IFNγ Induces DNA Methylation–Silenced GPR109A Expression via pSTAT1/p300 and H3K18 Acetylation in Colon Cancer

Kankana Bardhan, Amy V. Paschall, Dafeng Yang, May R. Chen, Priscilla S. Simon, Yangzom D. Bhattia, Pamela M. Martin, Muthusamy Thangaraju, Darren D. Browning, Vadivel Ganapathy, Christopher M. Heaton, Keni Gu, Jeffrey R. Lee, and Kebin Liu

Abstract

Short-chain fatty acids, metabolites produced by colonic microbiota from fermentation of dietary fiber, act as anti-inflammatory agents in the intestinal tract to suppress proinflammatory diseases. GPR109A is the receptor for short-chain fatty acids. The functions of GPR109A have been the subject of extensive studies; however, the molecular mechanisms underlying GPR109A expression is largely unknown. We show that GPR109A is highly expressed in normal human colon tissues, but is silenced in human colon carcinoma cells. The GPR109A promoter DNA is methylated in human colon carcinoma. Strikingly, we observed that IFNγ, a cytokine secreted by activated T cells, activates GPR109A transcription without altering its promoter DNA methylation. Colon carcinoma grows significantly faster in IFNγ-deficient mice than in wild-type mice in an orthotopic colon cancer mouse model. A positive correlation was observed between GPR109A protein level and tumor-infiltrating T cells in human colon carcinoma specimens, and IFNγ expression level is higher in human colon carcinoma tissues than in normal colon tissues. We further demonstrated that IFNγ rapidly activates pSTAT1 that binds to the promoter of p300 to activate its transcription. p300 then binds to the GPR109A promoter to induce H3K18 hyperacetylation, resulting in chromatin remodeling in the methylated GPR109A promoter. The IFNγ-activated pSTAT1 then directly binds to the methylated but hyperacetylated GPR109A promoter to activate its transcription. Overall, our data indicate that GPR109A acts as a tumor suppressor in colon cancer, and the host immune system might use IFNγ to counteract DNA methylation–mediated GPR109A silencing as a mechanism to suppress tumor development. Cancer Immunol Res; 3(7); 795–805. © 2015 AACR.

Introduction

Short-chain fatty acids, metabolites produced by colonic bacteria from fermentation of dietary fiber, are the essential energy source for colonocytes. Without these short-chain fatty acids for energy, colonocytes undergo autophagy and die. In addition, short-chain fatty acids, such as butyrate, also play a histone deacetylase (HDAC) inhibitor that mediates both normal and anti-inflammatory response in the intestinal tract (2). GPR109A is a G-protein-coupled receptor for short-chain fatty acids (2–6). It has been shown that inhibition of DNA methylation increases GPR109A expression in human colon carcinoma cells, suggesting that GPR109A is silenced by DNA methylation (2). Although the function of GPR109A has been extensively studied in normal cells and cancer cells, the molecular mechanisms underlying GPR109A expression are unknown.

IFNγ is a proinflammatory cytokine secreted primarily by activated T cells (7). IFNγ functions through signal transducer and activator of transcription 1 (STAT1) to regulate the expression of its target genes. It has been reported that IFNγ secretion is elevated in the peripheral blood (8), and IFNγ expression level is increased in the inflamed colonic mucosa tissues in patients with ulcerative colitis (9). The expression and activation level of STAT1 is also significantly increased in colonic tissues of ulcerative colitis patients (10). Furthermore, chronic IFNγ signaling increases Cox2 and inducible nitric oxide synthase expression to promote inflammation-dependent spontaneous colon cancer development (11). These observations thus suggest that chronic IFNγ signaling plays a key role in human ulcerative colitis pathogenesis and in inflammation-dependent spontaneous colon cancer development (8, 9, 11–13). However, it is apparent that the IFNγ signaling pathway is a two-edged sword. Although chronic IFNγ signaling promotes inflammation-dependent colon cancer development, the best known function of IFNγ in the tumor microenvironment is tumor suppression (7, 14–16).

We report here a novel mechanism underlying the regulation of GPR109A expression in colon cancer cells. Our data reveal that although the GPR109A promoter is methylated in human colon carcinoma cells, exposure of tumor cells to IFNγ reverses...
DNA methylation–mediated GPR109A silencing both in vitro and in vivo without altering the methylation status of the GPR109A promoter.

