Original Paper

A New Participant in the Pathogenesis of Alcoholic Gastritis: Pyroptosis

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Key Words

Alcoholic gastritis • Pyroptosis • Caspase-1 • IL-1 β • Ac-yvad-cmk

Abstract

Background/Aims: Alcohol abuse exerts deleterious effects on the internal organs of the body, and alcohol-related gastritis is a common disease for which prompt treatment is essential to prevent the condition from growing worse. However, the therapeutic methods have some adverse effects. Determining the pathogenic mechanisms of alcoholic gastritis is therefore essential. **Methods:** The MTT assay was developed in order to determine the optimal concentration of alcohol needed to treat gastric mucosal cells. The effects of alcohol on the gastric mucosal cells were determined by qRT-PCR and western blot. The release of IL-1 β and IL-18 were determined by ELISA assay. The immunofluorescence assay was used to detect caspase-1 activation levels, while immunohistochemical assay and HE staining were performed to identify the effectiveness of the caspase-1 inhibitor on alcoholic gastritis. The TUNEL assay was used to determine DNA fragmentation. **Results:** Here, we clarified that ethanol treatment could cause cell DNA damage, activate caspase-1, and promote the generation and release of IL-1 β and IL-18. In other words, ethanol could induce pyroptosis. Interestingly, a caspase-1 inhibitor could significantly suppress pyroptosis, decrease the release of inflammatory cytokines induced by ethanol, and cause no side effects *in vivo* and *in vitro*. **Conclusion:** Collectively, our results showed that pyroptosis is involved in the pathogenesis of alcohol-induced gastritis and that caspase-1 inhibitor Ac-yvad-cmk could effectively decrease the damage caused by alcohol, making it a potentially promising agent for the treatment of alcoholic gastritis.

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Introduction

Drinking alcoholic beverages is a common feature of social gatherings, and ethanol is the main ingredient in all kinds of alcoholic beverages. The consumption of ethanol is related to approximately 60 different types of diseases [1-4]. It is well known that alcohol abuse may cause acute erosive hemorrhagic gastritis, and long-term drinking could cause stomach disorders and chronic atrophic gastritis [5-7]. In general, gastritis is mainly due to the imbalance between aggressive and defensive factors of the gastric mucosa, which causes a variety of issues, from local defects to active inflammation [8-11]. This kind of chronic inflammation is increasingly recognized to have significant tumor-promoting potential [12, 13]. The precise regulation of inflammation progression is crucial for chronic inflammation disorders and for suppressing the depravation of pathogenetic conditions. Current therapeutic agents of gastritis are usually used to inhibit gastric acid secretion and to stimulate mucosal defense mechanisms [14-18]. However, these strategies generally fail due to hypersensitivity, gynecomastia, impotence, arrhythmia, and hematopoietic changes [19-21]. Therefore, exploring the mechanisms of gastritis and finding new therapeutic agents are vitally important.

Pyroptosis is a form of caspase-1- or caspase-11-dependent programmed cell death. In this process, a host cells recognize certain danger signals and produce cytokines, leading it to swell, burst, and ultimately die [22, 23]. The main molecular characteristic of pyroptosis is the activation of caspase-1 and the subsequent production and release of interleukin-1 β (IL-1 β) and interleukin-18 (IL-18)[8]. Caspase-1 is an inflammatory caspase that is essential for canonical inflammasome-mediated pyroptosis and cytokine maturation [24]. Caspase-11 (also known as caspase-4 or -5 in humans), another inflammatory caspase, is the core component of non-canonical inflammasome. Caspase-11 directly acts as a receptor of cytosolic bacterial lipopolysaccharide (LPS) and is activated by binding to LPS[25]. Caspase-11 can trigger pyroptosis, but its effects on IL-1 β and IL-18 maturation are indirect, because this process requires the NLRP3-dependent activation of caspase-1[26, 27]. Pyroptosis induces pathological inflammation, which is involved in the pathogenesis of multiple inflammatory diseases in several organs, including bowel [28], brain [29], heart [30] and kidney [31]. In gastritis, inflammation and mucosal cell death are also present. However, whether pyroptosis participates in the pathogenesis of gastritis remains unknown. Caspase-1 is a key enzyme of pyroptosis, and we therefore mainly used the caspase-1 inhibitor, Ac-yvad-cmk, to regulate pyroptosis [32-34]. The present study was thus designed to explore the role of pyroptosis in alcoholic gastritis.

