

Resistance to Camptothecin-Induced Apoptosis in Cells Expressing Dominant-Negative Gi2-alpha

We noted resistance to the induction of apoptosis in Gi2-alpha^{-/-} mouse colonic epithelium following treatment with azoxymethane in vivo (Fig. 4). To test whether the loss of Gi2-alpha signaling has a direct effect on colonic epithelial cell apoptosis in the absence of other cell types, we treated RKO and T84 cells with 5 μ M camptothecin (CPT). CPT is the prototype of a family of chemotherapeutic agents used to treat colon cancer that induce apoptosis by inhibiting topo-isomerase I, which leads to activation of caspase cascades.²¹ We assessed apoptosis using 3 complementary assays. Surprisingly, the overall basal caspase-3 activity level was somewhat higher in the G203T-expressing T84 cells than in control lines, while there was minimal basal caspase-3 activity in the RKO cell lines. In the absence of Gi2-alpha signaling, alterations in cell death in the absence of DNA damage agents may be directly relevant to tumor formation in vivo. In the presence of 5 μ M camptothecin, both T84 and RKO cells expressing G203T-Gi2-alpha had significantly lower caspase-3 activity than vector-control cells. The addition of 20 μ M arachidonic acid 16 hours prior to CPT treatment potentiated the caspase-3 activity in both control and Gi2-alpha-expressing cells, consistent with a proapoptotic role for arachidonate in colonic epithelium.

To corroborate these findings, we also ran Western blots for cleaved caspase-3 on the camptothecin-treated RKO cells. A time-course study revealed that the onset of cleaved caspase-3 production in response to camptothecin treatment was \approx 6 hours. The amount of cleaved caspase-3 species at 17 and 19 kDa was greater following camptothecin treatment in RKO-Neo cells than in RKO-G203T cells at both 6 and 24 hours (Fig. 8C). Similarly, treatment of RKO cells grown on coverslips and treated with 5 μ M CPT led to a greater percentage of RKO-Neo cells that were immunoreactive by TUNEL staining compared to RKO-G023T (Fig. 8D). Collectively, the data indicate that the overexpression of dominant-negative Gi2-alpha renders colon cancer cells more resistant to the induction of apoptosis by camptothecin treatment.

DISCUSSION

In our colony, we find that the penetrance of colitis is nearly 100% by 7 weeks of age. The original description likened the colitis to that seen in UC, noting the exclusively colonic localization of inflammation, and the worst colitis being present in the distal colon.¹² We find that the histopathologic features are somewhat more CD-like, given the patchy distribution of inflammation that is often the most severe in the cecum, in addition to transmural disease and serositis. However, there are important features of CD that are not seen in *Gia2*^{-/-} mice, which lack mucosal granulomas, prominent fissuring, and ileitis.

The most striking feature of our analysis is the incidence, location, histology, and multifocality of the colon cancers that arise in *Gi2-alpha*^{-/-} mice. Of the 32 mice older than 6 months at the time of sacrifice, nearly 80% of the animals had colon cancer. More than 75% of the cancers were located in the cecum or ascending colon and were multifocal. Many of these cancers arose out of areas of nonpolypoid dysplasia, although some invasive lesions are overlaid by inflamed but nondysplastic epithelium. These tumors displayed mucinous differentiation nearly 30% of the time. Together these features are very reminiscent of those seen in human patients with IBD-associated colon cancer.^{22–24} Choi and Zelig³ reviewed the features of IBD-associated cancers in 80 patients (28 CD and 52 UC)—50% of the CD and 36% of the UC cancers were located in the right colon; 22% of the CD patients and 15% of the UC patients had mucinous cancers, and 12% of all patients had multiple synchronous tumors.

There are several animal models of colitis that also develop colon cancer. IL-10 KO mice, like *Gi2-alpha*^{-/-} mice, develop transmural disease with a high incidence of colon cancer (60% at 6 months, $n = 10$),²⁵ although the distribution within the colon was not described further. The mucinous differentiation and relatively low-grade features seen in some *Gi2-alpha*^{-/-} tumors are also seen in IL-10^{-/-} mice. In the initial description of colon cancers in IL-10^{-/-} mice, lesions with these features were not counted as adenocarcinomas, but rather thought to represent colitis cystica profunda, an uncommon nonneoplastic lesion that can mimic colon cancer.²⁶ It is unlikely that all of the mucinous tumors in *Gi2-alpha*^{-/-} mice represent this benign entity, as several of these mucinous tumors are invasive into adjacent structures such as the pancreas. It should also be noted that the majority of adenocarcinomas that arise are in fact non-mucinous and are composed of irregular, poorly organized glands invading into an inflamed, desmoplastic stroma.

