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CXCR2 Inhibition Enhances Sulindac-Mediated Suppression of Colon Cancer Development

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Abstract

Small chemical compound sulindac has been approved as a preventive approach against colon cancer for its effectiveness in treatment of precancerous adenoma. Due to its severe toxicities in the cardiovascular, gastrointestinal and renal systems, however, a combination of low-dose sulindac with other chemopreventive agents has been sought after as an alternative therapeutic strategy that could increase its effectiveness, while minimizing its adverse effects. In order to identify the promising alternative approach, we investigated the therapeutic potential of targeting the Interleukin (IL)-8/CXCR2 pathway in colon cancer treatment using both loss-of-function (CXCR2 knockout) and gain-of-function (IL-8 overexpression) mouse models, as the IL-8/CXCR2 pathway has been shown to be activated in intestinal tumors of both human and experimental animals. We found that deletion of CXCR2 gene and ectopic expression of IL-8 suppresses and enhances, respectively, intestinal tumor development caused by a mutation in the APC gene. Moreover, a single copy deletion of CXCR2 gene resulted in abrogation of COX-2 and Gro- α upregulation in intestinal tumors caused by the APC mutation. Moreover, a single copy (heterozygote) deletion of CXCR2 gene was sufficient to synergize with a low-dose sulindac treatment in suppressing APCmin-induced intestinal polyposis. Together, our study provides a therapeutic justification of combined inhibition of CXCR2 and sulindac treatment in colon cancer prevention.

Keywords

Interleukin-8; CXCR2; adenomatous polyposis coli; colon cancer; sulindac

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INTRODUCTION

Germline mutations in the adenomatous polyposis coli (APC) gene have been reported to induce familial adenomatous polyposis (FAP), an inherited colorectal cancer syndrome that is characterized by hundreds of adenomatous polyps in the colon ^{1, 2}. Although FAP accounts for only ~ 1% of colon cancer, somatic mutations in the APC gene have been associated with the majority of colorectal cancer incidence ³. Like mutations associated with FAP, mutations in the murine homolog of the APC gene, such as multiple intestinal neoplasia (min) ⁴ allele, can develop a large number of polyps in the intestine at an early age, and thus mouse model harboring the min allele, named *Apc^{min}* mice, has served as an excellent experimental tool to study human colon cancer ⁵. Importantly, transcriptional profiling studies using the intestinal polyps from *Apc^{min}* mice and from human FAP patients have commonly demonstrated upregulation of several inflammation-related genes, including CXCR2, GRO- α , IL-8, and PTGS2/COX-2 ⁶. GRO- α is a secreted growth factor that, like IL-8, binds to and activates CXCR2, and plays a major role in inflammation by acting as a chemo-attractant for neutrophils and other immune cells. CXCR2, a high affinity receptor for IL-8 and Gro- α , is a G-protein-coupled receptor expressed in a broad range of cells such as keratinocytes, fibroblasts, endothelial cells as well as various cancer cells including melanoma and colon cancer cells ⁷. Tumor-derived IL-8 can induce tumor growth, survival, invasion, angiogenesis, metastases, resistance and recurrence. Multiple effects of IL-8/CXCR2 signaling in tumor cells and their microenvironments suggest that targeting of IL-8 and its receptors may effectively inhibit tumor development and progression, and sensitize tumors to chemotherapeutic regimen ⁸. We have recently reported that ectopic expression of IL-8 in the tumor microenvironment enhances colon cancer growth and metastasis, and the absence of CXCR2 in the tumor microenvironment inhibited colon cancer cell growth ^{9, 10}, further validating the therapeutic potential of IL-8/CXCR2 pathway in colon cancer treatment. In the current study, we verified clinical significance of IL-8/CXCR2 pathway as a target using mouse models. We show that a single copy deletion of CXCR2 gene was sufficient in reducing polyp development in the intestine of *Apc^{min}* mice, and delivers a synergistic suppression of polyp formation, when combined with a low-dose sulindac. Together, our study provides an *in vivo* therapeutic validation of using CXCR2 inhibitors in combination with sulindac for treatment and prevention of colorectal cancer.

MATERIALS AND METHODS

Animals

All animal experiments have been approved by the University of Southern California Institutional Animal Care and Use Committee (IACUC). Generation of the keratin-14-based human IL-8 transgenic mouse line (K14-hIL8) was previously described ¹⁰. CXCR2 knockout mice ¹¹ and *Apc^{min}* mice (C57BL/6) were purchased from the Jackson Laboratory (Bar Harbor, MN).

