Human Cancer Biology

PI3K/AKT Signaling Is Essential for Communication between Tissue-Infiltrating Mast Cells, Macrophages, and Epithelial Cells in Colitis-Induced Cancer

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Abstract

Purpose: To understand signaling pathways that shape inflamed tissue and predispose to cancer is critical for effective prevention and therapy for chronic inflammatory diseases. We have explored phosphoinositide 3-kinase (PI3K) activity in human inflammatory bowel diseases and mouse colitis models.

Experimental Design: We conducted immunostaining of phosphorylated AKT (pAKT) and unbiased high-throughput image acquisition and quantitative analysis of samples of noninflamed normal colon, colitis, dysplasia, and colorectal cancer. Mechanistic insights were gained from ex vivo studies of cell interactions, the piroxicam/IL-10−/− mouse model of progressive colitis, and use of the PI3K inhibitor LY294002.

Results: Progressive increase in densities of pAKT-positive tumor-associated macrophages (TAM) and increase in densities of mast cells in the colonic submucosa were noted with colitis and progression to dysplasia and cancer. Mast cells recruited macrophages in ex vivo migration assays, and both mast cells and TAMs promoted invasion of cancer cells. Pretreatment of mast cells with LY294002 blocked recruitment of TAMs. LY294002 inhibited mast cell and TAM-mediated tumor invasion, and in mice, blocked stromal PI3K, colitis, and cancer.

Conclusion: The PI3K/AKT pathway is active in cells infiltrating inflamed human colon tissue. This pathway sustains the recruitment of inflammatory cells through a positive feedback loop. The PI3K/AKT pathway is essential for tumor invasion and the malignant features of the piroxicam/IL-10−/− mouse model. LY294002 targets the PI3K pathway and hinders progressive colitis. These findings indicate that colitis and progression to cancer are dependent on stromal PI3K and sensitive to treatment with LY294002. Clin Cancer Res; 1–13. ©2013 AACR.

Introduction

Epithelial cells react to inflammation by increased mitotic activity, crypt architectural distortion, and ulcers, characteristics that in the chronic setting predispose to cellular transformation. Patients with ulcerative colitis or Crohn’s colitis who have persistent chronic inflammation of the colon for more than 8 to 10 years are in a very high risk of developing metastatic colorectal cancer (CRC). Approximately, 25% to 30% of patients with a history of pan-colitis for more than 30 years develop CRC, because of prolonged exposure of the colon to chronic inflammation (1–3). It is suggested that tissue-infiltrating proinflammatory cells drive the neoplastic changes in the inflamed colon leading to CRC.

In the piroxicam/IL-10 mouse model, phosphoinositide 3-kinase (PI3K) mediates activation of Akt and β-catenin in epithelial stem cells resulting in mitosis and crypt architectural changes that predispose to colitis (4). Furthermore, PI3K’s targeted ablation is protective (4, 5). The PI3K pathway is also etiologically linked with sporadic CRC, contributing to epithelial cell survival and proliferation (6, 7). Activated PI3K drives the transformation of well-differentiated epithelial cells to a less differentiated and more malignant phenotype (8–10).

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**Translational Relevance**

Stromal interactions that sustain chronic inflammation and predispose to cancer are poorly understood. Experimental models of colitis implicate the phosphoinositide 3-kinase (PI3K) pathway in its activation of gut enterocytes and tissue remodeling. Although mouse models are important for gaining mechanistic insights into diseases that affect us, validation of the findings in humans remains the only way to evaluate their clinical relevance. Here, we show that in contrast to mouse models of colitis, by far the greatest fraction of PI3K-active cells are tissue-infiltrating proinflammatory cells. We use a potent inhibitor of PI3K that is currently in clinical use in combination with ex vivo assays and animal modeling to elucidate the contribution of PI3K activity to the recruitment of inflammatory cells and predisposition to cancer. Our findings point to stromal interactions as the prime site of action of PI3K inhibitors in prevention and therapy for inflammation-induced colon cancer.

Mast cells (MC) play a central role in the inflammatory response associated with cancer (11). PI3K-driven pathways control all receptor-mediated activation, differentiation, survival, and homing of mast cells to their target tissues, and PI3K-deficient mice are devoid of mast cells (12). In addition, a range of chemoattractants activating G protein-coupled receptors (GPCR), receptor tyrosine kinases (RTK), and Toll-like/IL-1 receptors (TLR/IL1Rs) initiate tumor inflammation by activating PI3K in tumor-associated macrophages (TAM; ref. 13). Knowledge of the cellular source of PI3K activity in healthy, inflamed, and tumor tissues is therefore important for understanding how PI3K activity causes colitis and predisposes to cancer. This knowledge will lead to understanding the mode of action of PI3K-targeting drugs that are currently being tested for prevention and treatment of cancer (14).

We used paraffin-embedded human tissue specimens as well as fresh surgical tissue to study the tissue distribution of PI3K-active cells in the course of progression of colitis to CRC. We investigated interactions between 2 major proinflammatory tissue-infiltrating cells with known dependence on PI3K, mast cells, and TAMs. Furthermore, we looked at their contribution to tumor growth and invasion. LY294002 is a chemical inhibitor of PI3K that has been used to control experimental colitis and colon cancer (4, 15). LY294002 was used to interrupt PI3K activity and gain mechanistic insight. We report that in contrast to mouse tissue, in inflamed human colonic tissue, PI3K activity is most abundant among proinflammatory cells within the stroma. PI3K activity and phosphorylation of AKT underlie both the escalation of inflammation as well as the proliferation and invasion of epithelial cells. These processes are interrupted by LY294002. Our observations emphasize the value of targeting stromal PI3K activity for effective prevention of colitis and therapy for CRC.