In all segments of the colon, 100% of *Smad3*-KO mice develop multifocal adenocarcinomas in a *Helicobacter* infection-dependent manner.²⁷ Review of the histopathologic spectrum of tumors observed in *Smad3* mutant mice²⁸ reveals striking similarity to those seen in *Gi2-alpha*^{-/-} mice. We have performed *Helicobacter* species-specific PCR on stool samples from our colony and found products specific for *H. bilis*, *H. hepaticus*, and *H. rodentium* (data not shown). We have also previously identified resistance to TGF β signaling in T-cells from *Gi2-alpha*^{-/-} mice,²⁹ although there are no differences in *Smad3* expression between WT and *Gi2-alpha* littermates. Because TGF β is a negative regulator of colonic epithelial proliferation,²⁸ it will be interesting to see whether there is decreased responsiveness to TGF β in cells overexpressing dominant-negative *Gi2-alpha*.

The features of colon cancer in *Gi2-alpha*^{-/-} mice are also strongly reminiscent of those seen in patients with hereditary nonpolyposis colon cancer (HNPCC), who develop multifocal right-sided mucinous or medullary-type adenocarcinomas arising out of nonpolypoid (villous) adenomatous mucosa.³⁰ All these features (lymphocytic infiltration, proximal location, mucinous differentiation) have been shown to be independent predictors of a tumor being MSI-H (defined as having microsatellite instability at $\geq 30\%$ of the tested microsatellite markers).³¹ Although no direct link between *Gi2-alpha* signaling and mismatch repair has been

identified, there is emerging evidence that microsatellite instability is commonly found not only in IBD-associated cancers¹⁴ but in the surrounding nondysplastic mucosa as well.³² If the tumors in Gi2-alpha^{-/-} mice are found to exhibit MSI and whether MSI is a particular feature of this model or is a common feature in animal models of chronic colitis, remains to be seen.

A widely held principle in colitis-associated carcinogenesis is that carcinomas arise in areas of inflammation. Indeed, this is true in Gi2-alpha^{-/-} mice. However, the rapid onset of dysplasia and cancer (as early as 12 weeks of age) so soon after the development of colitis prompted us to ask whether the loss of Gi2-alpha has a direct effect on epithelial proliferation and/or apoptosis, in the absence of an inflammatory milieu. We found that the Gi2-alpha^{-/-} epithelium is hyperproliferative in the absence of colitis (Fig. 3). One limitation of this result is that there is evidence of widespread immune activation in Gi2-alpha^{-/-} mice before the onset of histologically identifiable colitis,³³ so inflammatory mediators might disrupt epithelial growth regulation in “precolitic” mice. We therefore evaluated proliferative and apoptotic indices in dominant-negative Gi2-alpha-expressing colon cancer cells in vitro and found comparable responses to those seen in vivo (Fig. 6, Fig 8). These data support the contention that Gi2-alpha signaling is a direct negative regulator of colonic epithelial cell growth.

The Gi2-alpha^{-/-} model of colitis-associated colon cancer poses a paradox. The loss of Gi2-alpha results in decreased epithelial (Fig. 7) and stromal¹¹ arachidonic acid release, leading to decreased mucosal PGE₂ levels, yet the epithelium is hyperproliferative. How might decreased arachidonate be related to the inhibition of apoptosis? Arachidonate has been shown to enhance the activity of intestinal sphingomyelinases that produce proapoptotic ceramides,³⁴ and inhibition of AA release has clear effects on apoptosis and carcinogenesis in vivo. Animals deficient in cPLA₂ develop a 5.5-fold greater tumor burden in the azoxymethane model of induced colon carcinogenesis due to significantly decreased colonic epithelial cell apoptosis seen by TUNEL assay, despite lower tissue PGE₂ levels.¹⁸ We are currently testing whether cell lines deficient in Gi2-alpha harbor decreased sphingomyelinase activity and ceramide generation which may underlie the observed antiapoptotic phenotype.