Gene expression study

Total RNA was isolated from mouse tissues as previously described ^{10, 12}. Expression of mouse Cox-2 and Gro- α were measured by quantitative real-time RT-PCR using Taqman

primer and probe sets purchased from Integrated DNA Technologies, Inc (Coralville, Iowa). Sequences for forward primer, reverse primer and probe of each gene are Cox-2 (CTC ACG AAG GAA CTC AGC AC/ GGA TTG GAA CAG CAA GGA TTT G/ 6-FAM-TC GGA AGA G/Zen/C ATC GCA GAG GTG-IABkFQ) and Gro- α (CAG ACG GTG CCA TCA GAG/ AAC CGA AGT CAT AGC CAC AC/ 6-FAM-TG CTA AAA G/Zen/G TGT CCC CAA GTA ACG G-IABkFQ).

Tumorigenesis study

Apc^{min} mice were mated with CXCR2^{+/-} mice to obtain *Apc^{min/+}/CXCR2^{+/-}* mice, which were further mated to generate *Apc^{min/+}/CXCR2^{-/-}* mice. Mice were sacrificed at 13 weeks of age and the entire gastrointestinal track was collected. Number and size of tumors were measured from the end of pyloric sphincter to the rectum under the microscope. The intestines were divided into the small and large intestines for further analyses. Tumors larger than 0.5 mm were identified and counted for their numbers, and the length of the large intestines was measured after excluding the cecum. Sulindac (Sigma-Aldrich, St. Louis, MO) was continuously administered to the mice through drinking water (10 mg/kg/day) for 9 weeks starting from postnatal week 4 to 13 (from day 28 to 91).

Statistical Analyses

Sizes and numbers of polyps in the small and large intestines by APC^{min} and treatment were analyzed using non-parametric methods such as Mann-Whitney U tests or Kruskal-Wallis tests whenever appropriate. The analyses were performed using the SAS statistical package version 9.3 (SAS Institute Inc., Cary, North Carolina, USA). Gene expression data were analyzed using Student t-test using Microsoft Excel 2010. All reported *p* values were unpaired two-sided at a significance level of 0.05 or less.

RESULT AND DISCUSSION

To investigate the role of CXCR2 in the APC^{min} mutation-induced polyposis, we bred *Apc^{min/+}* mouse (C57BL/6) with heterozygote CXCR2 mouse (C57BL/6) to produce compound mutant mice having APC^{min} mutation in CXCR2 wild-type (WT), heterozygote (Het) or homozygote (KO) knockout backgrounds. At 13 weeks of age, we detected a statistically significant reduction of total number of APC^{min} -induced tumors in the small intestines of both CXCR2 Het and KO groups (Fig.1A), indicating that deletion of CXCR2 in *Apc^{min/+}* mouse can significantly inhibit polyp development. Notably, the reduction in number was consistently found in all size tumors, and also comparably detected in both heterozygote and homozygote CXCR2 KO mice, suggesting that a single copy deletion of CXCR2 gene is sufficient enough to reduce tumor formation. In comparison, CXCR2 deletion did not inhibit development of large intestinal polyp in a statistically significant degree (Fig.1B). We attribute this to the overall inefficiency in APC^{min} -induced polyp formation in the large intestine, compared to the small intestine^{13, 14}. We next studied the molecular mechanism underlying the inhibition of APC^{min} -induced tumor formation by CXCR2 deletion. Because previous studies demonstrated both upregulation of COX-2 and Gro- α by APC^{min} mutation and their promoting roles in APC^{min} -induced tumor formation¹⁵, we determined the expression of these two inflammatory genes in APC^{min} -

induced tumors in the small intestines of CXCR2 WT, Het and KO mice. COX-2 and Gro- α were expectedly upregulated in the APC^{min}-induced tumor in the small intestine. Importantly, however, CXCR2 deletion prevented the upregulation of COX-2 and Gro- α in the APC^{min}-induced tumors in CXCR2 Het and KO mice (Fig.1C,D). Therefore, we conclude that downregulation of COX-2 and Gro- α may have significantly contributed to the reduced polyp formation observed in CXCR2 deletion mice. Taken together, our data demonstrate that loss of CXCR2 function results in a strong inhibition of APC^{min}-induced tumor development as well as upregulation of COX-2 and Gro- α .