**Materials and Methods**

**Tissue and tumor specimens**

Paraffin-embedded specimens of normal, noninflamed colon from 8 patients who had surgery for nonmalignant lesions such as colon antever venous malformation or diverticular disease were used as controls (normal group). In addition, surgical specimens from 12 patients with ulcerative colitis with active colitis (colitis group), 7 patients with ulcerative colitis with active colitis and dysplasia (dysplasia group), and 7 patients with ulcerative colitis with colitis and invasive CRC (cancer group) were obtained from Rush University Medical Center (Chicago, IL). All procedures were approved by Rush University Medical Center Institutional Review Boards.

**Mice**

Interleukin (IL)-10−/− mice and C57LB6 mice were obtained from Jackson Laboratories. Mice were maintained under specific pathogen-free conditions at Northwestern University Animal Care Facility, and the Animal Care Usage Committee of Northwestern University approved all experiments. IL-10−/− mice (6-weeks old) were transferred to conventional housing and allowed 1 week to acclimate. Two groups were formed with 10 mice per group, called control and treatment group. Both groups of mice received 60 mg/kg body weight of piroxicam from day 0 to 7 and 80 mg/kg body weight piroxicam from day 8 to 14. From day 15 to 35, the treatment group received 50 mg/kg LY294002 intraperitoneal injections dissolved in 20% dimethyl sulfoxide (DMSO; Sigma) every other day. The untreated group received DMSO from day 15 to 35, as described in an earlier study (4). Mice at 56 days postcommencement of piroxicam treatment were sacrificed and used for histologic evaluation.

**Cell culture**

LAD-2 cells were grown in stempro medium (Sigma-Aldrich) with 100 ng/mL stem cell factor (SCF) at 37°C. LAD-2 conditioned media was prepared as described earlier (16, 17). Gut-derived murine mast cells (GMMC) were grown from the gut of C57LB6 mice (18).

**Conditioned medium**

For the production of conditioned medium, 2 × 10⁶/mL LAD-2 mast cell or GMMCs or 1.5 × 10⁶/750 μL tumor-infiltrating leukocytes (TILs) were treated with carrier DMSO or 10 μmol/L LY294002 for 1 hour, washed 4 times with serum-free medium and kept in fresh culture for 1 week (LAD-2 mast cell or GMMCs) and 72 hours (TILs) at 37°C and 5% CO₂. The conditioned medium was removed, filtered using 0.22-μm filters, and used for medium transfer experiments.

**Immunofluorescence and immunohistochemistry**

Four-micrometer thick paraffin-embedded sections were treated with Target retrieval solution (Dako) and incubated...
with anti-tryptase (Neomarkers), anti-CD68 (Dako), anti-P-Akt\(^{T308}\) (Cell Signaling Technology), or anti-bromodeoxyuridine (BrdUrd; Accurate Chemical), followed by anti-rabbit or mouse horseradish peroxidase (HRP)–labeled polymer (Dako), 3',3'-diaminobenzidine (DAB) substrate (Dako), and counterstained with hematoxylin (for immunohistochemistry) and anti-mouse Alexafluor-594 and anti-rabbit Alexafluor-488 followed by 4',6-diamidino-2-phenyindole (DAPI; Invitrogen; for immunofluorescence). Ten-micrometer frozen sections were fixed in cold methanol, blocked with 1% bovine serum albumin (BSA), incubated with anti-MAC-1, α-M chain (Santa Cruz Biotechnology), and anti-P-Akt\(^{T308}\) (Cell Signaling Technology) followed by anti-mouse Alexafluor-594 (Molecular probes), anti-rabbit Alexafluor-488 (Molecular probes), and DAPI (Invitrogen). Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay was conducted as per manufacturer’s instruction (Millipore). Chloroacetate esterase (CAE) staining was conducted as described earlier (18).

**TissueGnostics**

TissueGnostics Tissue/Cell High Throughput Imaging and Analysis System and a semi-automated image acquisition microscope were used to acquire \(\times200\) magnification bright field and fluorescence images throughout the section for all imaging experiments. Images were stitched in Adobe Photoshop Program and analyzed using ImageJ software.

**Immunoblotting**

Whole-cell extracts were prepared in radioimmunoprecipitation assay (RIPA) buffer [10 mmol/L Tris–Cl (pH 7.5), 500 mmol/L NaCl, 0.1% SDS, 1% NP-40, 1% sodium deoxycholate, 2 mmol/L EDTA, and 1% protease, phosphatase I and II inhibitor cocktail (Sigma)]. Proteins (30 μg) were separated by SDS-PAGE and transferred following standard protocols. Immunoreactive proteins were detected with antibodies to phospho-AKT\(^{T308}\), phospho-AKT\(^{S473}\), total AKT (Cell Signaling), and β-actin (Sigma) using the HRP-conjugated secondary antibodies and SuperSignal chemiluminescent reagent (Thermo Scientific).