Materials and Methods

Cell culture and treatment

GES-1 cells were obtained from ATCC. The cells were grown in RPMI-1640 (HyClone, Logan, UT, USA) containing 10% fetal bovine serum (Biological Industries, Kibbutz Beit Haemek, Israel) and incubated at 37°C in humidified air with 5% CO₂. After seeding in a 96-well plate, the cells were treated with ethanol at concentrations of 0, 0.5%, 1%, 2%, 4%, 8% and 16%. An MTT assay was used to determine the cell viability. To explore the effects of ethanol and caspase-1 inhibitor (100 μ M), cells were treated with either ethanol or both ethanol and the caspase-1 inhibitor.

MTT assay

To measure the cell viability, 1 $\times$ 10⁴ cells per well were seeded into a 96-well plate and treated as previously described. After treatment, 10 μ l MTT reagents (0.5 mg/ml) was added to each well and incubated for 4 h at 37°C. The formazine granulars in the wells were dissolved with 150 μ l dimethyl sulphoxide (DMSO), and the absorbance at 570 nm was measured using a microplate reader.

TUNEL staining

DNA damage was detected by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining using an apoptotic cell detection kit following the manufacturer's directions (Promega, Madison, WI, USA).

HE staining

Hematoxylin and eosin (HE) staining was used to observe the histological changes. Gastric tissues from mice or gastric cancer tissues and adjacent normal tissues from humans were fixed in 4% paraformaldehyde and embedded in paraffin. The samples were cut into 5- μ m-thick sections and stained with HE.

Immunofluorescence staining

For immunofluorescence staining, cultured cells were fixed with 4% buffered paraformaldehyde. The cells were then washed in PBS and incubated with blocking solution (1% BSA and 0.1% Triton-X in PBS). Subsequently, the cells were incubated with primary antibodies against caspase-1 overnight at 4°C, followed by incubation with the secondary antibody (Invitrogen) for 1 h at room temperature. The nuclei were stained using 4',6-diamidino-2-phenylindole (DAPI; Beyotime, Shanghai, China) for 20 min at room temperature. The images were captured using a fluorescence microscope.

Immunohistochemical staining

For immunohistochemical analysis, frozen gastric section specimens were fixed with 4% buffered paraformaldehyde and embedded in paraffin. Specimens were dehydrated by an ascending series of ethanol and cleared with xylene. All sections were immunostained with primary antibodies against caspase-1, IL-1 β and IL-18 at 4°C overnight. After incubation with secondary antibodies, the sections were stained with diaminobenzidine.

Enzyme-linked immunosorbent assay.

The culture medium was collected for the measurement of IL-1 β and IL-18 using an ELISA kit (uscn-SEA064R and uscn-SEA563Ra) according to the manufacturer's instructions [35].

Mouse model of alcoholic gastritis

Male Kungming mice (25-30 g) were obtained from the Experimental Animal Center of Harbin Medical University. These mice were divided into three groups with five mice in each group. In the control and the ethanol groups, the mice were separately treated by gavage with saline or 2% ethanol (20 ml/kg/d). In the caspase-1 inhibitor and ethanol group, mice were given 2% ethanol or 2% ethanol with caspase-1 inhibitor (20 mg/kg/d) intraperitoneally. At 15 d after treatment, the mice were anesthetized and euthanized by cervical dislocation. The stomachs were harvested and rinsed with saline and used for the following detection. All experimental protocols were pre-approved by the Experimental Animal Ethics Committee of Harbin Medical University, China. The animals use was confirmed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

qRT-PCR

The total RNAs from cells or tissues were extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA). The extracted RNA was reverse-transcribed into double-strand cDNA with a reverse transcription kit (Toyobo, Osaka, Japan). Subsequently, the SYBR Green PCR Master Mix Kit (Applied Biosystems, Calif, USA) was used to quantify the mRNA expression level of caspase-1 and IL-1 β . Real time PCR was performed with the 7500 FAST real time PCR System (Applied Biosystems, CA, USA), using GAPDH as a control. The forward and reverse primer sequences for caspase-1 are 5'-TTTCCGCAAGGTTTCGATTTC-3' and 5'-GGCATCTGCGCTCTACCATC-3', respectively. For IL-1 β , the forward primer was 5'-ATGATGGCTTATTACAGTGGCAA-3', and the reverse primer was 5'-GTCGGAGATTCTAGCTGGA-3'. For IL-18, the forward primer was 5'-CAAGGAATTGTCTCCAGTGC-3', and the reverse primer was 5'-CAGCCGCTTTAGCAGCCA-3'.

