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Role of MCP-1 in Alcohol-induced Aggressiveness of Colorectal Cancer Cells

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Abstract

Epidemiological studies demonstrate that alcohol consumption is associated with an increased risk of colorectal cancer (CRC). In addition to promoting carcinogenesis, alcohol may also accelerate the progression of existing CRC. We hypothesized that alcohol may enhance the aggressiveness of CRC. In this study, we investigated the effect of alcohol on the migration/invasion and metastasis of CRC. Alcohol increased the migration/invasion of colorectal cancer cells (DLD1, HCT116, HT29 and SW480) in a concentration-dependent manner. Among these colon cancer cell lines, HCT116 cells were most responsive while HT29 cells were the least responsive to ethanol-stimulated cell migration/invasion. These *in vitro* results were supported by animal studies which demonstrated that ethanol enhanced the metastasis of colorectal cancer cells to the liver and lung. Monocyte chemoattractant protein-1 (MCP-1) is a chemokine that plays an important role in regulating tumor microenvironment and metastasis. Alcohol increased the expression of MCP-1 and its receptor CCR2 at both protein and mRNA levels. The pattern of alcohol-induced alterations in MCP-1 expression was consistent with its effect on migration/invasion; HCT116 cells displayed the highest up-regulation of MCP-1/CCR2 in response to alcohol exposure. An antagonist of CCR2 blocked alcohol-stimulated migration. Alcohol caused an initial cytosolic accumulation of β -catenin and its subsequent nuclear translocation by inhibiting GSK3 β activity. Alcohol stimulated the activity of MCP-1 gene promoter in a β -catenin-dependent manner. Furthermore, knock-down of MCP-1/CCR2 or β -catenin was sufficient to inhibit alcohol-induced cell migration/invasion. Together, these results suggested that alcohol may promote the metastasis of CRC through modulating GSK3 β / β -catenin/MCP-1 pathway.

Keywords

Chemokines; ethanol; metastasis; tumor promotion; Wnt signaling

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Introduction

Chronic alcohol consumption is implicated as an etiological factor in the genesis of many types of cancer, including the respiratory tract, gastrointestinal tract, liver and breast [1-5]. Alcohol and its major metabolite, acetaldehyde, are listed as Group 1 human carcinogens by the International Agency for Research on Cancer (IARC). Colorectal cancer (CRC) is the second most common cause of cancer death in developed countries among men (after lung cancer) and the third most common among women [6;7]. Epidemiological study demonstrated that heavy and/or chronic alcohol consumption increases the risk of colon cancer [8-13]. In addition to acting as a risk factor for carcinogenesis, epidemiological studies also indicate that alcohol consumption is often associated with advanced and aggressive tumors [14-16]. Metastasis of CRC is the main cause of CRC mortality. Recent studies demonstrate an increase in incidents of CRC in younger adults (20-40 years) with advanced stages and lower survival rate [17-21]. Heavily drinking is a serious problem among young adults in the U.S. Therefore, it is important to determine whether alcohol promotes the aggressiveness of CRC and understand the underlying mechanisms. In this study, we investigated the effect of alcohol on CRC migration/invasion and metastasis using both *in vitro* and *in vivo* models.

Chemokines are a family of secreted cytokines which consists of more than 50 types. Chemokines bind to G-protein-coupled chemokine receptors and are involved in myriad physiological and pathological processes. Chemokines play an important role in tumorigenesis and cancer progression, such as tumor cell growth/survival, angiogenesis and metastasis [22;23]. Monocyte chemoattractant protein 1 (MCP-1), also known as chemokine (C-C motif) ligand 2 (CCL2), is one of the critical chemokines involved in all stages of tumor development, including tumor initiation and metastasis. Serum levels of MCP-1 are positively correlated with tumor stage and grade in breast and bladder cancer patients [24-26]. Recent studies suggest that MCP-1 and its receptor play an important role in colon carcinogenesis and CSC progression [27-29]. In this study, we further investigated the role of MCP-1 in alcohol-mediated CSC aggressiveness and the mechanism underlying alcohol-induced MCP-1 activation.

