The Absence of LPA_2 Attenuates Tumor Formation in an Experimental Model of Colitis-Associated Cancer

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Background & Aims: **Chronic inflammation is a risk factor for colon cancer (CC). Lysophosphatidic acid (LPA), a naturally produced phospholipid, mediates multiple effects that are vital to disease process, including inflammation and cancer. The expression of LPA receptor 2 (LPA_2) is up-regulated in several types of cancer, including ovarian and colon cancer, but the importance of LPA and LPA_2 in the development and progression of CC is unclear. In this study, we sought to determine whether LPA and LPA_2 regulate the progression of CC in vivo.** ***Methods:*** **We examined the potential role of LPA in CC progression by administering LPA to mice heterozygous for the adenomatous polyposis coli (Apc) allele. We determined the loss of LPA_2 function in tumorigenesis in the colon by treating mice with genetic deletion of LPA_2 ($LPA_2^{-/-}$) with azoxymethane and dextran sulfate sodium.** ***Results:*** **We found that LPA increased tumor incidence in $Apc^{min/+}$ mice. $LPA_2^{-/-}$ mice showed reduced mucosal damage and fewer tumors than wild-type (WT) mice. Reduced epithelial cell proliferation and decreases in β-catenin, Krüppel-like factor 5, and cyclooxygenase-2 expression were observed in $LPA_2^{-/-}$ mice. Unlike WT mice, induction of monocyte chemoattractant protein-1 and macrophage migration inhibitory factor was significantly attenuated in $LPA_2^{-/-}$ mice with reduced infiltration by macrophages.** ***Conclusions:*** **These results show that LPA is capable of promoting tumorigenesis in the colon. The absence of LPA_2 attenuates several effects that contribute to cancer progression in vivo, and, hence, the current study identifies LPA_2 as an important modulator of CC.**

Colon cancer (CC) is the fourth most common cancer, and inflammation is an established risk factor for CC.[1] Patients with inflammatory bowel disease are at increased risk for CC, and the mortality in patients diagnosed with CC in the setting of inflammatory bowel disease is higher than for sporadic colorectal cancer.[1]

Lysophosphatidic acid (LPA) is an extracellular lipid mediator that evokes multiple growth factor-like effects in almost every cell type.[2,3] LPA mediates its effects primarily by coupling to a family of G-protein-coupled receptors: LPA_1–LPA_5.[4] The initial indication that LPA could contribute to tumorigenesis came from studies showing that LPA increases cell proliferation and motility.[5] Subsequently, overexpression of LPA_2 in ovarian cancer cells has suggested that aberrant LPA-LPA_2 signaling axis might have a tumor-promoting activity in ovarian cancer.[6,7] Recent evidence shows that deregulation of LPA_2 or LPA_3 is more commonly found in other types of cancer.[8–10]

Mice deficient in individual LPA receptors are available.[11–13] Whereas mice with targeted deletion of LPA_1 or LPA_3 show receptor-specific defects such as craniofacial deformity or delayed implantation of embryos,[11,13] genetic ablation of LPA_2 in mice ($LPA_2^{-/-}$) does not cause any obvious phenotypic defect.[12] Moreover, transgenic expression of LPA_2 in ovaries did not result in ovarian malignancy in mice.[14] Although the absence of tumors in LPA_2 transgenic mice implies a nonessential or redundant role of LPA_2 in tumorigenesis, a body of in vitro experimental evidence tends to suggest the insufficiency of LPA-LPA_2-signaling axis to induce malignancy in ovary.[14] Therefore, the significance of LPA_2 in tumorigenesis remains unclear, and specific pathways affected are not known.

Azoxymethane (AOM), a metabolite of 1,2-dimethylhydrazine, has been used widely to induce the formation of precancerous epithelial lesions, aberrant crypt foci (ACF).[15] Together with AOM, repeated oral administration of dextran sulfate sodium (DSS) is widely employed to induce acute inflammatory reaction and ulceration in the entire colon and accelerates and increases the incidence of colon carcinogenesis.[16] The potential role of LPA in inflammation has extensively been studied,[17] and chronic inflammation increases the risk of CC.[1] In this study, we investigated the loss of LPA_2 function in colitis-associated cancer (CAC). Our study revealed that the extent of colon carcinogenesis was markedly decreased in

Abbreviations used in this paper: ACF, aberrant crypt foci; AOM, azoxymethane; Apc, adenomatous polyposis coli; LPA, lysophosphatidic acid; CAC, colitis-associated cancer; CC, colon cancer; COX-2, cyclooxygenase-2; DSS, dextran sulfate sodium; KLF5, Krüppel-like factor 5; MCP-1, monocyte chemoattractant protein 1; MIF, macrophage migration inhibitory factor; PG, prostaglandin.

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0016-5085/09/$36.00
doi:10.1053/j.gastro.2009.01.002

$LPA_2^{-/-}$ mice with decreased epithelial cell proliferation and infiltration of inflammatory leukocytes.