Western blot analysis

The total protein was extracted from the cells using RIPA buffer (Thermo, Shanghai, China) containing phenylmethanesulfonylfluoride (PMSF) (Beyotime, China). The total protein concentrations were measured with a Bradford assay and Protein Assay Kit (Bio-Rad, Hercules, CA, USA). Thirty μ g of the protein lysate was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto PVDF membranes (Millipore, Billerica, Massachusetts, USA). The PVDF membranes were blocked with 5% BSA in 0.05% Tween 20-TBS for 1 hour and incubated with the corresponding primary antibody. The mixtures were then diluted in blocking buffer overnight at 4°C. Dilutions for primary antibodies were as follows: anti-caspase-1 (1:1,000, Cell Signaling, 2225), anti-IL-1 β and anti-IL-18 (1:400, Santa Cruz Biotech). After extensive washing with TBST, anti-rabbit IgG-HRP secondary antibody (1:5,000, Santa Cruz Biotech) was added. Protein bands were visualized using the enhanced chemiluminescence technique with SuperSignal West Pico chemiluminescent substrate (Thermo Fisher Scientific, USA).

Statistical Analysis

All statistical analyses were performed using the SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). One-way ANOVA was conducted for normally distributed data. All data were expressed as the mean $\pm$ SD. Statistical significance was set at $P < 0.05$.

Results

Ethanol decreases cell viability in a concentration-dependent manner.

Chronic alcohol consumption is highly associated with gastric disease and ulcers, which may progress to gastric cancer. Alcohol-induced gastric mucosal injury can be mediated by various cellular molecules such as cyclooxygenase (COX), lipoxygenase (LOX), cytokines, and oxygen-derived free radicals [36, 37]. To clarify the pathogenesis of alcoholic gastritis, we first detected the effects of different concentrations of ethanol on the survival of GES-1 cells to define the optimal concentration of our assays. The cells were treated with ethanol at a concentration of 0, 0.5%, 1%, 2%, 4%, 8% and 16%. After 24 hours, viability detection of the cells showed the survival rate was decreased in a concentration-dependent manner (Fig. 1A).

Fig. 1. Ethanol treatment induced cell viability decreases and caspase-1 activation in GES-1 cells. A. Ethanol decreased the viability of GES-1 cells. B. Ethanol increased the mRNA expression of caspase-1. C. Immunofluorescence staining of caspase-1. D. Ethanol increased the protein expression of caspase-1. Data are representative of five independent experiments and represent the mean $\pm$ SD. * $P < 0.05$ vs. 0 ethanol group.

Ethanol induces pyroptosis in gastric mucosal cells.

Ethanol perfusion caused gastric mucosa damage [38] and induced oxidative stress and inflammation in the gastric tissues [39]. As part of the inflammation progress, pyroptosis is involved in many chronic diseases [40-43]. To clarify whether pyroptosis is included in gastritis pathogenic mechanisms, we detected the expression of

caspase-1 and inflammatory cytokines. In a preliminary experiment, we found that 1% and 2% ethanol could significantly increase the amount of active caspase-1 with little effect on the viability of cells. Therefore, we chose concentrations of 1% and 2% for the following experiments. An immunofluorescence assay showed that caspase-1 co-localized with the nucleus in ethanol-treated cells. The greater the concentration of ethanol, the more cells that showed co-localization with the nucleus. However, in the normal cells, caspase-1 did not show this co-localization (Fig. 1B), suggesting that ethanol may activate caspase-1 in a concentration-dependent manner. To further clarify this phenomenon, we carried out real time PCR and western blot analysis to detect the mRNA and protein expression levels of caspase-1 (Fig. 1C, D). The results showed that both the mRNA and protein levels were increased in the ethanol-treated cells, especially in the 2% ethanol-treated cells.

DNA damage occurs during pyroptosis. TUNEL staining was used to further confirm the effect of ethanol on the cells. The results showed that the number of TUNEL positive cell increased after treatment with ethanol in a concentration dependent manner (Fig. 2).