Materials and Methods

Materials

MTT assay kit was purchased from Roche Molecular Biochemicals (Indianapolis, IN). Transwells were purchased from Becton Dickinson Labware (Franklin lakes, NJ). Matrigel Invasion Chambers were purchased from BD Biosciences (Bedford, MA). Alcohol (200 Proof) was obtained from Fisher Scientific (Pittsburgh, PA). MCP-1 Human ELISA Kit was purchased from Invitrogen Corporation (Carlsbad, CA). Anti-human MCP-1 antibody and CCR2 antagonist (CCR2-I) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Recombinant Human MCP-1/CCL2 was purchased from Biolegend (San Diego, CA). Anti- β -catenin, anti-non phospho β -catenin (Ser33/37/Thr41), anti-phospho-GSK3 β and anti-GSK3 β antibodies were purchased from Cell Signaling Technology Inc. (Beverly, MA). β -catenin siRNA, MCP-1 siRNA and CCR2 siRNA were purchased from Santa Cruz

Biotechnology (Santa Cruz, CA). Anti-Ref-1 antibody was provided by Dr. Xianglin Shi (University of Kentucky, Lexington, KY). NE-PER Nuclear and Cytoplasmic Extraction Kit was purchased from Thermo Scientific (Rockford, IL). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Cell culture and treatments

Human colorectal cancer cell lines: DLD-1, HCT116, HT29 and SW480 were provided by Dr. Zhuo Zhang (University of Kentucky, Lexington, KY). All of these cell lines were cultured in DMEM medium containing 10% fetal bovine serum and 1% antibiotic-antimycotic (Invitrogen Corporation, Carlsbad, CA) at 37°C with 5% CO₂. For alcohol exposure, a method utilizing sealed containers was used to maintain alcohol concentrations in the culture medium [30]. With this method, alcohol concentrations in the culture medium can be accurately maintained. For most experiments, a physiologically relevant concentration of alcohol (43.4 mM or 200 mg/dl) was used. To block CCR2 signaling, CCR2 antagonist (CCR-I, 20 nM) was added into cells 2 hours prior to ethanol or MCP-1 exposure.

Cell invasion and migration

Cell invasion was assayed using Matrigel Invasion Chambers (BD Biosciences). Briefly, cells were placed on the upper compartment of invasion chambers and treated with alcohol or MCP-1 in the presence or absence of CCR2 antagonist (CCR2-I). Culture medium containing 10% FBS was added into the lower compartment of invasion chambers and served as chemoattractants for the cells. Cells were maintained in the invasion chambers overnight. The invaded cells were fixed in 3.7% paraformaldehyde and stained with 0.5% crystal violet in 2% ethanol. Membranes were washed and the dye was eluted with 10% acetic acid. Absorbance was measured at 595 nm using a microtiterplate reader (Beckman coulter).

Cell migration was analyzed using a Transwell Migration System (Costar). Briefly, cells were plated into upper chambers (Transwells with 8.0 µm pore size) in serum free medium and treated with alcohol or MCP-1 in the presence or absence of CCR2 antagonist (CCR2-I). The lower compartment of the chamber contained regular medium containing 10% FBS. The chambers were cultured at 37°C in 5% CO₂ for 12 hours. Migrated cells were fixed and stained with 0.5% crystal violet, followed by dye elution and absorbance measurement as described above.

MTT assay

The number of viable cells in culture was determined by the MTT assay. Briefly, the cells were plated into 96-well plates and exposed to alcohol or MCP-1 for indicated times. After the treatment, 10 µl of MTT reagent was added into each well and the plates were incubated at 37°C for 4 hours. The cultures were solubilized and spectrophotometric absorbance was measured at 595 nm using a microtiterplate reader (Beckman coulter).

Cell fractionation and immunoblotting

After treatments, cells were washed twice in ice-cold PBS, lysed in RIPA buffer (15 mM NaCl, 50 mM Tris, 1% NP-40, 0.5% sodium deoxycholate, 2 mM EGTA, 1 mM sodium orthovanadate, 1 mM phenylmethanesulfonyl fluoride, 5 µg/ml aprotinin and 2 µg/ml leupeptin). For preparation of nuclear and cytoplasmic extracts, the NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific) were used to prepare nuclear and cytoplasmic extracts, respectively. Samples were separated by centrifugation at 10,000 rpm for 10 min at 4°C, and then resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were transferred to nitrocellulose membranes. The membranes were probed with the indicated primary antibodies, followed by the appropriate horseradish peroxidase-conjugated secondary antibodies, and developed by enhanced chemiluminescence.