**β-Hexosaminidase release (mast cell degranulation) assay**

For mouse degranulation, GMMCs were stimulated overnight using mouse anti-DNP immunoglobulin E (IgE) 1 μg/ml concentration. On the next day, cells were harvested, excess IgE was washed with Tyrode buffer, and treated for 120 minutes either with 10 μmol/L LY294002 or the carrier and subsequently challenged with DNP-BSA from Sigma at 100 ng/ml for 30 minutes. The supernatant was collected and stored at 4°C and the pellet was lyzed with 0.1% Triton X. The 20 μL of supernatant or pellet lysate were incubated with 1 mmol/L 4-nitrophenyl N-acetyl-β-D-glucosaminide (PNAG) for 60 minutes at 37°C and the reaction was stopped with 200 μL carbonate buffer (0.1 mol/L, pH 10). β-Hexosaminidase release in the supernatant was measured at 405 absorbance and interpreted as the percentage of total cellular (lysate + supernatant) β-hexosaminidase. For β-hexosaminidase release spectrophotometric kit from Millipore was used.

**Macrophage migration assay**

Blood mononuclear cells were prepared using Ficoll-Paque from GE Healthcare. CD11b\(^+\) cells were isolated using biotinylated anti-CD11b, α-M chain (BD Biosciences), streptavidin magnetic beads (Miltenyi Biotec), and LS column (Miltenyi Biotec) and purity checked using flow cytometry. For the migration assay, CD11b\(^+\) cells were resuspended in serum-free RPMI at 10^6/ml concentration in 22.5 μL and seeded in triplicates in the top wells of 5 μm uncoated 96-well Chemo TX system (Neuro Probe). The bottom well was loaded with 29 μL of Stempro medium without (negative control) or with 100 ng/mL SCF (positive control), or LAD-2 mast cell conditioned medium without or with 10 μmol/L LY294002. Alternatively, LAD-2 mast cells were pretreated with 10 μmol/L LY294002 and washed before conditioning medium. After 3 hours of incubation at 37°C, the migrated CD11b\(^+\) cells were counted with Trypan blue on hemocytometer. Assays were in triplicate.

**Colon cancer epithelial cell proliferation assay**

A total of, 1 × 10^4 colon cancer epithelial HT-29 cells were seeded in triplicates in 100 μL per well in a 96-well plate with McCoy’s 5A medium for 24 hours at 37°C and 5% CO\(_2\). On the next day, the medium was removed and 100 μL of either McCoy’s 5A or Stempro with 100 ng/mL SCF or LAD-2 mast cell conditioned medium or LAD-2 mast cell conditioned medium with 10 μmol/L LY294002 or conditioned medium obtained after 10 μmol/L LY294002 treatment of LAD-2 mast cells were added and incubated for 24, 48, and 72 hours at 37°C and 5% CO\(_2\). A total of 0.5 mCi of[^1]H Thymidine was added to each well and incubated for 6 hours after 24, 48, and 72 hours time points. HT-29 cell proliferation was measured using a scintillation counter (LKB RackBeta; Wallac). Mouse CT44 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) and used in a similar setup to the HT-29 proliferation assay.

**Colon cancer epithelial cell invasion assay**

A total of 3 × 10^4 HT-29 colon cancer epithelial cells were seeded in triplicates in 500 μL per well of McCoy’s 5A in the top wells (inserts) in a 24-well (12) insert plate (BD BioCoat). The bottom well contained the same media as used for the migration assay. After 48 hours at 37°C and 5% CO\(_2\), the noninvaded cells were removed. After fixing the membrane and staining with Diff quick, invading cells were counted using a bright field microscope.

To obtain TILs, surgical colon cancer tissue samples from 3 patients bearing ulcerative colitis-associated colon cancer were washed with DMEM (with 0.5% penicillin/streptomycin, 10 μg/mL gentamycin sulfate), minced with surgical...
blades, digested using 750 U/mL type IV collagenase (Worthington Biochemical), 500 U/mg hyaluronidase (Sigma), 0.1 μg/mL DNase (Sigma), and subjected to Percoll gradient centrifugation (40%–80%). The interphase was collected for invasion assays (16) and incubated in complete RPMI-1640 with 10% FBS with or without 10 μmol/L LY294002 at 37°C, followed by 2 washes with complete RPMI. Immunohistochemical staining revealed the mast cells percentage (41.75% ± 2.394%) and TAMs percentage (31.25% ± 2.562%) in the TIL population (data not shown). In setup 1, $3 \times 10^4$ carboxyfluorescein succinimidyl ester–labeled HT-29 cells were seeded in triplicates in the top wells (inserts) of an invasion assay plate, and in the lower chamber conditioned medium from $1.5 \times 10^6$ TILs was plated. In setup 2, $3 \times 10^4$ carboxyfluorescein succinimidyl ester–labeled HT-29 cells were seeded either alone, or in 1:1 ratio with TILs untreated or treated with 10 μmol/L LY294002 in the top wells, whereas the bottom wells were filled with McCoy’s 5A. After 48 hours at 37°C and 5% CO2, tumor invasion was manually recorded and quantified as earlier. Alternatively, $3 \times 10^4$ CT44 cells were seeded in top wells in serum free DMEM in triplicates, whereas in bottom wells, conditioned medium obtained from untreated or LY294002 pretreated primary mouse mast cells or carrier medium was added in the invasion assay chamber (BD Biocoat).