We also detected the generation and release of IL-1 β and IL-18, which are the downstream cytokines of caspase-1. GES-1 cells were treated with either ethanol (1% and 2%) or solvent. An ELISA assay showed that ethanol treatment induced the release of IL-1 β and IL-18 (Fig. 3A & B). The mRNA and protein detection showed that ethanol could increase IL-1 β and IL-18 expression levels in a concentration-dependent manner (Fig. 3C-F). These results indicated that ethanol (1% and 2%) could induce pyroptosis in a concentration-dependent manner.

The inhibition of caspase-1 suppresses the inflammatory response induced by ethanol

We use siRNA and inhibitor to inhibit the function of caspase-1. Ac-yvad-cmk, a caspase-1 inhibitor, could inhibit the activation of caspase-1. Cells were divided into five groups, including the control group, the si-NC group, the ethanol + si-NC group, the ethanol + si-caspase-1 group and the ethanol+caspase-1 inhibitor group. Then, we carried out the immunofluorescence experiment (Fig. 4A). Compared with the group treated with ethanol group, si-caspase-1 or caspase-1 inhibitor significantly decreased the co-localization of caspase-1 and the nucleus, suggesting that si-caspase-1 and Ac-yvad-cmk could suppress caspase-1 activation. TUNEL assay revealed that the inhibition of caspase-1 could protect against ethanol induced DNA damage (Fig. 4B & C). The ELISA assay showed that the inhibition of caspase-1 attenuated the release of IL-1 β and IL-18 (Fig. 4D & E). We then extracted the mRNA and protein and carried out real time PCR and western blot assays. Our results were consistent with the immunofluorescence observations. si-caspase-1 and Ac-yvad-cmk significantly suppressed the caspase-1, IL-1 β and IL-18 mRNA expression levels (Fig. 4F-H), as well as the protein expression levels (Fig. 4I-L). These data indicated that the caspase-1 inhibitor could inhibit pyroptosis caused by ethanol, thereby weakening the inflammation responses.

Fig. 2. The effect of ethanol on DNA damage in GES-1 cells. A. Representative TUNEL assay images. B. The percentage of apoptotic cells. Data are representative of five independent experiments and represent the mean $\pm$ SD. * $P < 0.05$ vs. 0 ethanol group.

Fig. 3. Ethanol promotes the generation and release of IL-1 β and IL-18. A & B. The release of IL-1 β and IL-18. C & D. The mRNA expression of IL-1 β and IL-18. E & F. The protein expression of IL-1 β and IL-18. Data are representative of five independent experiments and represent the mean $\pm$ SD. * $P < 0.05$ vs. 0 ethanol group.

Fig. 4. Caspase-1 knockdown attenuates the GES-1 cell injury induced by ethanol. A. Immunofluorescence staining of caspase-1. B. DNA damage was detected using the TUNEL assay in GES-1 cells. C-E. The mRNA expression of caspase-1, IL-1 β and IL-18. F. Representative western blot bands. G-J. The protein expression of caspase-1, IL-1 β and IL-18. Data are representative of five independent experiments and represent the mean $\pm$ SD. * $P < 0.05$ vs. control group. # $P < 0.05$ vs. ethanol group.

Caspase-1 inhibitor protects against alcoholic gastritis in mice without obvious side effects

To explore the therapeutic potential of the caspase-1 inhibitor *in vivo*, mice were divided into three groups, including the control group, the ethanol group and the ethanol+caspase-1 inhibitor group. We isolated the gastric epithelial tissues of these three groups for HE staining and detected inflammatory cell evasion level in these three groups. Our results showed that a significant decrease in mouse gastric mucosal inflammation was observed in the ethanol and Ac-yvad-cmk treated group, compared with the ethanol treated group. This decrease was almost equal to the normal group (Fig. 5A). Next, we detected caspase-1, IL-1 β and IL-18 expression via immunohistochemical assays using the gastric epithelial tissues of these three groups. The results showed that caspase-1, IL-1 β and IL-18 were activated in the ethanol-treated groups, and the caspase-1 inhibitor Ac-yvad-cmk significantly decreased the activation levels (Fig. 5B-D). To further clarify the role of the caspase-1 inhibitor in suppressing the inflammatory response, we extracted the mRNA from gastric tissues. The real time PCR results showed that caspase-1 inhibitor significantly decreased the elevation of the caspase-1, IL-1 β and IL-18 mRNA expression levels induced by ethanol (Fig. 5E-G). Next, we detected the protein expression level of these three groups. The results showed that the caspase-1 inhibitor decreased the translation levels of caspase-1, IL-1 β and IL-18 (Fig. 5H-K). Importantly, the caspase-1 inhibitor showed no significant adverse effects on gastric tissues from mice and GES-1 cells (Fig. 6).