Quantification of MCP-1 and CCR2 mRNA

The expression of MCP-1 and CCR2 mRNAs were analyzed by quantitative real-time PCR or RT-PCR. Briefly, after ethanol treatment, total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. cDNA was then synthesized from 1 microgram RNA, respectively, using the Reverse Transcription System (New England Biolabs) according to the manufacturer's instructions. Real-time PCR was performed using the LightCycler SYBR Green I Master kit (Roche Applied Science, Mannheim, Germany) on Roche LightCycler Real-Time PCR system (Roche). The data were analyzed using $2^{-\Delta\Delta Ct}$ method. The relative expression levels of mRNA were normalized to the housekeeping gene GAPDH and compared with control groups. MCP-1 or CCR2 cDNA was amplified using DreamTag Green PCR Master Mix (Thermo Scientific) according to the manufacturer's instructions. The following reverse (R) and forward (F) Primers were used: GAPDHF: 5'-CTCTCTGCTCCTCTGTTCGAC-3', GAPDHR: 5'-TGAGCGATGTGGCTCGGCT-3', MCP-1F: 5'-TCATAGCAGCCACCTT C ATTC-3' and MCP-1R: 5'-CATGGAATCCTGAACCCACTT-3', CCR2F: 5'-AGAGGCA TAGGGCAGTGAGA, CCR2R: 3'-ACTCCTGGACCTCCACACAC. Each experiment was replicated at least 3 times.

siRNA and cell transfection

Transient transfection of β -catenin siRNA (β -Cat si), MCP-1 siRNA (MCP si) or CCR2 siRNA (CCR si) (San Cruz Biotech) was performed using a Neon Transfection System (Invitrogen Corporation, Carlsbad, CA) according to the manufacturer's protocol. Briefly, HCT116 cells were electroporated and incubated with control (scramble) siRNA (con si), β -Cat si, MCP si or CCR2 si. Forty eight hours after transfection, cells were exposed to alcohol (200 mg/dl) and assayed for cell migration.

Xenografts and metastasis analysis

All procedures were performed in accordance with the guidelines set by the National Institutes of Health (NIH) *Guide for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committee. Each of the Balb/c nude mice (10 weeks old) received subcutaneous inoculation of HCT116 cells (10^6 in 100 µl

PBS). Mice were divided into two groups and fed with standard chow diet ad libitum. The mice in the alcohol-exposed group were given 2% alcohol in their drinking water for a 12 hour-period during the night starting at 8:00 pm, and then replaced with water without alcohol at 8:00 am for the remaining 12 hours each day for 4 weeks. The mice in the control group were provided with regular drinking water only. The blood alcohol concentration (BEC) was determined using an Analox AM1 Alcohol Analyzer (Analox Instruments, Lunenburg, MA) as previously described [31]. This paradigm of alcohol exposure resulted in blood alcohol concentration of 40-50 mg/dl which is equivalent to modest alcohol consumption in humans. Metastases in the liver and lung were analyzed as previously described [31]. The initial experiment (n = 5 for EtOH group; n = 6 for control group) examined the effect of alcohol on lung metastasis. One mouse in the EtOH group died leaving only 11 animals (5 for EtOH and 6 for control). The subsequent experiment (n = 6 for EtOH group; n = 6 for control group) examined both livers and lungs.

Statistical analysis

Differences among treatment groups were tested using analysis of variance (ANOVA). Differences in which *p* was less than 0.05 were considered statistically significant. In cases where significant differences were detected, specific *post hoc* comparisons between treatment groups were examined with Student-Newman-Keuls tests. The prevalence of liver/lung metastasis between control and alcohol-treated groups was determined by the Fisher exact test.