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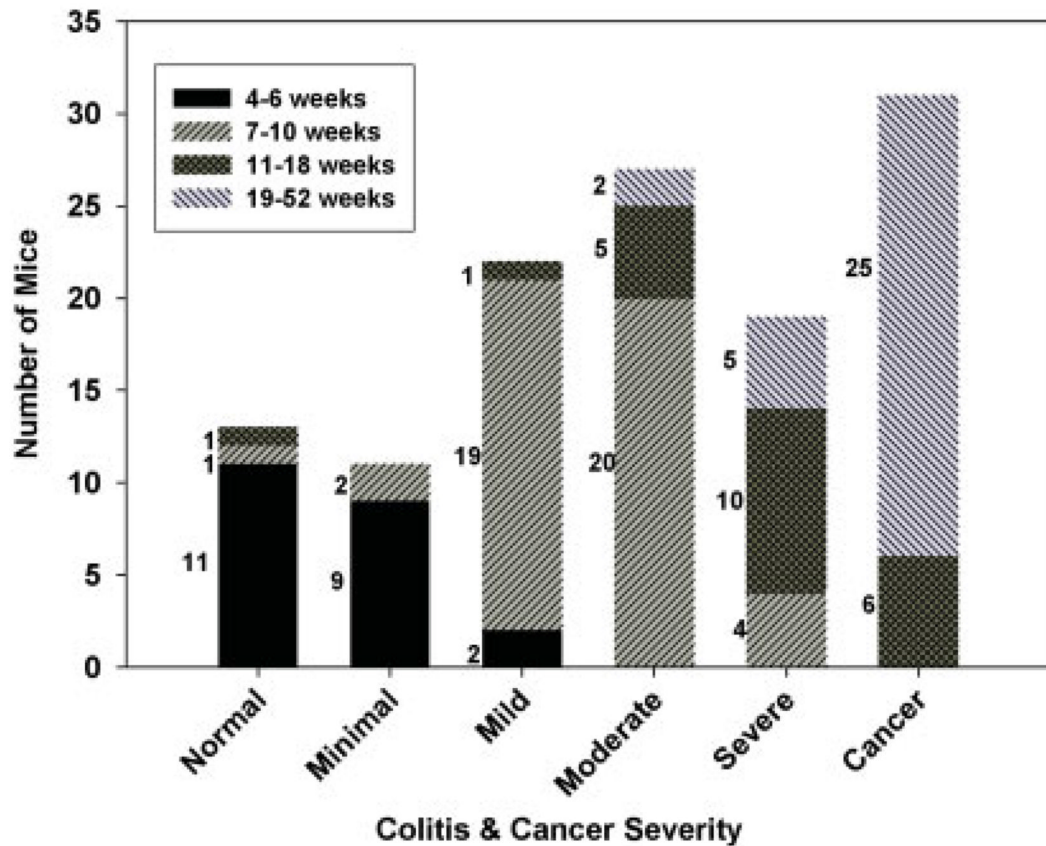
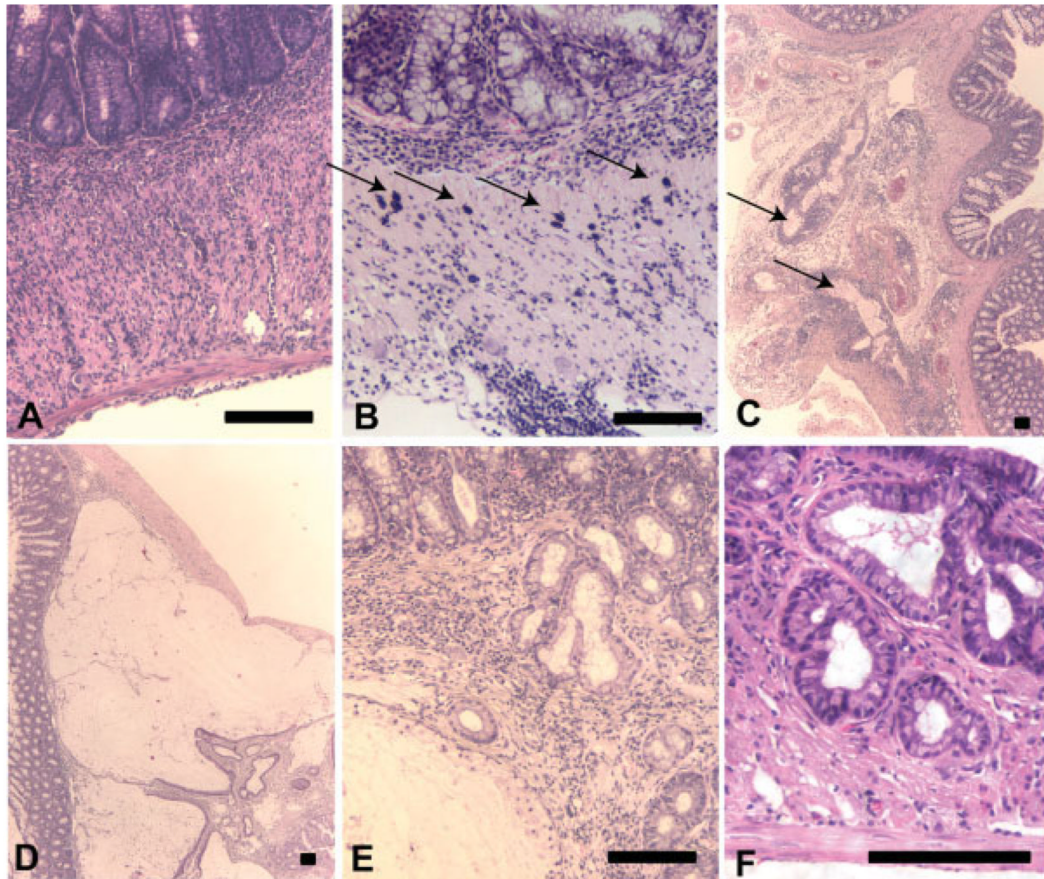