**Statistical analysis**

All experiments were repeated 3 times and 10 mice were used in each group. Comparison of groups was assessed using the Student t test or ANOVA, where appropriate. For multiple comparisons, data were analyzed using ANOVA. P values less than 0.05 were considered statistically significant.

**Results**

**Bone marrow–derived pAKT-positive cells progressively increase in colitis, dysplasia, and colon cancer**

To understand the spatial distribution and kinetics of PI3K activity in situ during progression from colitis to cancer, human surgical specimens were separated into 4 groups according to their histopathologic and clinical findings, namely: (i) no colitis no dysplasia (designated “normal” in this study), (ii) ulcerative colitis without dysplasia (colitis), (iii) ulcerative colitis with dysplasia (dysplasia), and (iv) ulcerative colitis with invasive CRC (invasive cancer; Fig. 1A and Supplementary Table S1–S4). The study
was distributed according to mucosal and submucosal findings (Fig. 1B and C). For mucosal tissue, data were analyzed from the muscularis mucosa extending to the lumen, including epithelium, lamina propria, and the muscularis mucosa itself. Tissue underneath muscularis mucosa was considered submucosal (Fig. 1). Phosphorylated AKT (pAKT) cells were detected by immunohistology in mucosa (Fig. 2A, B, and F) and submucosa (Fig. 2C and G). The mean frequencies of epithelial pAKT cells in mucosa did not show significant differences when comparing normal (0.59 ± 0.23) to colitis (0.74 ± 0.13) to dysplasia (0.69 ± 0.13) and to invasive cancer (1.10 ± 0.17; Fig. 2A). The frequency of stromal pAKT cells infiltrating the mucosa in all cases outnumbered pAKT epithelial cells (compare Fig. 2A and B). Significant increases in pAKT cells were detected in the stroma of the mucosa when progressed from "normal" (2.33 ± 0.65) to colitis tissue (6.83 ± 1.12; *, P < 0.05), but thereafter plateaued (Fig. 2B) with no significant differences from colitis to dysplasia (5.81 ± 1.27) to invasive cancer (8.27 ± 1.48). In contrast, the frequency of pAKT cells steadily and significantly increased in the submucosa with each transition from "normal" (2.33 ± 0.65), to colitis (6.56 ± 0.80; *, P < 0.05), to dysplasia (16.19 ± 4.70; *, P < 0.05) and finally to
cancer (37.87 ± 7.39; *, P < 0.05), with cancer having the highest density of pAKT+ cells (Fig. 2C).

To validate the presence of TAMs in tissue samples, we stained histologic sections with antibodies to pan-macrophage antigen CD11b (Mac1; ref. 19) and CD68 (20, 21). On the basis of mouse modeling, it has been suggested that chemoattractants, growth factors, and pathogen-associated molecular patterns initiate tumor inflammation by activating PI3K in CD11b+ myeloid cells (13). To test this notion, we did double immunofluorescence staining for pAKT and macrophage markers (Fig. 2D and E and Supplementary Fig. S3A and S3B). There was abundant colocalization of pAKT with CD68 (Fig. 2D and E). Densities of pAKT+ CD68+ TAMs increased in the mucosa when disease progressed from normal (2.33 ± 0.56) to colitis (4.77 ± 0.96) but did not increase further in dysplasia (5.81 ± 1.27) and cancer (6.27 ± 1.14; Fig. 2D and H). In contrast, the densities of pAKT+ CD68+ TAMs increased steadily from normal (1.56 ± 0.44) to colitis (4.83 ± 0.67), dysplasia (12.58 ± 3.83), and cancer (34.54 ± 4.56) in the submucosa (Fig. 2E and I). Total CD68+ cell densities (inclusive of all pAKT+) also increased progressively in the submucosa from normal (2.13 ± 0.45), colitis (6.18 ± 0.84), dysplasia (18.81 ± 4.23) to cancer (38.62 ± 4.51; data not shown). TAM identity was validated by additional immunostainings for total Mac1+ and Mac1+pAKT+ cells (data not shown).

Mast cells increase with progression to colitis and to cancer and recruit TAMs in a PI3K-dependent manner