Results

Alcohol increases migration/invasion and metastasis of colorectal cancer cells

We first examined the effect of alcohol (0, 43.4 or 86.8 mM) on the migration of four CRC lines (DLD1, HCT116, HT29 and SW480). Generally, alcohol increased the migration of all these CRC lines, although the sensitivity to alcohol varied (Fig. 1A). In HCT116 cells, alcohol stimulated cell migration in a concentration dependent manner. Alcohol also enhanced the invasion of DLD1, HCT116 and SW480 cells, but not HT29 cells (Fig. 1B). Among these cells, HCT116 cells were most responsive to alcohol-stimulated migration/invasion while HT29 cells were the least. MTT assay indicated that alcohol at lower concentrations (21.7-43.4 mM or 100-200 mg/dl) had little effect on cell viability, but slightly reduced cell viability at a higher concentration (86.8 mM or 400 mg/dl) (data not shown). The *in vitro* data suggested that alcohol increased the aggressiveness of CRC. To determine whether alcohol indeed increased CRC aggressiveness in a physiologically relevant condition, we used a xenograft mouse model to investigate CRC metastasis. We initially examined CRC metastasis in the lung (6 mice in control and 5 mice in alcohol-exposed group). In the second set of experiments (6 mice in control and 6 mice in alcohol-exposed group), we studied CRC metastasis in both liver and lung. The data were presented in Table 1 and Fig. 1C. Five out of six mice in the alcohol-exposed group showed liver metastasis in comparison to the control in which only one out of 6 mice had liver metastasis (Table 1). Nine out eleven mice in alcohol-exposed groups exhibited lung metastasis in comparison to the control in which three out of twelve mice had lung metastasis. Therefore, alcohol exposure for 4 weeks significantly increased CRC metastasis in the liver and lung.

Alcohol increases MCP-1 expression and function

MCP-1 is implicated in the tumorigenesis and the aggressiveness of CRC [27;32], [28]. Since alcohol increased the aggressiveness of CRC, we sought to determine whether alcohol affected MCP-1 expression in CRCs. DLD1, HCT116, HT29 and SW480 cells were exposed to alcohol (43.4 mM or 200 mg/dl) for 0, 3, 6 or 12 hours and mRNA levels of MCP-1 were measured by RT-PCR. All cells expressed MCP-1 and responded to alcohol exposure by increasing MCP-1 expression. Among these cells, HCT116 cells were more sensitive to alcohol and displayed the highest increase of MCP-1 expression following alcohol exposure (Fig. 2A). Alcohol also increased the expression of MCP-1 receptor, CCR2 in DLD-1, HCT116 and SW480 cells; but the time course of alcohol-induced CCR2 up-regulation lagged behind its effect on MCP-1. Alcohol increased MCP-1 secretion in the condition medium collected from HCT116 and HT29 cells (Fig. 2B). Time course study indicated that alcohol-induced increase in MCP-1 mRNA started at 1 hour following alcohol exposure and peaked at 3 hours in HCT116 cells (Fig. 2C). ELISA study indicated that alcohol-enhanced MCP-1 secretion was observed at 3 hours and peaked at 6 hours following ethanol exposure (Fig. 2D). This observation was confirmed by immunoblotting analysis which also showed an upregulation of MCP-1 protein following alcohol exposure (Fig. 2E). Together, these data indicated that alcohol increased MCP-1 expression.

MCP-1 increases the migration/invasion of colorectal cancer cells

Since HCT116 cells were sensitive to alcohol, the subsequent experiments focused on this cell line. To establish the role of MCP-1 in CRC aggressiveness, we first examined the effect of MCP-1 on the migration/invasion of HCT116. In this experiment, HCT116 cells were treated with MCP-1 (0, 5, 10 or 20 ng/ml) overnight and evaluated for their migration/invasion potential. As shown in Fig. 3, MCP-1 at 10-20 ng/ml significantly increased the migration/invasion of HCT116 cells. However, we did not observe a dose-dependent effect of MCP-1 at this concentration range. MCP-1 treatment did not change the number/viability of HCT116 cells (data not shown). MCP-1 interacts with its receptor, CCR2, to execute its biological function and a recent study indicates that reduced metastasis and extravasation are observed in CCR2 deficient mice [28]. We therefore sought to determine whether blocking CCR2 signaling can inhibit MCP-1 or alcohol-stimulated migration/invasion. To inhibit MCP-1/CCR2 signaling, a CCR2 antagonist (CCR2-I) was added to the cultures 2 hours prior to MCP-1 or alcohol exposure. As shown in Figs 3C and D, CCR2-I significantly inhibited MCP-1- and alcohol-stimulated migration of HCT116 cells. Together, these data suggested that alcohol-stimulated migration/invasion may be mediated by MCP-1/CCR2 signaling.