FIGURE 1.

Natural history of colitis and colon cancer in $Gi2\text{-}\alpha^{-/-}$ mice. Whole colons from 123 $Gi2\text{-}\alpha^{-/-}$ mice were stratified by age into 4 groups and analyzed for the presence and severity of colitis and colon cancer. The number to the left of each bar reflect the number of mice in each age group with a given degree of colitis. Nearly all $Gi2\text{-}\alpha^{-/-}$ animals have at least mild colitis by 7 weeks of age and begin to develop colon cancer as early as 12 weeks of age. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

**FIGURE 2.**

Histopathologic features of colitis and cancer in *Gi2-alpha*^{-/-} mice. Mice with moderate to severe colitis often develop transmural inflammation and serositis (A,C). Some colon specimens contain numerous intramuscular mast cells (B, arrows), and/or prominent lymphoplasmacytic cuffing of serosal lymphatic channels (C). Mucinous carcinomas are characterized by large mucin pools that dissect through the muscularis; note the overlying nonneoplastic mucosa at left (D). Many of the carcinomas are characterized by very well-differentiated glands with small basal nuclei and mucin vacuoles (shown invading into muscle in E,F.) Scale bars = 100 μ m. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

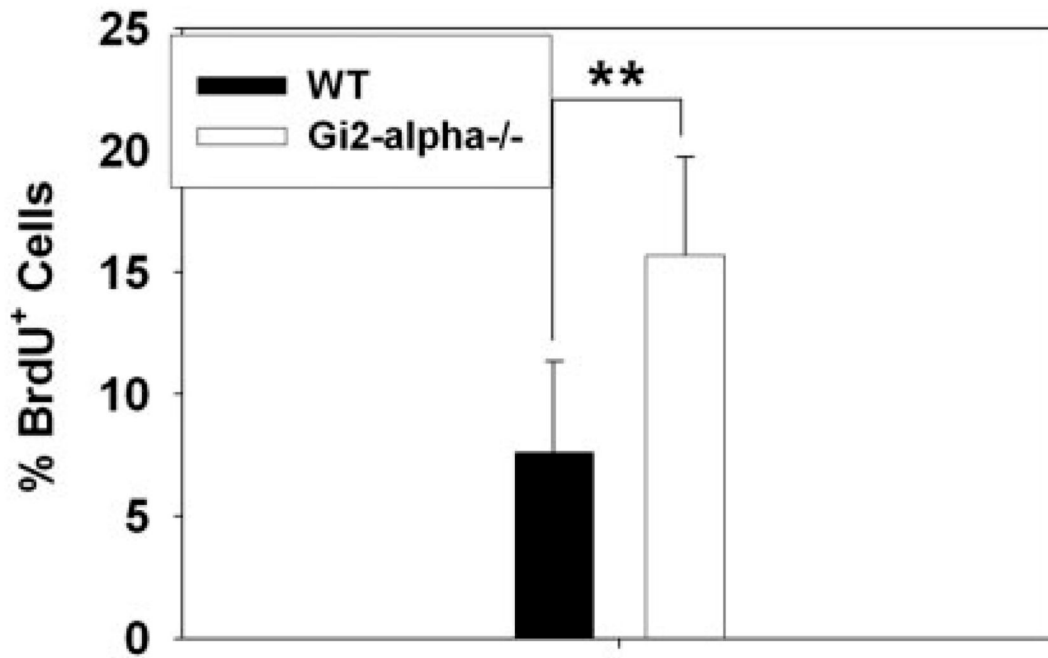


FIGURE 3. Gi2-alpha^{-/-} colonic epithelium is hyperproliferative before the onset of colitis. Groups of age-matched colitis-free 6-week-old ($n = 8$) WT and Gi2-alpha^{-/-} mice were injected i.p. with BrdU and the labeled tissues assessed for BrdU incorporation by immunofluorescent staining. The percentage of BrdU⁺ colonic epithelial cells in 10 well-oriented crypts per mouse are expressed as a mean \pm SD. ** $P < 0.01$. The data from the 6-week-old experiments are pooled from 2 separate iterations of the experiment.

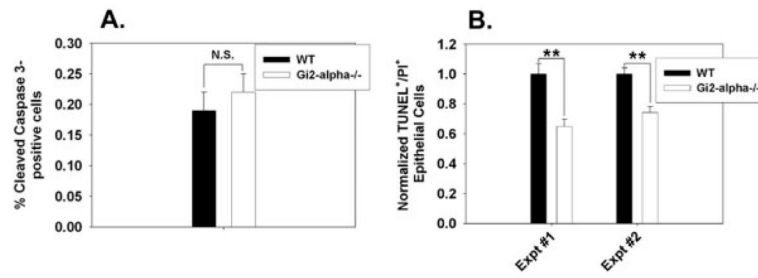


FIGURE 4.

Resistance to short-term azoxymethane-induced apoptosis in Gi2-alpha^{-/-} colonic epithelium. WT and Gi2-alpha^{-/-} mice ($n = 3$ each and 4 each, in replicate experiments) were left untreated (A), or given i.p. injections of 15 mg/kg azoxymethane and sacrificed 6 hours later. Untreated tissues were stained for cleaved-caspase-3 and azoxymethane-treated tissues were stained for TUNEL. The TUNEL results (normalized mean \pm SEM) of 2 experiments are shown. ** $P < 0.001$.

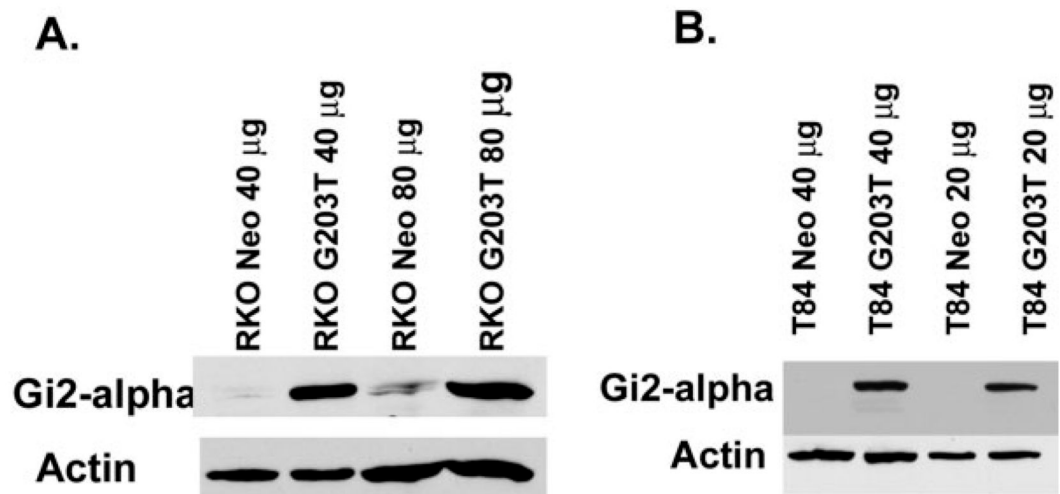


FIGURE 5.