We next studied the effect of gain of CXCR2 function by ectopically expressing IL-8 in APC^{min}-induced tumor development and regulation of COX-2 and Gro- α . We crossed Apc^{min/+} mice with a transgenic mouse model that expresses human IL-8 from the direction of Keratin-14 promoter (K14-hIL8)¹⁰. The K14 promoter is active mainly in keratinocytes of the skin and additional tissues including tongue and esophagus¹⁶. Human IL-8 has been shown to bind to and activate mouse CXCR2, although at a much lower efficiency compared to human CXCR2¹⁷. K14-hIL8 mice have been shown to produce human IL-8 protein (50~200 pg/mL) in the serum^{10, 12} (Supplemental Fig. 1) and to promote inflammatory responses in the skin, as determined based on dermal delayed-type hypersensitivity assays¹². Since the mouse genome does not contain any gene that is homologous to human IL-8 gene¹⁸, expression of human IL-8 confers an ectopic stimulation to APC^{min}-induced tumor as well as the tumor microenvironment. Indeed, the compound mice with Apc^{min/+} and K14-hIL8 alleles developed significantly more tumors in the small intestine, compared to the littermates harboring APC^{min} mutation alone (Fig.2A). Of note, the total number of polyp was reduced in this compound mice, most likely due to mixed strain backgrounds resulted from the out-breeding between Apc^{min/+} mouse (C57BL/6) and K14-hIL8 (mixed background). Reduction in polyp numbers in outbred strains has previously been reported^{4, 19}. Number of polyps in the large intestines did not show any statistical difference between the two groups (Fig.2B). Expression of COX-2 and Gro- α was not further increased in tumors of Apc^{min/+}/K14-hIL8 mice, compared to the littermates harboring APC^{min} mutation alone (data not shown). Taken together, ectopic expression of IL-8 promoted the APC^{min}-induced tumor development by activating the CXCR2 pathway.

Sulindac, a non-selective COX-1 and COX-2 inhibitor that has been known to prevent colon tumor cell growth and polyp formation, is a FDA-approved NSAID against acute treatment of inflammatory conditions and effective in regression of precancerous adenoma^{20, 21}. However, because of its severe toxicities in the cardiovascular, gastrointestinal and renal systems, combination of sulindac at a lower dose with other chemopreventive agents has been considered as an attractive alternative strategy that would increase its effectiveness, while minimizing the adverse effects^{22, 23}. Therefore, we asked whether CXCR2 inhibition with a concurrent treatment with sulindac provides a synergistic suppression of APC^{min}-induced tumor development. To address this possibility, Apc^{min/+}/CXCR2 WT and Apc^{min/+}/CXCR2 Het littermates were treated with a low dose sulindac (10 mg/kg/day) through a buffered drinking water for 9-weeks from day 28 to 91 of their ages. Of note, many previous studies have used sulindac at doses higher than 20 mg/kg/day^{2, 13, 14, 24}. While sulindac treatment and a single copy deletion of CXCR2 alone reduced total number of polyps in the small intestine by 31% and 19%, respectively, the combined conditions

reduced the intestinal polyp formation by 57% (Fig.3A), demonstrating an enhanced efficacy in reducing tumor formation in the small intestine. No statistical change was detected in the large intestinal polyp formation (data not shown). In addition, upregulation of COX-2 and Gro- α by APC^{min} allele was comparably suppressed by CXCR2 deletion and/or sulindac treatment, either alone or together (Fig.3B,C). It was unexpected to see some rebound in the Gro- α expression when sulindac was treated on Apc^{min/+}/CXCR2 Het background (Fig.3B,C), which may be a result of an *in vivo* compensation mechanism. Importantly, the interaction plot for total polyp number generated based on our statistical analyses indicates that the effect of CXCR2 deletion is independent from with the effect of sulindac treatment in reducing APC^{min} - induced tumor formation (Fig.3D). These data suggest that, although CXCR2 deletion has contributed to downregulation of COX-2 and Gro- α , it may have more profoundly affected additional pathways other than the COX-2 pathway, in suppressing the intestinal polyp formation. An alternative, but not mutually exclusive, explanation of these data is that sulindac treatment and CXCR2 deletion operate in different cellular compartments, namely tumor vs. microenvironment. In fact, previous studies have demonstrated that CXCR2 is predominantly expressed in stromal tissues, whereas COX-2 is expressed in tumors much stronger than in the stromal cells²⁵. Supporting this notion, one of the most prominent phenotypes of CXCR2 KO mice is impaired neutrophil recruitment^{26, 27} and a recent study has demonstrated the importance of CXCR2 and neutrophil recruitment in colon cancer development²⁸. We also determined macrophage recruitment and found that CXCR2 Het and KO showed a decreased number of macrophage in the tumor stromal area (Supplemental Figs. 2–4).