Alcohol increases MCP-1 expression through regulating GSK3 β / β -catenin pathway

There are multiple signaling pathways that may participate in the regulation of MCP-1 expression; these include ERK, JNK, PKC and PI3K/Akt/GSK3 β [33-35]. Several transcription regulators, such as NF κ B, AP-1 and β -catenin are involved in this regulation [24;36]. The activity of GSK-3 β is positively regulated by its phosphorylation of at Tyr216 [pGSK3 β (Tyr216)] and negatively by its phosphorylation at serine 9 [pGSK3 β (Ser9)]. We showed that alcohol inhibited GSK3 β activity by stimulating pGSK3 β (Ser9) and inhibiting

pGSK3 β Tyr216 (Fig. 4A). GSK-3 β negatively regulates β -catenin levels; the activation of GSK-3 β phosphorylates β -catenin at Ser33, Ser37 and Tyr41 which leads to the degradation of β -catenin. In contrast, inhibition of GSK-3 β stabilizes β -catenin, causing the accumulation of cytosolic β -catenin and subsequent nuclear translocation. Next, we examined the expression level of β -catenin. An antibody detecting only non-phosphorylated β -catenin was used in this study. Alcohol increased protein levels of non-phosphorylated β -catenin (Fig. 4A). Alcohol induced an initial increase of β -catenin in the cytoplasm; which started at 10 min and lasted for 1 hour (Fig. 4B). At 30 min after alcohol exposure, β -catenin level in the nuclear fraction increased, indicating that the β -catenin accumulated in the cytoplasm then translocated to the nucleus (Fig. 4B). The results suggested that alcohol inhibited GSK3 β activity and stabilized β -catenin, causing its translocation to the nucleus.

There is a β -catenin binding site (5'-CTTTGTA-3') on the enhancer region of human MCP-1 gene which is located 1399 bp upstream of the transcription initiation site [24]. To investigate the role of β -catenin in alcohol-stimulated MCP-1 gene transcription, we constructed a luciferase vector containing a mutated β -catenin binding site (CTTTGGC) on the enhancer region of human MCP-1 gene. HCT116 cells were transfected with luciferase vectors containing either the wild type MCP-1 gene promoter or MCP-1 gene promoter with mutated β -catenin binding site on. Forty eight hours after the transfection, cells were exposed with alcohol for 6 hours and assayed for luciferase activity. As shown in Fig. 4C, alcohol stimulation of luciferase activity was observed in the cells carrying wild type MCP-1 gene promoter, but not in cells transfected with a mutated promoter (Fig. 4C). These results indicated that β -catenin played an important role in alcohol-induced MCP-1 gene transcription.

Gene silencing of MCP-1/CCR2 or β -catenin attenuates alcohol-stimulated cell migration

To establish the roles of MCP-1/CCR2 and β -catenin in alcohol-stimulated migration, we knocked down the expression of MCP-1/CCR2 and β -catenin in HCT116 cells with respective siRNAs. Downregulation of MCP-1/CCR2 and β -catenin was verified by immunoblotting analysis (Data not shown). Forty eight hours after the transfection of siRNA, cells were exposed to alcohol and assayed for cell migration. As shown in Fig. 5, alcohol-stimulated migration of HCT116 cells was blocked by the treatment of siRNAs for MCP-1/CCR2 or β -catenin. These results indicated that MCP-1/CCR2 and β -catenin signaling played an important role in alcohol-stimulated migration of colorectal cancer cells.