Mast cells are sentinel cells that are activated early in the process of intestinal carcinogenesis, and contribute to cancer initiation (11). In mouse models of cancer, mast cells orchestrate further inflammatory reactions by mobilizing TAMs (18, 22, 23). Previously, we provided evidence for mast cell recruitment of TAMs in human pancreatic cancer (16). To relate tissue mast cell densities to mobilization of TAMs in colitis progression to cancer, we stained paraffin-embedded tissues for mast cell-tryptase (Fig. 3). Images from 50 fields of vision were recorded for quantification by TissueGnostics high-throughput imaging microscope for each sample of mucosa and submucosa. Mast cells were detected in mucosa and submucosa (Fig. 3A–D) from samples with colitis, dysplasia, and cancer. Relative densities and subtissue distributions of mast cell mirrored that of pAKT+ cells. In other words, mast cell frequencies in mucosa increased significantly from normal colon (4.75 ± 0.56) to colitis (14.17 ± 1.82; *, P < 0.05), but did not increase further as the disease progressed from colitis to invasive cancer (colitis, 14.17 ± 1.82; dysplasia, 15.54 ± 3.07; invasive cancer, 19.44 ± 3.74; Fig. 3A). In contrast, mean mast cell frequencies in submucosa increased steadily as the disease progressed from normal (6.34 ± 0.99) to colitis (12.35 ± 1.86; *, P < 0.05), to dysplasia (33.54 ± 8.55;
Mast cells orchestrate secondary inflammatory reactions by recruiting other bone marrow–derived cells (11, 24) such as TAMs that critically contribute to CRC progression (25). We postulated that PI3K activity in mast cell is needed for their chemotactic potential. To test this hypothesis, we preincubated human LAD-2 mast cell in the presence or absence of LY294002, and tested inhibition of PI3K activity by Western blot analysis of phospho-proteins. Proteins separated by gel electrophoresis and transferred to membrane were reacted with antibodies to phospho-AKT T308, phospho-AKT S473, total AKT (Cell Signaling) and β-actin (Sigma). This analysis showed that preincubation of mast cell for 1 hour with 10 μmol/L of LY294002, hindered T308-phosphorylation of AKT by 1.74±0.15-fold and S473 by 4.01±0.38-fold. (Fig. 4A and B). Next, we treated LAD-2 mast cell with 10 μmol/L of LY294002 and washed the cells before putting them back into culture to collect conditioned medium. The chemotactic activity of conditioned media was then tested by measuring migration of CD11b+ macrophages freshly prepared from human peripheral blood mononuclear cells (PBMC) through Chemo TX system. To assay migration of macrophages, we used the Chemo TX 5-μm pore size migration assay system. CD11b+ macrophages were loaded in the top chamber and the conditioned media from LAD-2 mast cell were loaded in the bottom chamber. There was a significant migration of the CD11b+ macrophages to the bottom chamber containing conditioned medium from untreated mast cell as compared with similar setups where regular nonconditioned medium was used for comparison (*, P<0.05; Fig. 4D). Pretreatment of LAD-2 mast cell with 10 μmol/L of LY294002 abrogated the bioactivity of the conditioned medium in this assay, and thus CD11b+ macrophage migration (*, P<0.05; Fig. 4D; and Supplementary Fig. S2). PI3K activity is essential for differentiation of mast cells, as well as their long-term survival and function (12). Mature mast cells produce various biologically active mediators, which are released either by secretion or by degranulation. In particular, it has been reported that in mouse models of cancer, inhibiting mast cell degranulation abrogates tumor-promoting properties of mast cells (26). Hence, we decided to test the impact of different concentrations (5 and 10 μmol/L) of LY294002 on mast cell degranulation, and for this purpose we used human LAD-2 mast cell. Treatment of LAD-2 mast cell with LY294002 inhibited degranulation—the β-hexosaminidase release (%) in carrier-treated/control (72.38±5.78) was reduced after 5 μmol/L LY294002 (47.76±6.43; *, P<0.05) and 10 μmol/L LY294002 treatment (39.82±4.39; *, P<0.05; Fig. 4E).

LY294002 inhibits mast cell tumor-promoting properties

Next, we hypothesized that mast cell produce soluble factors that enhance the proliferation and invasion of epithelial cancer cells (16) and investigated the role of PI3K in the proliferative response of HT-29 colon cancer cells to human LAD-2 mast cell (27). LAD-2 mast cell conditioned medium enhanced the rate of proliferation of HT-29 cells progressively at 24, 48, and 72 hours (*, P<0.05; Fig. 4F–H). Next, we tested the effect of inhibition of PI3K by treating LAD-2 mast cell with 10 μmol/L LY294002 and preparing conditioned medium from washed cells. Preincubation with LY294002 significantly reduced the ability of the LAD-2 conditioned medium to stimulate proliferation of HT-29 cells, measured at 3 separate time points (*, P<0.05; Fig. 4F–H). We then tested the response of HT-29 cells against direct treatment with the PI3K inhibitor. LY294002 has direct inhibitory effects on the tumor cells. However, the direct inhibitory effect of LY294002 was less significant at 24- and 48-hour time point in comparison with the LY294002 pretreated LAD-2 conditioned medium, whereas at 72 hours similar trend was seen, but data were not significant (Fig. 4F–H). These observations suggest that PI3K activity in mast cell contributes to tumor proliferation and its inhibition by LY294002 is a critical event in suppression of tumor growth.

We further investigated the possibility that PI3K activity and phosphorylation of AKT in mast cell contributes to tumor invasion. To test this hypothesis, we conducted in vitro invasion assays with the HT-29 colon cancer cells in the presence or absence of LAD-2 conditioned medium. Because, LY294002-treated LAD-2 conditioned media attenuates HT-29 proliferation by 40% at 48 hours, we normalized the HT-29–invaded cell count (reduced the cell number by 40% in Control/Stempro+SCF and LAD-2 conditioned media groups for analysis and graphical representation). There was a significant increase in mean HT-29 cell invasion/well in Matrigel in response to LAD-2 MC conditioned medium (64.80±6.92; *, P<0.05) in comparison with the control (11.40±1.03; *, P<0.05; Fig. 4I; Supplementary Fig. S3A). Invasion was attenuated when the conditioned medium was obtained from LAD-2 mast cell that had been previously treated with 10 μmol/L LY294002 as described earlier (17.67±1.45; *, P<0.05; Fig. 4I; Supplementary Fig. S3A). As with the proliferation response, LY294002 had direct inhibitory effect on tumor cell invasion. However, even in the presence of this inhibitor, LAD-2 conditioned medium elicited a significant invasion response in the tumor cells (38.67±4.91; *, P<0.05; Fig. 4I). These observations strongly suggest that mast cells promote tumor invasion and that this property of mast cells is partially PI3K-dependent. Thus, release of tumor-promoting agents by mast cells and potential contribution of mast cells to tumor growth and invasion were blocked by LY294002.