**Materials and Methods**

**Human cell lines and tissue specimens**

Human colon cancer cell lines SW480, SW620, SW116, and T84, and mouse colon carcinoma cell line CT26 were obtained from the American Type Culture Collection (ATCC). The ATCC has characterized these cells by morphology, immunology, DNA fingerprint, and cytogenetics. Deidentified human colon carcinoma specimens were obtained from the Georgia Regents University Medical Center and University Hospital with approval by the Georgia Regents University and University Hospital Human Assurance Committee.

**Mouse tumor model**

IFNγ knockout (KO) mice (129S7(B6)-Ifngtm1Ts/J) and age-matched wild-type (WT) control mice (BALB/cj) were obtained from The Jackson Laboratory. Mice were anesthetized under constant flow of oxygen and isofluorane. A small abdominal incision was made to expose the cecum. Tumor cells (1 × 10⁸ cells in 20 μL saline) were injected into the cecal wall on the serosal side. The wound was sealed with a wound clip. The use of mice and surgery procedures was approved by Georgia Regents University Institutional Animal Care and Use Committee.

**RT-PCR analysis**

Total RNA was isolated from cells using Trizol (Invitrogen) according to the manufacturer’s instructions, and used for the first strand cDNA synthesis using the Moloney murine leukemia virus (MMLV) reverse transcriptase (Promega). The cDNA was then used as the template for PCR amplification. RT-PCR was conducted as previously described (17). The sequences of primers are listed in Supplementary Table S1.

**Analysis of gene expression with RT-PCR array**

Total RNA was isolated from freshly dissected human colon carcinoma specimens and matched adjacent normal colon tissues. RNAs from 3 patients were pooled and used for cDNA probe preparation using the RT2 First Strand Kit (Cat# 330401; Qiagen). The Human Inflammammasomes PCR Arrays (Qiagen; Cat# PAHS-0972) were used to analyze the inflammation-related gene expression using real-time RT-PCR according to the manufacturer’s instructions.

**Western blot analysis**

Western blotting analysis was performed as previously described (16). The blot was probed with antibodies specific for pSTAT1 (Cat# 612133; BD Biosciences), H3K9ac (Cat# 9649; Cell Signaling), H3K18Ac (Cat# 9675; Cell Signaling), H3K27ac (Cat# 4753; Cell Signaling), H3 (Cat# 4499; Cell Signaling), p300 (Cat# sc-584; Santa Cruz Biotechnology), GPR109A (Cat# sc-134583; Santa Cruz Biotechnology), and β-actin (Cat# A1978; Sigma-Aldrich).

**Cell treatment**

For demethylation of DNA, cells were treated for 3 days with 5′-aza-deoxycytidine (Sigma) at a final concentration of 1 μg/mL. For IFNγ treatment, cells were cultured in the presence of recombinant IFNγ (R & D Systems) at a final concentration of 100 U/mL.

**DNA methylation analysis**

Genomic DNA was purified using the DNaseasy Tissue Kit (Qiagen) according to the manufacturer’s instructions. Sodium bisulfite treatment of genomic DNA was carried out using the DNA Modification Kit (Zymo Research) according to the manufacturer’s instructions. Methylation-sensitive PCR (MS-PCR) and DNA sequencing were carried out as previously described (18). For DNA sequencing, the bisulfite-modified genomic DNA was used as the template for PCR amplification of the human GPR109A promoter region. The amplified DNA fragments were cloned into pCR2.1 vector (Invitrogen), and individual clones were sequenced. DNA methylation was analyzed using QUMA program as previously described (19). All primer sequences are listed in Supplementary Table S1.

**Immunohistochemistry**

Immunohistochemical staining was performed at the Georgia Pathology Service. CD4- and CD8-specific antibodies were obtained from Dako (Cat# IS649 and IS623; DAKO). GPR109A-specific antibody has been previously described (20). The density of peritumoral lymphocytes was documented for the percentage of tumor staining was documented.