Discussion

We demonstrate here that alcohol promotes metastasis and migration/invasion of CRCs *in vitro* and *in vivo*, suggesting that alcohol may promote the aggressiveness of existing CRC in addition to increasing the risk of CRC carcinogenesis. Alcohol upregulates the expression of MCP-1 through modulating GSK3 β / β -catenin pathway and blocking MCP-1-mediated signaling effectively mitigates alcohol-enhanced migration/invasion of CRCs, suggesting MCP-1 may mediate alcohol-promoted progression of CRC (Fig. 5B).

MCP-1 has been implicated in various aspects of tumorigenesis and cancer progression, such as angiogenesis, tumor growth and metastasis [25;27;29;31]. MCP-1 plays an

important role in intestine inflammation and tumorigenesis/progression of colon cancer [27;32;37]. In this study, alcohol increases the migration of all CRCs tested (DLD1, HCT116, HT29 and SW480) (Fig. 1). Alcohol also enhances the invasion of these cells except HT29 cells. Among these cells, HCT116 cells are most responsive to alcohol-enhanced cell migration/invasion while HT29 cells were the least. This is generally consistent with the profile of alcohol-induced upregulation of MCP-1/CCR2; alcohol causes the most drastic increase of MCP-1/CCR2 expression in HCT116 cells. It appears that HT29 cells do not express CCR2.

Alcohol increases MCP-1/CCR2 expression at both mRNA and protein levels; it also stimulates its secretion. We have previously demonstrated that alcohol enhanced MCP-1 production in breast cancer cells which in turn promoted mammary tumor growth and angiogenesis [31]. Therefore, alcohol-mediated activation of MCP-1/CCR2 signaling pathway may not be specific for CRCs. Since MCP-1/CCR2 is important for intestine inflammatory response, alcohol stimulation of MCP-1/CCR2 may be implicated in some intestinal abnormalities associated with heavy alcohol consumption. For example, alcohol consumption is positively associated with inflammatory bowel disease (IBD) [38] and MCP-1 is a known mediator for IBD [27;39]. It is therefore possible that alcohol may promote IBD by activating the MCP-1/CCR2 pathway. In addition, patients with IBD have an increased risk of developing colon cancer. Alcohol-mediated alteration of MCP-1/CCR2 may be implicated the gut inflammation and the carcinogenesis of colon cancer.

Our *in vitro* data indicate that alcohol stimulates the activity of MCP-1 gene promoter, suggesting that alcohol may directly affect the transcription of MCP-1 gene (Fig. 4). Several transcription factors, such as NF κ B, AP-1 and Sp1, are involved in the regulation of MCP-1 gene transcription [36;40;41]. In addition, β -catenin is also shown to regulate MCP-1 gene transcription and there is a β -catenin binding site on human MCP-1 promoter located at 1399 bp upstream of the transcription initiation site [24]. We demonstrate here that alcohol exposure increases the accumulation of β -catenin in the cytoplasm and its subsequent translocation to the nucleus. Alcohol activates MCP-1 gene promoter containing wild type β -catenin binding site, but fails to activate MCP-1 gene promoter containing mutated β -catenin binding site (Fig. 5). Additionally, downregulation of β -catenin or MCP-1 by siRNA effectively inhibits alcohol-induced cell migration. Since NF κ B is also a critical transcription factor regulating MCP-1 transcription, we examined the effect of alcohol on MCP-1 gene promoter containing NF κ B binding sites. Alcohol does not affect the activity of MCP-1 gene promoter containing either wild type or mutated NF κ B binding sites (data not shown). In some cases, NF κ B is reported to negatively regulate MCP-1 expression. For example, NF κ B is shown to repress MCP-1 expression in response to hypoxia and alcohol can induce hypoxia [42-44]. Therefore NF κ B may not be involved in alcohol stimulation of MCP-1 gene transcription. Together, these data indicate that β -catenin/MCP-1 pathway is critical for alcohol promotion of CRC aggressiveness (Fig. 5B).