Next, we measured the ability of TILs isolated from CRC tumors to promote tumor cell invasion. TILs were checked for phosphorylation at the Threonine 308 residue (pAKT-T308) and the Serine 473 residue of AKT (pAKT-S473). Of note, 10 μmol/L LY294002 treatment significantly attenuated pAKT-T308 1.62±0.05-fold and pAKT-S473 3.53±0.17-fold in comparison with carrier-treated pAKT-T308 and pAKT-S473 (*, P<0.05; Fig. 4A and C). By pretreating the TILs with LY294002, we tested the dependence of tumor
invasion promoting activity on PI3K. To address this question, we isolated TILs from fresh surgical specimens derived from tumors of patients with colitis-associated colon cancer. Two different setups were used. In the first setup, we added tumor cells in the top chamber and conditioned medium from the TILs (carrier or 10 μmol/L LY294002 pretreated) in the bottom chamber. In the second setup, we plated the bottom well with conditioned medium derived from a coculture of the TILs with the tumor cells, again pretreated with carrier or 10 μmol/L LY294002. After normalization of HT-29 cell counts in Stempro+SCF, TILs conditioned media and HT-29+TILs study groups, we found mean invasion of HT-29 cells into Matrigel was significantly enhanced by the TILs in both experimental setups (76.80 ± 5.67 for setup-1 and 133.80 ± 7.99 for setup-2; *, P < 0.05; Fig. 4I and K; Supplementary Fig. S3B). Pretreatment of the TILs with 10 μmol/L LY294002 significantly inhibited tumor invasion relative to treatment with conditioned media from carrier-treated TILs or coculture with carrier-treated TILs, respectively (26.67 ± 6.93 for setup-1 and 57.00 ± 4.72 for setup-2; *, P < 0.05; Fig. 4I and K; Supplementary Fig. S3B). These results are compatible with those obtained with mast cell conditioned medium and show that the PI3K inhibitor LY294002 inhibits production of mediators of tumor invasion by TILs, including TAMs and mast cells.

LY294002 treatment inhibits mast cells, colitis, and cancer development in the IL-10−/− piroxicam mouse model

To further validate our in vitro observations and to see if PI3K/AKT play central roles in the in vivo progression of colonic inflammation into colon cancer, we treated cancer-prone colitis mice with LY294002. IL-10−/− mice, when treated with piroxicam, develop colitis with ulcers, followed by invasive cancer by day 56 (mean invasive lesions 2.30 ± 0.26; Fig. 5A and F; ref. 4). LY294002 pretreatment reduced the incidence of invasive cancer in this model (0.100 ± 0.10; *, P < 0.05; Fig. 5A and F). Reduced frequency of BrdUrd-positive cells (*, P < 0.05; Fig. 5B and G), increased apoptosis as measured by TUNEL (*, P < 0.05; Fig. 5C and H), and reduced the pAKT levels within the crypt-epithelium (*, P < 0.05; Fig. 5D and I), and stroma (*, P < 0.05; Fig. 5E and J).

We had reported earlier a causative role for focal mastocytosis and preneoplasia in the mouse intestine (11, 18). Thus, we used CAE staining to study the in vivo impact of LY294002 on mast cells infiltrating the gut tissue. CAE is a cytochemical staining that stains mast cells and granulocytes (28). We found that LY294002 treatment inhibited the mean frequencies of tissue-infiltrating CAE+ cells (0.26 ± 0.06 in comparison with control untreated mice (0.98 ± 0.09; *, P < 0.05; Fig. 6A and F). Moreover, LY294002 treatment significantly attenuated mast cell degradation in situ (purple mast cells; % mean, 30.12 ± 2.98), found predominantly in the submucosa (site of invasion) of the non-LY294002–treated mice (85.02 ± 1.57; *, P < 0.05; Fig. 6B and G).

We used in vitro assays to validate inhibition of degranulation in gut-derived primary mouse mast cells by LY294002. The β-hexosaminidase release (%) in carrier-treated GMMCs (33.75 ± 0.49) dropped after 5 μmol/L LY294002 (11.28 ± 0.47; *, P < 0.05) and 10 μmol/L LY294002 (6.86 ± 0.39; *, P < 0.05; Fig. 6C) treatment. Next, we used the pretreated conditioned medium (with 10 μmol/L LY294002 or carrier) obtained from mouse mast cells to study the effect of PI3K inhibition on mouse mast cells in the context of CT44 mouse colon cancer proliferation and invasion. We found that, LY294002 pretreated conditioned medium significantly attenuated the mean CT44 cell proliferation counts (8580.00 ± 1009) in comparison with conditioned medium obtained from carrier-treated mouse mast cells at 24 hours time points (12910.00 ± 678.20; *, P < 0.05; Fig. 6D). However, at 48- and 72-hour time points there was no significant difference between the 2 groups (data not shown). Similarly, the mean CT44 cell invasion per well after normalization (CT44 invasion count number in carrier-treated study group only was normalized by reducing 33.53% as at 24 hours the CT44 proliferation was attenuated by 33.53%) was significantly attenuated by 10 μmol/L LY294002 pretreated conditioned medium (303.70 ± 16.70) in comparison with conditioned medium from carrier-pretreated mouse mast cells ([516.70 ± 45.18; *, P < 0.05; Fig. 6E and H). These observations show that PI3K and phosphorylation of AKT are critical for mast cell functions that promote cancer and LY294002 inhibits these functions.