**Chromatin immunoprecipitation assay**

Chromatin immunoprecipitation (ChIP) assays were carried out using anti-pSTAT1 antibody (Cat# sc-8394; Santa Cruz Biotechnology), anti-HDAC1 (Cat# sc-7872; Santa Cruz Biotechnology), and protein A-agarose beads (Millipore) as previously described (16). The GPR109A promoter DNA was detected by PCR using gene-specific primers. All PCR primer sequences are listed in Supplementary Table S1.

**Protein–DNA interaction assay**

DNA–protein interaction was determined by electrophoresis mobility shift assay (EMSA) as previously described (19). The probe sequences are listed in Supplementary Table S1.

**Gene silencing**

T84 cells were transiently transfected with scrambled siRNA (Dharmacon), p300-specific siRNA (Cat# sc-29431; Santa Cruz Biotechnology), and STAT1-specific siRNA (Qiagen; cat# SIO2662324; SI02662884), respectively, using lipofectamine 2000 (Invitrogen) overnight.

**Chromatin remodeling assay**

Cells (5 × 10⁶) were harvested and washed in cold PBS, and then resuspended in nuclear extraction buffer (25 mmol/L Tris-HCL, pH 8.0, 50 mmol/L KCl, 5 mmol/L MgCl₂, 8% glycerol, 0.5% NP-40, and protease/phosphatase inhibitors) and incubated on ice for 10 minutes. Then the cells were homogenized in a glass homogenizer, and spun down at 1,000 g for 5 minutes. Cell pellets were washed once with DNase buffer, spun down at 1,000 g for 5 minutes, and then washed once with nucleus storage buffer (NSB; 50% glycerol,
Human GPR109A methylated in all 5 colon cancer specimens examined (Fig. 1B). With a p value of less than 0.05, the difference is statistically significant.

Results

GPR109A is silenced by DNA methylation in human colon carcinoma

Analysis of GPR109A mRNA levels from matched normal colon tissues and colon carcinoma tissues indicates that GPR109A is highly expressed in normal human colon epithelial cells but is silenced in human colon carcinoma cells. GPR109A expression is approximately 1.4-fold to 150-fold higher in the normal colon tissues as compared with that in the matched colon carcinoma tissues among the six matched pairs of tissues analyzed (Fig. 1A).

To determine whether the GPR109A promoter is methylated in vivo in colon carcinoma cells, genomic DNA was isolated from human colon carcinoma tissues of 5 colon cancer patients. The MethPrimer program was used to design MS-PCR primers, and MS-PCR analysis of the bisulfite-modified genomic DNA indicated that the GPR109A promoter is methylated in all 5 colon cancer specimens examined (Fig. 1B). To determine whether GPR109A expression can be reactivated by inhibition of DNA methylation, tumor cells were treated with 5'-aza-deoxycytidine (Aza-dC) and analyzed for GPR109A expression. Consistent with the heavy DNA methylation level, Aza-dC treatment dramatically increased GPR109A expression in human colon carcinoma cells in a dose-dependent manner (Fig. 1C).

Inhibition of DNA methylation upregulates GPR109A expression in human colon carcinoma cells (2). However, DNA sequence analysis indicates that there are no classical CpG islands in the human GPR109A gene promoter region (Fig. 1D). Taken together, these data demonstrate that GPR109A is silenced in human colon carcinoma cells by methylation of its promoter DNA.

STAT1 directly binds to the GPR109A promoter region to activate transcription from the methylated promoter

While analyzing the inflammation-related gene expression in colon carcinoma cells, we observed, surprisingly, that GPR109A was upregulated by IFNγ, a proinflammatory cytokine secreted by activated T cells. RT-PCR analysis of four human colon carcinoma cell lines revealed that GPR109A was dramatically upregulated by IFNγ despite its promoter DNA being methylated (Fig. 2A). Analysis of GPR109A expression kinetics revealed that GPR109A mRNA starts to increase 3 to 6 hours after IFNγ treatment and continued to increase even 24 hours after the treatment (Fig. 2A). Furthermore, IFNγ at a concentration as low as 10 μg/mL can reregulate GPR109A expression (Fig. 2A).

Flow cytometry analysis revealed that IFNγR is expressed on the cell surface of all 4 cancer cell lines examined (Fig. 2B). Western blotting analysis indicated that pSTAT1, the key mediator of the IFNγ signaling pathway, is rapidly activated by IFNγ through phosphorylation in human colon carcinoma cells. pSTAT1 was detected as early as 10 minutes after IFNγ treatment (Fig. 2C).