It appears that alcohol-induced upregulation of β -catenin is mediated by GSK3 β which regulates β -catenin degradation. GSK3 β is constitutively active in cells and forms a complex with adenomatous polyposis coli (APC) and scaffold protein Axin in the absence of Wnt/Wingless signal. Phosphorylation of APC by GSK3 β provides a docking site for β -

catenin binding. GSK3 β phosphorylates β -catenin leading to its degradation by ubiquitin-proteasome pathway [45]. GSK3 β is a well-known target of alcohol [46]. We show here that alcohol inhibits GSK3 β activity by stimulating pGSK3 β (Ser9) and suppressing pGSK3 β (Tyr216), which may cause cytosolic accumulation of β -catenin and its subsequent translocation to the nucleus (Fig. 4).

This is the first study to show GSK3 β / β -catenin is involved in MCP-1 gene transcription in colorectal cancers. Dysregulation of Wnt/ β -catenin signaling has been frequently associated with gastrointestinal cancers and are implicated in colorectal tumorigenesis as well as growth and maintenance of colon cancer stem cells [47-50]. Abnormal expression of many Wnt/ β -catenin targeted genes, such as c-myc, c-jun, claudin-1, MMP7 and VEGF, has been identified in colon cancers [51-55]. However, little is known about Wnt/ β -catenin signaling and its regulation of the chemokine system in the intestine. A previous study shows that β -catenin/TCF-4 upregulates MCP-1 expression in response to losing of E-cadherin in advanced breast cancer cells [24]. GSK3 β is reported to regulate MCP-1 expression in macrophage-like cells [35]. Here, we demonstrate that GSK3 β / β -catenin signaling is involved in the regulation of MCP-1 expression in colon cancer in response to alcohol exposure. MCP-1 plays an important role in gastrointestinal inflammation and involved in colorectal tumorigenesis [27;56]. Dysregulation of Wnt/ β -catenin signaling is a hall mark of colon cancer. More than 90% of colorectal cancers have a mutation in the components of Wnt/ β -catenin signaling pathway [50]. In addition, dysregulation of GSK3 β / β -catenin is associated with poor prognosis in colon cancer patients [57]. Our study not only provides a new insight into the mechanisms of alcohol-induced promotion of colon cancer, but also helps to understand how MCP-1 is regulated during colorectal carcinogenesis/progression.

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Abbreviations

AMVD	
CCL2	chemokine (C-C motif) ligand 2
CCR2	CC chemokine receptor 2
CCR2A	CCR2 antagonist
CRC	Colorectal cancer
MCP-1	Monocyte chemoattractant protein-1

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Figure 1.
Effect of alcohol on the migration/invasion and metastasis of colorectal cancer cells. Colorectal cancer cells (DLD1, HCT116, HT29 and SW480) were exposed to alcohol (0, 21.7, 43.4 and 86.8 mM) overnight and assayed for migration (**A**) and invasion (**B**) as described in the Materials and Methods. Each data point was the mean $\pm$ SEM of three independent experiments and presented relative to the control values. * denotes significant difference from control groups. **C**: HCT116 cells ($10^6/100 \mu\text{l}$ PBS) were inoculated into nude Balb/c mice. Animals were exposed to alcohol in drinking water as described in the Materials and Methods. After 4 weeks of alcohol exposure, mice were sacrificed and analyzed for tumor metastasis. The image shows lung metastases.

Figure 2. Effect of alcohol on MCP-1 expression and secretion. **A:** Colorectal cancer cells (DLD1, HCT116, HT29 and SW480) were exposed to alcohol (43.4 mM or 200 mg/ml) for indicated time (0, 3, 6 and 12 hours). mRNA levels of MCP-1 and CCR2 were analyzed by RT-PCR and GAPDH mRNA was shown as a loading control. **B:** Colorectal cancer cells were exposed to alcohol (43.4 mM) for 6 hours and conditioned medium was collected and assayed for secreted MCP-1 by ELISA. **C:** HCT116 cells were exposed to alcohol (43.4 mM) for indicated times (0, 1, 3, 6 and 12 hours). mRNA level of MCP-1 was measured by

real-time PCR. The alteration in expression was presented relative to the control level. **D**: HCT116 cells were exposed to alcohol (43.4 mM) for indicated times and secreted MCP-1 in conditioned medium was assayed as described above. **E**: HCT116 cells were exposed to alcohol (43.4 mM) for indicated times and the expression of MCP-1 was analyzed by immunoblotting. The expression of actin was shown as a loading control. Each data point was the mean $\pm$ SEM of three independent experiments and presented relative to the control values. * denotes significant difference from controls.