Validation of dominant-negative (203T-Gi2-alpha) expressing colon cancer cell lines. Total cellular protein from vector-transfected or G203T-Gi2-alpha-transfected RKO cells (A) or T84 cells (B) was immunoblotted with anti-Gi2-alpha antibodies, demonstrating overexpression of Gi2-alpha in the G203T-h Gi2-alpha-transfected cells

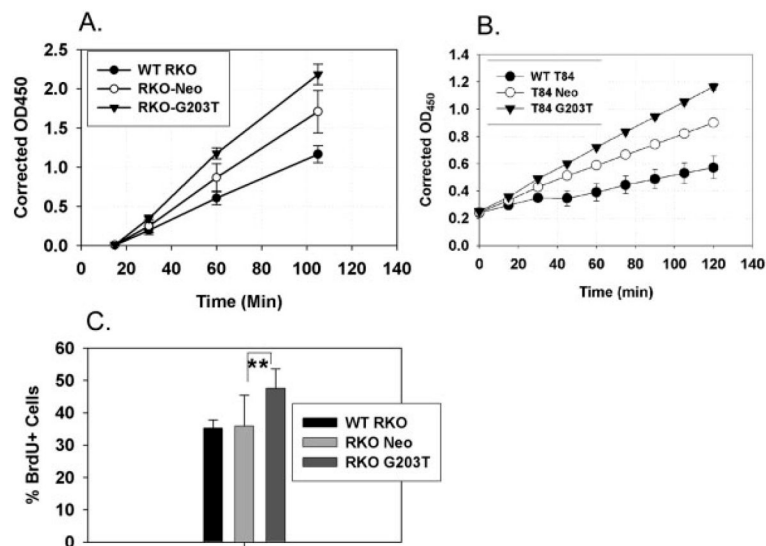


FIGURE 6. Proliferation of G203T-h Gi2-alpha-expressing human colon cancer cells. Triplicate wells of 1×10^5 RKO (A) or T84 (B) cells expressing nothing (WT), empty neomycin resistance vector (Neo), or dominant-negative Gi2-alpha (G203T) were starved overnight then incubated with $10 \mu\text{L}$ /well of WST-1 reagent. Data shown represent the mean \pm SD at each timepoint and are representative of at least 3 separate experiments. In C, 6 high-power fields containing at least 200 of each of the RKO clones were assessed for BrdU incorporation. G203T- Gi2-alpha-expressing RKO cells exhibited significantly (** $P = 0.0004$) higher levels of labeling compared to vector control or WT RKO cells.

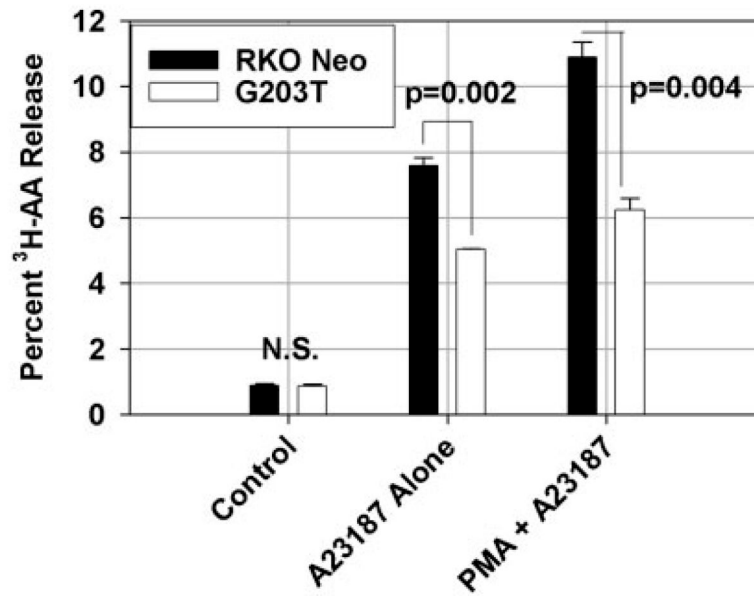


FIGURE 7.