Analysis of the human GPR109A gene promoter revealed the presence of two gamma activation sequence (GAS) elements in the region (Fig. 2D). ChIP analysis with pSTAT1-specific mAb showed that IFNγ-activated pSTAT1 is associated with both GAS sites in the GPR109A promoter chromatin (Fig. 2D). Furthermore, EMSA indicated that pSTAT1 binds to the GAS DNA element directly (Fig. 2E). IFNγ functions through both STAT1-dependent and STAT1-independent signaling pathways (21). To determine whether IFNγ-induced GPR109A expression depends on STAT1, STAT1 was silenced using specific siRNAs in human colon carcinoma cells (Supplementary Fig. S1). Analysis of the human GPR109A expression revealed that silencing STAT1 abolished IFNγ-induced GPR109A expression (Supplementary Fig. S1). These observations thus indicate that IFNγ upregulates GPR109A expression through direct binding of pSTAT1 to the methylated GPR109A promoter DNA to activate its transcription.

To determine whether other cytokines induce GPR109A expression, T84 cells were treated with IFNα, IFNβ, TNFα, IL23, and GM-CSF for 24 hours, and analyzed for GPR109A expression. None of these cytokines upregulated GPR109A in human colon carcinoma cells (Supplementary Fig. S2).

IFNγ regulates GPR109A expression in colon carcinoma cells in vivo

IFNγ is primarily produced by activated T cells in vivo. To determine the expression levels of GPR109A and T-cell infiltration in human colon carcinoma, we analyzed 18 human colon carcinoma specimens using immunohistochemical staining with GPR109A, CD4+, and CD8-specific antibodies. The stained tissues were then analyzed by two pathologists (C.M. Heaton and J.R. Lee). The images are presented in Fig. 3 and Supplementary Figs. S3 to S5. The staining scores are presented in Supplementary Table S2. Four colon carcinoma specimens (patients 7, 9, 10, and 13) showed no detectable GPR109A protein level. Tumor tissues from 2 of these 4 patients (patients 7 and 9) also had minimal to no CD4+ and CD8+ T-cell infiltrations. However, tumor specimen from patient 10 had...
microenvironment (Supplementary Table S2).

To determine the inflammation gene expression profiles, we analyzed the expression levels of inflammation-related genes in human colon carcinomas and matched adjacent normal colon tissues using PCR arrays. Real-time RT-PCR analysis identified 41 inflammation-related genes that are differentially expressed in normal colon tissues and in colon carcinoma tissues. Among these 41 genes, the expression level of IFNγ is about 4 times higher in carcinoma tissues than in the adjacent normal colon tissues (Supplementary Table S3).

To determine functionally whether IFNγ activates GPR109A transcription from the methylated promoter under pathophysiological conditions, we established mouse colon carcinoma CT26 cell orthotopic transplant models in WT and IFNγ KO mice. The gpr109a promoter is methylated in CT26 cells (Fig. 4A); however, IFNγ treatment dramatically increased