Figure 3.

Effect of MCP-1 on the migration/invasion of colorectal cancer cells. **A** and **B**: HCT 116 cells were treated with MCP-1 (0, 5, 10 or 20 ng/ml) for overnight and assayed for cell migration (**A**) and invasion (**B**). **C**: HCT116 cells were pretreated either with DMSO (control) or CCR2 antagonist (20 μ M) for 2 hours and then exposed to MCP-1 (10 ng/ml) overnight. After that, cell migration was assayed as described above. **D**: HCT116 cells were pretreated either with DMSO (control) or CCR2 antagonist (20 μ M) for 2 hours and exposed to alcohol (43.4 mM) overnight, then assayed for cell migration. Each data point was the mean $\pm$ SEM of three independent experiments and presented relative to the controls. *

denotes significant difference from control groups. # denotes significant difference from MCP-1- or alcohol-treated groups.

Figure 4.

Effect of alcohol on GSK3 β and β -catenin. **A:** HCT116 cells were exposed to alcohol (200 mg/dl) for 10 min, 30 min, 1 hour, 3 hours and 6 hours. The expression of GSK3 β , phosphorylated GSK-3 β at serine 9 or tyrosine 216, β -catenin and non-phosphorylated β -catenin was analyzed by immunoblotting as described in the Materials and Methods. The expression of actin was used as a loading control. **B:** cytoplasmic and nuclear fractions were prepared and non-phosphorylated β -catenin (Ser33/37/Thr41) (Cell Signaling Technology Inc) was analyzed by immunoblotting as described in the Methods and Materials. The

expression of actin or Ref-1 was used as a loading control for cytosolic or nuclear protein, respectively. C: HCT116 cells were transfected with luciferase reporter carrying either wild-type MCP-1 gene promoter (MCP-1 Wt) or β -catenin-mutated promoter (β -cat Mut) for 48 hours. Cells were exposed to alcohol (43.4 mM) for 6 hours, and then assayed for luciferase activity. Each data point was the mean $\pm$ SEM of three independent experiments and presented relative to the controls. * denotes significant difference from control groups. # denotes significant difference from MCP-1 Wt groups treated with alcohol.

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A

B

Figure 5.

Effect of siRNAs for MCP-1/CCR2 and β -catenin on alcohol-stimulated cell migration. **A:** HCT116 cells were transfected with the scramble siRNA (con si), β -catenin siRNA (β -Cat si), MCP-1 siRNA (MCP-1 si) or CCR2 siRNA (CCR2 si), for 48 hours, and exposed to alcohol (43.4 mM) overnight. After that, cell migration was assayed. Each data point was the mean $\pm$ SEM of three independent experiments and presented relative to the controls. * denotes significant difference from the control without alcohol exposure. # denotes significant difference from the control with alcohol exposure. **B:** Schematic of the signaling

pathway that is involved in alcohol-induced CSC metastasis. Alcohol inactivates GSK3 β , resulting in nuclear translocation of β -catenin. β -catenin activates MCP-1 gene transcription by stimulating MCP-1 gene promoter. High expression of MCP-1 may enhance CSC metastasis.

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Table 1

Effect of alcohol on the metastasis of colorectal cancer cells.

	Mice	Metastasis to Liver
Control	6	1
EtOH	6	5
		$p=0.04$

	Mice	Metastasis to Lung
Control	12	3
EtOH	11	9
		$p=0.01$

HCT116 cells ($10^6/100 \mu\text{l}$ PBS) were inoculated into nude Balb/c mice (12 in control and 11 in alcohol-exposed group). Animals were exposed to alcohol in drinking water as described in the Materials and Methods. After 4 weeks of alcohol exposure, mice were sacrificed and analyzed for tumor metastasis. The difference between control and alcohol-exposed group was determined by Fisher exact test.