Figure 4. LY294002 treatment attenuates mast cell (MC) degranulation and mast cells associated macrophage migration. HT-29 tumor proliferation and invasion. A, immunoblot of total and phosphorylated AKT in LAD-2 mast cells and TILs untreated and treated with 10 μmol/L LY294002. Bar graphs indicate quantitation of the (B) ratio of LAD-2 MC band intensity with LAD-2 MC treated with 10 μmol/L LY294002, indicating fold inhibition of phosphorylation by LY294002 using ImageJ software. D, mean ± SE CD11b migration in response to Stempro + SCF (control/LAD-2 base growth medium), conditioned media (CM; LAD-2 conditioned medium), conditioned media + 10 μmol/L LY294002 (conditioned media with 10 μmol/L LY294002), and 10 μmol/L LY pretreated conditioned media (conditioned media obtained after treatment of LAD-2 cells with 10 μmol/L LY294002). E, percentage β-hexosaminidase release from LAD-2 mast cells before and after treatment of 5 or 10 μmol/L LY294002. Mean ± SE HT-29 proliferation (F) after 24 hours, (G) after 48 hours, and (H) after 72 hours of treatment of McCoy’s 5A (negative control), Stempro + SCF (internal control for LAD-2 conditioned media), conditioned media (LAD-2 conditioned media), conditioned media + 10 μmol/L LY294002 (conditioned media with 10 μmol/L LY294002), and 10 μmol/L LY pretreated conditioned media (conditioned media obtained after treatment of LAD-2 cells with 10 μmol/L LY294002). I, quantification of mean ± SE invaded HT-29 cells per well or chamber in response to Stempro + SCF (internal control for LAD-2 conditioned media), LAD-2 conditioned media, LAD-2 conditioned media + 10 μmol/L LY294002 (conditioned media with 10 μmol/L LY294002), and 10 μmol/L LY pretreated conditioned media (conditioned media obtained after treatment of LAD-2 cells with 10 μmol/L LY294002). J, quantitation of mean ± SE invaded HT-29 cells per well in the experimental setup-1 (HT-29 in top chamber and conditioned media from TILs with or without 10 μmol/L LY294002 pretreatment in bottom well), K, quantitation of mean ± SE invaded HT-29 cells per well in the experimental setup-2 (HT-29 in 1:1 coculture with TILs with or without 10 μmol/L LY294002 pretreatment in top chamber). *, P < 0.05 represents the result of ANOVA. c.p.m., counts per minute.
Discussion

Inflammation plays a pivotal role in the initiation and progression of colon cancer (11, 29). Chronic inflammation in patients with ulcerative colitis increases the risk of rapidly progressing CRC (30, 31). It is known that PI3K activity significantly increases in CRC and is associated with poor prognosis (32, 33). Mouse models of ulcerative colitis have shown that PI3K activity is critical for progression to cancer (4, 18, 34). However, much of what is known is focused on the role of PI3K signaling in tumor cells; furthermore, the relevance to inflammation-driven colon cancer in humans remains unclear (4, 18, 34). By *in situ* staining of human surgical specimens, we found that PI3K activity overlapped abundantly with tissue-infiltrating mast cells and macrophages and tumor-infiltrating cells. Increasing densities of PI3K-stained cells as well as mast cells and TAMs in the submucosa of colon tissues were noted in patients with ulcerative colitis as inflammation progressed to cancer. These reached a peak in the submucosal areas of preneoplasia and cancer. Earlier studies have shown the presence of mast cell and TAMs in the subepithelial region of the mucosa in colitis (35, 36) and the stromal tumor front in CRC (37, 38). It is in these locales that mast cells and TAMs have been shown to promote tumor growth via angiogenesis and proinflammatory growth factors (11, 25). Using *in vitro* experiments with human-derived cell lines, we found that mast cells readily mobilize macrophages and that conditioned medium from both promoted invasion of tumor cells into Matrigel. LY294002 attenuated macrophage migration and tumor invasion. These results were validated and extended in the mouse model of experimental colitis and colon cancer, piroxicam-treated IL-10−/− mice. LY294002 treatment of the mice significantly inhibited PI3K activity and reduced infiltrating mast cell and TAMs, epithelial proliferation, and progression to cancer. Also, *in vivo* LY294002 treatment of mice attenuated mast cell degranulation in the colonic tissue and more specifically in the submucosa, and *in vitro* pretreatment of gut-derived mouse mast cells with LY294002 reduced epithelial cell proliferation and invasion in response to mast cell conditioned medium. These results indicate that PI3K activity in the tumor stroma and submucosa contributes toward cancer progression. Mast cells and macrophages escalate inflammation that predisposes to cancer, whereas cancer-associated inflammation promotes tumor proliferation and invasion. In preneoplasia, increased PI3K activity of epithelial cells is more likely to be caused by inflammation than mutation. Our observations suggest that the tissue and tumor microenvironments are the primary factors.
targets of action of LY294002. Both mast cells and TAMs are inhibited by treatment with LY294002.