Figure 1.
The human GPR109A promoter is methylated and GPR109A expression is silenced in human colon carcinoma cells. A, GPR109A expression level in matched pairs of human normal colon and colon carcinoma tissues. Colon carcinoma tissues and adjacent normal tissues were collected from 6 patients, and analyzed for GPR109A expression by RT-PCR. GAPDH was used as normalization control. Bottom: the GPR109A levels were quantified using the NIH J program. The ratio of GPR109A versus GAPDH in patient #1 was arbitrarily set at 1. The GPR109A expression levels of the remaining five specimens were then normalized based on patient #1. B, methylation status of the GPR109A gene promoter in human colon carcinoma specimens. Genomic DNA was isolated from colon carcinoma specimens of 5 colon cancer patients and modified with bisulfate. The modified DNA was then analyzed by MS-PCR (U, unmethylated; M, methylated). Numbers above the figure are patient codes. C, inhibition of DNA methylation increases GPR109A expression. SW116 and T84 cells were treated with Aza-dC for 3 days at the indicated doses and analyzed for GPR109A expression level by semiquantitative RT-PCR (top) and real-time RT-PCR (bottom). The GPR109A expression levels of untreated cells were arbitrarily set at 1. Column, mean; bar, SD. D, methylation level of the human GPR109A gene promoter in human colon carcinoma cell lines. The human GPR109A gene DNA sequence was exported from the human genome database and analyzed for CpG islands using MethyPrimer computer program. Top, the human GPR109A gene promoter structure. Vertical bars under the line indicate location of CpG dinucleotides, and the number under the line indicates nucleotide number relative to GPR109A transcription initiation site (+1). Bottom, methylation level of the GPR109A gene promoter in the indicated cell lines. Genomic DNA was modified with bisulfite. The indicated DNA fragment was amplified by PCR and cloned into pCR2.1 vector. Individual clones for each cell line were sequenced, and the methylation level of the cytosine in the CpGs was analyzed using QUMA computer program (open circle, unmethylated CpG; closed circle, methylated CpG).
IFNγ activates GPR109A transcription from the methylated GPR109A promoter. A, tumor cells were treated with IFNγ and analyzed for GPR109A expression level by RT-PCR (top). The GPR109A expression in T84 cells was also analyzed by real-time PCR (middle). Column, mean; bar, SD. Bottom, SW116 cells were treated with IFNγ at 10 and 100 U/ml for 24 hours and analyzed by Western blotting analysis. B, tumor cells were stained with IFNγR mAb and analyzed for cell surface IFNγR protein level. Gray area, IgG isotype control; solid line, IFNγR-specific staining. C, T84 cells were treated with IFNγ for the indicated time and analyzed for pSTAT1 protein level by Western blotting. D, the human GPR109A promoter structure. The GAS element locations and consensus sequences are shown under the bar. The ChIP PCR amplified regions are indicated above the bar. Bottom, SW116 and T84 cells were either untreated (−IFNγ) or treated with IFNγ (+IFNγ) for 6 hours and analyzed by ChIP using pSTAT1-specific mAb. Purified genomic DNA (gDNA) was used as a positive control for the PCR (left). The ChIP DNA was then analyzed by real-time PCR (right). E, T84 cells were either untreated or treated with IFNγ (+IFNγ) for 6 hours and used for nuclear extract preparation. The nuclear extracts were incubated with the GAS element–containing DNA probe in the absence or presence of IgG or pSTAT1 mAb and then analyzed for protein-DNA interaction by EMSA. A mutant GAS DNA probe (MT probe) was used as negative control.

*IFNγ Regulates GPR109A Transcription*

gpr109a mRNA levels in CT26 cells in vitro (Fig. 4A). A low-dose of CT26 (1 × 10⁴ cells/mouse) and a short tumor growth time (21 days) were used in this study to unmask the difference between WT and IFNγ KO mice. When surgically implanted into mouse cecum, CT26 tumors grew much faster in IFNγ KO mice than in WT mice (Fig. 4B). Analysis of the dissected tumor tissues revealed that mRNA levels of gpr109a are significantly higher in tumors grown in WT mice than that in IFNγ KO mice (Fig. 4C). Our data thus demonstrate that IFNγ activates gpr109a transcription from their methylated promoters in vivo.

IFNγ does not alter GPR109A promoter DNA methylation

IFNγ has been shown to induce DNA demethylation (22). To determine whether IFNγ upregulates GPR109A expression through inhibition of DNA methylation, human colon carcinoma cells were cultured in the absence or presence of IFNγ for 24 hours, and the DNA methylation level in the pSTAT1-binding consensus regions (Fig. 2D) of the GPR109A promoter was analyzed. DNA sequencing analysis indicated that the GPR109A promoter DNA is still methylated after IFNγ treatment in human colon carcinoma cells (Fig. 5).

Inhibition of HDACs enhances IFNγ-mediated GPR109A upregulation

Promoter DNA methylation of CpG islands often causes chromatin condensation to block transcription factor binding to the DNA, thereby silencing gene expression (23). However, histone acetylation may mediate the switch between repressive and permissive chromatin and thus dictate the functional state of genes (24–26). Our above observations that IFNγ upregulates GPR109A expression from the methylated GPR109A promoter without altering the promoter DNA methylation level suggest that histone acetylation might play a role in IFNγ-mediated GPR109A expression. Therefore, we hypothesized that inhibition of HDAC activity may enhance GPR109A transcription activation by IFNγ. To test this hypothesis, human
colon carcinoma cells were treated with either TSA or IFNγ, or both TSA and IFNγ together, and analyzed for GPR109A expression. RT-PCR analysis revealed that either Trichostatin A (TSA) or IFNγ treatment dramatically increased GPR109A expression (Fig. 6A). However, combined TSA and IFNγ treatment resulted in an even greater upregulation of GPR109A than either agent alone (Fig. 6A).