Materials and Methods

Mice

$LPA_2^{-/-}$ mice[12] were bred into C57BL/6 background for at least 10 generations. $LPA_2^{+/-}$ mice were crossbred to derive wild-type (WT) (ie, $LPA_2^{+/+}$), $LPA_2^{+/-}$, and $LPA_2^{-/-}$ littermates, and these littermates were used in all studies. Experiments with animals were carried out under approval by the Institutional Animal Care and Use Committee of Emory University (Atlanta, GA).

LPA Treatment

Six-week-old male C57BL/6 mice and mice heterozygous for the adenomatous polyposis coli (Apc) allele $(Apc)^{Min/+}$ mice (Jackson Laboratory, Bar Harbor, ME) were given 1 μg/kg LPA suspended in 0.1% bovine serum albumin (BSA) containing phosphate-buffered saline (PBS) (~0.06 μmol per mouse; Avantis Polar, Alabaster, AL) by placing LPA into the stomach using a 22-gauge gavage needle once every 3 days for 1 month. Control animals received the same volume of PBS/0.1 % BSA. Animals were killed 1 month after the last LPA administration. The small intestine was removed, flushed with ice-cold PBS, cut open longitudinally along the main axis, and examined under a dissecting microscope for the presence of adenomas.

AOM and DSS Treatment

WT, $LPA_2^{+/-}$, and $LPA_2^{-/-}$ littermates of 6- to 8-week-old age were injected with 10 mg/kg of AOM (Midwest Research Institute, Kansas City, MO) intraperitoneally at the beginning of the experiment. After 14 days, mice were given 3% DSS (Sigma–Aldrich, St. Louis, MO) in drinking water for 7 days, followed by a 2-week period of recovery with normal water. This was followed by another 1-week 3% DSS treatment and 2 weeks of normal water. Body weight of each mouse was measured and recorded daily. Mice were killed on day 36 or day 56. Colon was removed, flushed with ice-cold PBS, cut open longitudinally, and examined under a dissecting microscope for the presence of tumors as previously described.[16] Colonic tissues were fixed in 10% buffered formalin overnight for histologic analysis. Paraffin-embedded sections were stained with H&E for microscopic assessment of colitis.

AOM Treatment

$LPA_2^{-/-}$ and WT littermates of 5- to 6-week-old age received 4 weekly intraperitoneal injections of AOM at 10-mg/kg body weight. Four weeks after the last AOM injection, all mice were killed, and their colons were removed and fixed in 4% paraformaldehyde. Fixed tissues were stained with methylene blue and ACF were visualized by stereo microscopy. ACF were identified using a published set of criteria.[18]

Immunohistochemistry

See Supplementary Materials.

Myeloperoxidase Activity

See Supplementary Materials.

Quantitative Reverse-Transcription Polymerase Chain Reaction

See Supplementary Materials.

Statistical Analysis

Data are expressed as means ± SE. Statistical significance was determined by 1-way ANOVA. P values $< .05$ were considered significant.

Results

Effect of LPA On the Development of Adenomas in $Apc^{Min/+}$ Mice

Previous studies have shown that LPA_2 expression is elevated in human CC patients and cell lines.[9,10] Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) analysis showed that LPA_2 messenger RNA (mRNA) level was elevated 3.5 ± 0.78-fold (n = 6) in intestinal adenomas of $Apc^{Min/+}$ mice compared with normal intestinal tissue from WT controls (Figure 1*A*). There was a small statistically significant increase in LPA_1 in $Apc^{Min/+}$ mice ($P < .05$) as well, but LPA_3 expression was not different.

LPA plays a vital role in intestinal wound healing, cell proliferation, cell survival, cytokine induction, and regulation of ion transport.[9,10,19–22] We initially tested whether LPA regulates tumorigenesis in the colon by orally administering LPA to $Apc^{Min/+}$ mice. Although the stability of orally administered LPA may be a concern,[23] a previous study has shown that orally administered LPA is effective in inhibition of fluid secretion in the ileum, demonstrating the bioavailability of orally administered LPA in the intestine.[22] Administration of LPA increased the number of adenomas (60 ± 5.2, n = 9, $P < .001$) in $Apc^{Min/+}$ mice compared with $Apc^{Min/+}$ mice that received carrier (32 ± 6.7, n = 9) (Figure 2*B*). This result shows that LPA may potentiate tumorigenesis in the colon, although whether this effect was mediated by LPA_2 is unclear.

Reduced Tumor Incidence in $LPA_2^{-/-}$ Mice

We next sought to determine the importance of LPA_2 in colon tumorigenesis by characterizing the loss of LPA_2 function in vivo. To this end, we used a CAC model, which combines the treatment of mice with AOM and DSS (Figure 2*A*). AOM and DSS induced colitis in WT, $LPA_2^{+/-}$, and $LPA_2^{-/-}$ littermates as evidenced by the weight loss (Figure 2*B*) and appearance of soft stool, but a significant difference in weight loss between WT and $LPA_2^{-/-}$ mice was observed. Similarly, the clinical score,

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