The current study provides for the first time insight into the spatial distribution of PI3K activity, mast cell, and TAMs in human colon during progression from chronic inflammation to dysplasia and cancer. Our observations build upon previous reports on the prevalence of PI3K activity in human tumors and infiltration by mast cells and TAMs (9, 32, 39–42). Much of the attention to the role of PI3K in preneoplasia and cancer in the gastrointestinal tract has focused on gut epithelial cells. In particular, previous reports based on mouse modeling suggest that progression

Figure 6. LY294002 attenuates inflammation and mast cell (MC) degranulation in IL-10−/− mice in vivo, and tumor cell proliferation and invasion ex vivo. A, quantification of % mean ± SE CAE+ cells per total nuclei in LY294002 untreated and treated IL-10−/− mice. B, quantification of % mean ± SE in vivo mast cell degranulation per total mast cells in the colon of the LY294002 untreated or treated IL-10−/− mice (percentage in situ degranulation = total degranulating mast cells × 100/total mast cells in LY294002 untreated or treated IL-10−/−). C, quantification of percentage in vitro β-hexosaminidase release from GMMCs after treatment of carrier or 5 or 10 μmol/L LY294002. D, quantification of mean ± SE CT44 mouse colon cancer proliferation at 24-hour time point in response to carrier or 10 μmol/L LY294002 treated GMMC conditioned medium. E, quantification of mean ± SE CT44 cell invasion per well in response to carrier or 10 μmol/L LY294002 treated GMMC conditioned medium. F, CAE staining at ×100 and ×200 magnification, respectively, of IL-10−/− mice 56 days after piroxicam ± LY294002 treatment, small arrows indicate CAE+ cells. G, toluidine blue staining at ×100 and ×1,000 magnification, respectively, of IL-10−/− mice 56 days after piroxicam ± LY294002 treatment, large black arrow indicates magnified, degranulating, or nondegranulating mast cells. H, CT44 mouse colon cancer cell invasion in response to conditioned medium obtained either carrier or LY294002 treated GMMCs. Scale bar in F and top of G, 50 μm, scale bar for ×1,000 magnification for G, 20 μm; *, P < 0.05 represents the result of Student t test.
from colitis to cancer is associated with PI3K activity in crypt epithelial cells. In the IL-10–deficient mouse model, PI3K is required for induction of colitis, whereas PI3K’s targeted genetic ablation (4, 5) or treatment of mice with the broad PI3K inhibitors mesalamine (34) or LY294002 (4) protect against colitis. PI3K has been described as mediating proliferation and activation of AKT and β-catenin in epithelial stem cells resulting in crypt architectural changes that predispose to colitis (4). However, we show with unbiased in situ staining of human colonic tissue sections that the relative frequency of detectable PI3K active cells in the stroma and submucosa is more than 10-fold above that in epithelial cells and increases with colitis and cancer. In contrast, in the IL-10−/− mouse colon, this ratio is reversed. This difference between human and mouse tissue may have masked the significance of PI3K activity of tissue-infiltrating cells in predisposition to colitis and progression to cancer.

PI3K activity is essential for the differentiation, homing, and functions of a number of key proinflammatory cell types that drive colitis and progression to cancer. PI3K is essential for mast cell differentiation and function, to the extent that inhibition of PI3K by overexpression of the dominant negative inhibitor Dp85 leads to a significant decline in mast cell degranulation via antigen-induced Ca2+ signals (43, 44), and mice deficient for PI3K activity are devoid of mast cells (12, 45). Furthermore, earlier mouse studies have shown that proinflammatory macrophages with tumor-promoting properties have high levels of PI3K activity (13). We have shown in the past that mast cells are an inherent component of intestinal carcinogenesis in mouse models of hereditary polyposis (11, 18). Mast cells increase in ulcerative colitis and in colon cancer (11). Higher densities of mast cells correlate with active angiogenesis and poor clinical outcome for patients with CRC (39, 46, 47). We showed that treatment of mice with the PI3K inhibitor LY294002 blocks degranulation of mast cells.

Macrophages are among the major and most extensively proinflammatory bone marrow–derived cells that accumulate in tumors and contribute to CRC progression and invasion (22, 25, 48). We showed that pAKT levels steadily increased in the submucosa and colocalized with CD68+ TAMs as colitis progressed to invasive cancer. Furthermore, we showed that inhibition of PI3K activity hinders the ability of these cells to promote tumor cell proliferation and invasion. We found TAMs from patients with ulcerative colitis promoted HT-29 invasion, either through their secreted soluble factors in the conditioned medium (experimental setup-1) or direct cell–cell contact (experimental setup-2). Indeed, when these cells were cocultured, invasion was even more pronounced (experimental setup-2); LY294002 treatment of TILs reduced their ability to promote HT-29 invasion in both setups.

Tissue-infiltrating mast cells and macrophages are sensitive to inhibition of PI3K and are abundantly present in increasing numbers during progression to colitis and cancer. Therapies that target the PI3K pathway need to take into account that tumor cells may not be the primary target cells. Our findings show the role of PI3K in tumor-infiltrating cells and their communication with tumor cells, drawing attention to the role of PI3K signaling in the tissue and tumor environment in predisposition to cancer.

Disclosure of Potential Conflicts of Interest
A. Keshavarzian has ownership interest (including patents) in a pending patent with co-owner of a company called NutraBiotic. No potential conflicts of interest were disclosed by the other authors.

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References


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