It is known that HDAC1 mediates histone acetylation level in many gene promoters (27). Our observation that inhibition of HDAC activity dramatically increased IFNγ-induced GPR109A expression in human colon carcinoma cells led us to reason that IFNγ might regulate GPR109A expression by repressing HDAC1 expression. To test this hypothesis, we analyzed HDAC1 association with the GPR109A promoter. ChIP analysis showed that HDAC1 is associated with the chromatin at one of the two regions analyzed at the GPR109A promoter region (Fig. 6A). Consistent with the observation that HDAC1 expression is not regulated by IFNγ, IFNγ did not alter HDAC1 association with the GPR109A promoter chromatin (Fig. 6A).

p300 is the immediate early target of the IFNγ signaling pathway

Histone acetyltransferases (HAT) and HDACs antagonize each other through modification of the lysine side chains (27, 28). p300 is a HAT that often acts as a transcription cofactor (29–33). For example, p300 can mediate H3K18/27 acetylation (34–37). Therefore, we sought to determine whether IFNγ mediates p300 expression to regulate GPR109A expression. RT-PCR analysis revealed that IFNγ treatment indeed rapidly upregulates p300 expression level in human colon carcinoma cells. The increase in p300 mRNA and protein levels occurred just 10 minutes after IFNγ treatment and reached a plateau at 6 hours (Fig. 6B). Analysis of the human p300 gene promoter region identified two GAS sites (Fig. 6C), and ChIP analysis indicated that IFNγ-activated pSTAT1 is associated with one of the two GAS sites at the p300 promoter region in human colon carcinoma cells (Fig. 6C). Consistent with the ChIP results, EMSA indicated that pSTAT1 directly and specifically binds to the GAS DNA of the p300 promoter (Fig. 6D).

Taken together, our data indicated that p300 is an immediate early target of the IFNγ signaling pathway in human colon cancer cells.

IFNγ activates GPR109A transcription through p300 association with its promoter

To determine whether p300 directly mediates IFNγ regulation of GPR109A transcription, we first analyzed p300 association with the GPR109A promoter chromatin. ChIP analysis revealed that IFNγ treatment dramatically increased p300 association with the GPR109A promoter chromatin (Fig. 6E). Next, we sought to determine whether p300 mediates GPR109 expression. p300 was silenced with p300-specific siRNA in human colon carcinoma cells, and GPR109A expression levels were then analyzed. Real-time RT-PCR analysis revealed that silencing p300 diminished IFNγ-induced GPR109A (Fig. 6F). Thus, we conclude that p300 directly mediates pSTAT1-activated GPR109A transcription in human colon carcinoma cells.

IFNγ increases p300 expression to increase H3K18 hyperacetylation

Although p300 acetylates multiple lysine residues in both H3 and H4 (37), p300 has been shown to specifically mediate H3K18/27 acetylation (34, 36). We therefore analyzed the effects of IFNγ on H3K18/27 acetylation. H3K9ac was also included as a positive control. IFNγ treatment increased the global acetylation levels of H3K9, H3K18, and H3K27 in human colon cancer cells (Fig. 7A). However, repeated ChIP analysis revealed that H3K27 is not acetylated in the GPR109A promoter regions and that IFNγ treatment does not induce acetylation of H3K27 in the GPR109A promoter region in human colon cancer cells (Fig. 7B). H3K9 is constitutively acetylated in the GPR109A promoter region in human colon cancer cells, and IFNγ increases H3K9 acetylation and induces H3K18 acetylation in the GPR109A promoter region in human colon cancer cells. To determine whether IFNγ induces H3K18 hyperacetylation specifically through p300, p300 was silenced with p300-specific siRNA. Silencing p300 diminished
the IFNγ-induced acetylation in both H3K9 and H3K18 in the GPR109A promoter region in human colon cancer cells (Fig. 7C). Therefore, our data indicated that H3K9 is constitutively acetylated, whereas H3K18 is acetylated in response to IFNγ stimulation. Thus, our results suggest that GPR109A activation in response to IFNγ signaling induces p300-mediated H3K18 hyperacetylation in the GPR109A promoter region in human colon cancer cells.