In summary, we demonstrated that a single copy deletion of CXCR2 was sufficient to reduce APC^{min} - induced polyp formation and that activation of CXCR2 by ectopic expression of IL-8 promoted APC^{min} - induced tumor formation. These loss-of-function and gain-of-function studies consistently indicate the critical role of CXCR2 in APC^{min} - induced polyp formation. In addition, inhibition of CXCR2 and sulindac treatment provided an enhanced suppression to the intestinal tumor formation by targeting multiple pathways that are important for tumor formation. As several previous studies have shown that the IL-8 and CXCR2 are two of the most highly upregulated genes in colorectal cancer, we believe that targeting IL-8/CXCR2 pathway will play a key role in not only FAP, but also other sporadic colon cancers²⁹. It will be important to use chemical and biological inhibitors for IL-8 and CXCR2 and verify the results from the CXCR2 genetic model. Moreover, it will be necessary to evaluate the effect of IL-8/CXCR2 inhibition using other colon cancer mouse models including the Interleukin-10 knock out (KO)³⁰ and K-ras mutant (K-rasG12D)³¹ mouse models. As our recent study showed a significant anti-tumor effect of IL-8/CXCR2 against colon cancers, IL8/CXCR2 inhibition using chemical inhibitors such as a selective antagonist for CXCR1/2, SCH-527123^{32, 33} will be an excellent strategy for preventing colon cancers and also could be utilized as an adjuvant for the current chemotherapy against colon cancer. A limitation of the current study is that only tumors in the small intestine, but not those in the large intestine, showed the effects by the CXCR2 KO, overexpressed IL-8 and sulindac treatment, which may as well affect tumor development in the large intestine in human. This is mainly due to the fact that mutations in the APC gene develop tumors predominantly in the small, but not large, intestine in mice, while patients with FAP develop

adenomas in the large intestine^{13, 14}. Taken all together, the outcome of our animal studies confers a therapeutic justification for CXCR2 inhibition in prevention of colon cancers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Novelty & Impact Statements

This study defines the contributions of IL-8 and CXCR2 to intestinal adenoma development induced by an APC gene mutation using mouse models. Intestinal adenoma formation was suppressed by a single copy deletion of CXCR2 gene, but promoted by ectopic expression of IL-8. Moreover, inhibition of CXCR2 enhanced the efficacy of the FDA-approved cancer drug, sulindac. Together, this study provides a therapeutic justification of inhibition of IL-8/CXCR2 pathway combined with sulindac treatment for colon cancer prevention.

Figure 1. CXCR2 deletion reduces *APC*^{min}-induced tumor formation and expression of COX-2 and Gro-α
(A,B) Number of polyps in the small (A) and large (B) intestine of mice harboring *Apc*^{min} mutation in CXCR2 WT, Het and KO backgrounds. Number of mice, average number of polyps per mice, range of polyp size, and the median and average size of polyps are shown for the small and large intestines. All sizes are shown in millimeter. Expression of COX-2 (C) and Gro-α (D) was determined in each tumor group. *, *p* < 0.05; **, *p* < 0.01.

Figure 2. Ectopic expression of IL-8 promotes APC^{min}-induced tumor formation

(A) Transgenic expression of human IL-8 increased the number of polyps in the small intestine of *Apc^{min/+}* mouse. (B) No statistically significant change in the number of polyp was detected in the large intestine of the same mice. Number of mice, average number of polyps per mice, range of polyp size, and the median and average sizes of polyps are also shown. All sizes are shown in millimeter. **, $p < 0.01$.

Figure 3. Heterozygote deletion of CXCR2 synergizes sulindac-mediated suppression of APC^{min}-induced tumor formation in the small intestine

(A) Number of polyps in the small intestine of mice harboring *Apc^{min}* mutation in the CXCR2 WT and Het backgrounds that were treated or not by sulindac (10 mg/kg/day) for 9 weeks. Number of mice, average number of polyps per mice, range of polyp size, and the median and average sizes of polyps are also shown. All sizes are shown in millimeter.

Expression of COX-2 (B) and Gro-α (C) was determined in each tumor group. (D)

Interaction plat for total number of polyps in the small intestine.