Loss of Gi2-alpha signaling in RKO cells results in decreased epithelial arachidonic acid release. Two $\times 10^5$ RKO-Neo- or G203T-expressing cells were labeled overnight with 0.3 μ Ci/well ³H-arachidonic acid then treated for 30 minutes with 1 μ M A23187 alone or in combination with 200 nM PMA. ³H-ara-chidonic acid release into the supernatant was assayed by scintillation counting. The data shown are means \pm SD of duplicate measurements and are representative of 3 separate experiments.

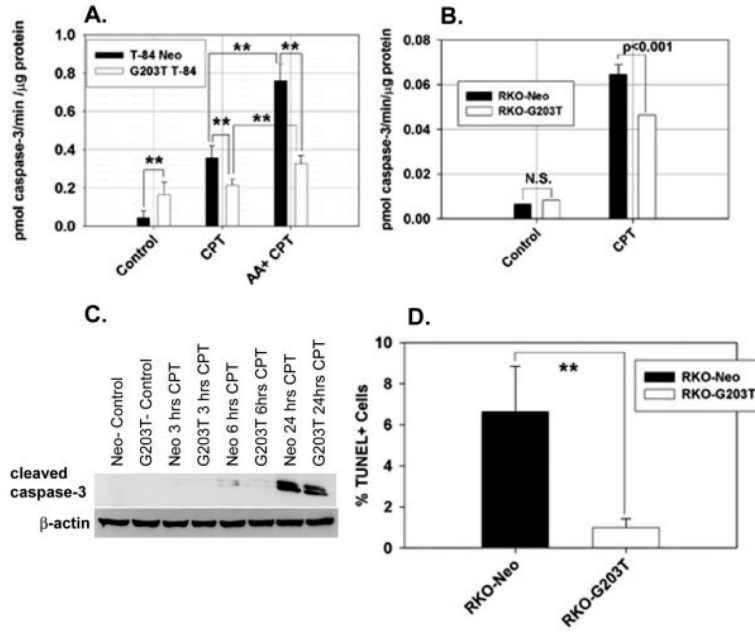


FIGURE 8. Resistance to campto-thecin-induced caspase-3 activation and apoptosis in cells expressing dominant-negative Gi2-alpha. T84 (A) and RKO (B) cells expressing control vector alone (Neo) or G203T- Gi2-alpha were treated with 5 μM camptothecin for 6 hours alone, or following overnight pretreatment with 20 μM ar-achidonicacid. The data shown are the mean ± SD of triplicate caspase-3 activity measurements and are representative of 3 separate experiments. C: 100 μg of the cell lysates from control and CPT-treated cells at 0,3,6, and 24 hours were separated and blotted with anti-activated caspase-3 Ab (recognizes 19 and 17 kDa fragments). D: CPT-treated Neo and Gi2-alpha-expressing cells were evaluated for apoptosis by TUNEL staining; the data represent the mean ± SD percentage of TUNEL+ cells in 10 medium-powerfields. **P < 0.001.

TABLE 1

Location and Histologic Features of Carcinomas from *Gi2-alpha-/-* Mice

Location	12–24 weeks of age (n = 9)				25–53 weeks of age (n = 22)				Overall:			
	Non-mucinous	Mucinous	Total No.	% of total	Non-mucinous	Mucinous	Total No.	% of total	Non-mucinous	Mucinous	Total No.	% of total
Cecum	13	0	13	65	22	5	27	56.3	35	5	40	58.8
Proximal	2	1	3	15	4	5	9	18.8	8	6	12	17.6
Mid	3	1	4	20	5	3	8	16.7	8	4	12	17.6
Distal	0	0	0	0	0	4	4	8.3	2	4	4	5.9
Totals	18	2	20	29.40%	31	17	48	70.60%	49	19	68	100%

Whole colons ($n = 123$) from *Gi2-alpha-/-* mice were evaluated for the presence and severity of colitis and colon cancer, and the data grouped into young (12–24 weeks of age) and old (25–53 weeks) age groups as shown. All cancers arise in areas of mucosa that contain at least moderate levels of colitis.