IFNγ induces GPR109A promoter chromatin remodeling

Our above findings raise the possibility that IFNγ induces chromatin remodeling at the GPR109A promoter region to convert a methylated DNA-mediated repressive chromatin configuration to a hyperacetylated and transcriptionally permissive one to facilitate pSTAT1 binding to activate GPR109A transcription. To test this hypothesis, we used limited DNaseI digestion to detect chromatin remodeling at the GPR109A promoter region. Human colon carcinoma cells were treated with IFNγ. Nuclei were then isolated from the cells and incubated with DNase I for various time periods. Genomic DNA was purified from the nuclei and used as the template for PCR amplification using the GPR109A promoter DNA-specific primers. Comparison of the PCR-amplified DNA fragment levels showed that the GPR109A promoter DNA methylation by MS-PCR. U, unmethylated; M, methylated. CT26 cells were also treated with IFNγ (100 U/mL) and analyzed for GPR109A expression level by real-time RT-PCR (right). Column, mean; bar, SD. B, CT26 cells (1 x 10^6/mouse) were surgically implanted into the cecal wall of WT (n = 9) and IFNγ KO (n = 7) mice. Tumor growth on the colon tissues was analyzed 21 days after tumor transplant. Left, representative image of WT and IFNγ KO mouse colon tissues showing colon tumor development (red arrows). Tumor volumes were quantified and presented in the right plot. C, colon carcinoma tissues as shown in B were dissected from the colon tissues of three WT and three IFNγ KO mice, respectively, and analyzed by real-time RT-PCR for GPR109A expression levels. Each column represents relative GPR109A expression level in one mouse.

Discussion

In colorectal cancer, genome-wide analysis showed that many of the methylated genes have known or predicted function in...
the suppression of tumorigenesis (38). Therefore, colon cancer cells use DNA methylation as a mechanism to silence tumor suppressor genes to advance the disease (39). Although there are no classical Cpg islands in the human GPR109A gene promoter region, we identified several Cpg dinucleotides in the region and observed that cytosines in these dinucleotides are methylated in human colon carcinoma cells. Analysis of six matched pairs of normal human colon tissues and colon carcinoma tissues revealed that GPR109A expression is indeed dramatically lower in the tumor tissues as compared with the adjacent normal colon tissues, which is consistent with the GPR109A promoter DNA methylation status. These observations thus indicate that GPR109A is silenced by its promoter DNA methylation in human colon carcinoma cells in vitro and in colon carcinoma tissues in vivo.

GPR109A is the receptor in colonocytes for the gut commensal bacterial metabolite butyrate (2) and mediates the butyrate-dependent anti-inflammatory effects to suppress colonic inflammation and inflammation-dependent colon cancer (2, 13). Therefore, it is not surprising that the GPR109A gene is silenced in human colon carcinoma cells. However, although the GPR109A promoter is methylated, GPR109A is expressed to some degree in
It has been shown that DNA methylation may render tumor cells resistant to IFN-induced apoptosis and that IFN-stimulated gene expression determines tumor cell sensitivity to DNA demethylation-induced apoptosis (43, 44). Strikingly, in the present study we observed that IFNγ can override the silencing effects of DNA methylation to activate GPR109A transcription without obvious demethylation of the GPR109A promoter DNA in human colon carcinoma cells. Current approach to reactivate DNA methylation–silenced tumor suppressor genes primarily relies on chemical inhibitors, mainly 5′-aza-dC that inhibits DNA methyltransferase (DNMT) activity (43, 45). This type of DNA methylation inhibitor is highly cytotoxic, and its use as chemotherapeutic agents in cancer therapy is associated with extensive toxicity (45). Our data suggest that immunotherapeutic approach, such as adoptive or active CTL immunotherapy (46), might be an alternative and yet effective and less toxic approach to activate DNA methylation–silenced expression of genes in cancer tissues. In addition, 5′-aza-dC is a general DNA methylation inhibitor that causes global DNA demethylation. In contrast, IFNγ-activated pSTAT1 only binds to specific DNA sequences (GAS element) to activate specific gene transcription. Therefore, IFNγ-activated expression of genes from DNA methylation–silenced promoters is gene specific; this approach is thus likely to be associated with low toxicity.

p300 is a histone acetyltransferase that also functions as a master transcriptional mediator in mammalian cells (27, 34, 47–49). In this study, we demonstrate that p300 is an immediate early target of the IFNγ signaling pathway. p300 transcription activation was detected 10 minutes after IFNγ treatment. STAT1 was also activated within 10 minutes after IFNγ treatment. Although it has been shown that pSTAT1 and p300 directly interact with each other (50), we observed that pSTAT1 physically binds to the p300 promoter region. Therefore, the likely signaling transduction cascade is that pSTAT1 is activated first, and it then binds to the p300 promoter to activate p300 transcription. Elevation of p300 enhances its association with the GPR109A promoters to induce H3K18 hyperacetylation, and chromatin remodeling, resulting in a permissive chromatin conformation at the GPR109A promoter to facilitate pSTAT1 binding to activate GPR109A transcription.

Based on the activation and expression kinetics of pSTAT1, p300, and GPR109A, we propose a model to illustrate IFNγ-induced GPR109A transcription activation from their methylated promoters. We propose that the GPR109A promoter is methylated and thus transcriptionally inactive in human colon carcinoma cells. Exposure of human colon carcinoma cells to IFNγ induces rapid STAT1 activation. Activated STAT1 directly binds to the p300 promoter region to rapidly activate p300 transcription. P300 then binds to the GPR109A promoters to induce H3K18 hyperacetylation and resultant chromatin remodeling to create a transcriptionally permissive chromatin at the GPR109A promoter regions without an obvious change in the methylation status of the promoter DNA. A transcriptionally permissive chromatin structure allows pSTAT1 binding to activate GPR109A transcription despite DNA methylation (Supplementary Fig. S7).
Authors' Contributions

Conception and design: K. Bardhan, M. Thangaraju, V. Ganapathy, K. Liu
Development of methodology: K. Bardhan, A.V. Paschall, M. Thangaraju
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K. Bardhan, A.V. Paschall, M.R. Chen, P.S. Simon, Y.D. Bhutta, K. Gu

Analysis and interpretation of data (e.g., statistical analysis, bio-statistics, computational analysis): K. Bardhan, A.V. Paschall, C.M. Heaton, K. Liu
Writing, review, and/or revision of the manuscript: K. Bardhan, D.D. Browning, V. Ganapathy, K. Liu
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): D. Yang, M.R. Chen, P.M. Martin, J.R. Lee, K. Liu
Study supervision: D.D. Browning, K. Liu

Acknowledgments

The authors thank Kimberly Smith and Kimnya Jones for their excellent technical assistance in immunohistochemical staining of tumor tissues.

Grant Support

This work was supported by NIH grants CA133085 and CA182518 and the VA merit review award I01BX001962 (to K. Liu).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received September 3, 2013; revised February 3, 2015; accepted February 23, 2015; published OnlineFirst March 3, 2015.

References


IFNγ Induces DNA Methylation–Silenced GPR109A Expression via pSTAT1/p300 and H3K18 Acetylation in Colon Cancer

Kankana Bardhan, Amy V. Paschall, Dafeng Yang, et al.


Updated version Access the most recent version of this article at: doi:10.1158/2326-6066.CIR-14-0164

Supplementary Material Access the most recent supplemental material at: http://cancerimmunolres.aacrjournals.org/content/suppl/2015/03/03/2326-6066.CIR-14-0164.DC1

Cited articles This article cites 50 articles, 23 of which you can access for free at: http://cancerimmunolres.aacrjournals.org/content/3/7/795.full#ref-list-1

Citing articles This article has been cited by 1 HighWire-hosted articles. Access the articles at: http://cancerimmunolres.aacrjournals.org/content/3/7/795.full#related-urls

E-mail alerts Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, use this link http://cancerimmunolres.aacrjournals.org/content/3/7/795. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.