Fig. 5. The caspase-1 inhibitor attenuates the gastric injury induced by ethanol in mice. A. Gastric histopathology of mice was analyzed with hematoxylin and eosin (HE) staining (magnification, 100 $\times$). B-D. Immunohistological staining of caspase-1, IL-1 β and IL-18 in gastric tissues from mice (magnification, 40 $\times$). E-G. The mRNA expression level of caspase-1, IL-1 β and IL-18. H. Representative western blot bands. I-K. The protein expression levels of caspase-1, IL-1 β and IL-18. Data are representative of five independent experiments and represent the mean $\pm$ SD. * $P < 0.05$ vs. the control group. # $P < 0.05$ vs. the ethanol group.

Fig. 6. The caspase-1 inhibitor showed no significant adverse effects on gastric tissues from mice and GES-1 cells. A. The histological characteristic of gastric tissues from mice treated with the caspase-1 inhibitor. B. The viability of GES-1 cells treated with the caspase-1 inhibitor. Data are representative of five independent experiments and represent the mean $\pm$ SD.

Fig. 7. Caspase-1, IL-1 β and IL-18 were upregulated in human gastric cancer tissues. A-C. Immunohistochemical staining of caspase-1, IL-1 β and IL-18 in gastric cancer tissues and adjacent normal tissues (magnification, 20 $\times$). D. The mRNA expression levels of caspase-1, IL-1 β , and IL-18. E. Representative western blot bands. F-H. The protein expression levels of caspase-1, IL-1 β and IL-18. Data are representative of five independent experiments and represent the mean $\pm$ SD. * $P < 0.05$ vs. Normal group.

Pyroptosis is included in gastric cancer

Previous studies have shown that atrophic gastritis is a probable forerunner of gastric cancer [44]. Since pyroptosis plays an important role in gastritis induced by ethanol, we next detected the activation levels of caspase-1, IL-1 β and IL-18 in gastric cancer cells to determine if pyroptosis was involved in gastric cancer progression. We carried out an immunohistochemical assay to detect the caspase-1 expression levels using the gastric cancer tissues and adjacent normal tissues, and a significant increase in the caspase-1 and IL-1 β and IL-18 expression levels was observed in the gastric cancer tissues compared with the adjacent normal tissues (Fig. 7A-C). We then detected the transcription levels of caspase-1, IL-1 β and IL-18, and our results showed that the caspase-1, IL-1 β and IL-18 mRNA expression levels were upregulated in gastric cancer tissues compared to the adjacent normal tissues (Fig. 7D). Furthermore, the protein expression of caspase-1, IL-1 β and IL-18 were higher in the cancer tissues than in the non-tumor tissues (Fig. 7E-H). Taken together, these results suggest that pyroptosis plays a role in the gastric cancer progression.

Discussion

Chronic gastritis is a multistep, progressive and life-long inflammatory process [45-49], and the available treatments for this disease remain deficient. Increased levels of inflammation with the release of large amounts of cytokines are the main features of gastritis [50-53]. It has become clear that bacteria have caused gastritis in the overwhelming majority of the cases recorded since 1982 [54]. However, before that, studies have shown that alcohol causes chronic gastritis and the severity of the mucosal lesion is directly related to the duration of excess drinking [55, 56]. Here, we showed that ethanol could induce pyroptosis in GES-1 cells, and this was characterized by DNA damage, the activation of caspase-1 and the release of IL-1 β and IL-18. These changes were reduced by treatment with a caspase-1 inhibitor. Pyroptosis is a highly inflammatory form of programmed cell death, which is triggered by caspase-1 activation and associated with pro-inflammatory cytokine production. Accumulating evidence has revealed the important role of pyroptosis in various pathophysiological conditions, including microbial infection, nervous diseases and heart diseases [57-60].

Our study identified the involvement of pyroptosis in the pathogenesis of alcoholic gastritis. We established a model for alcoholic gastritis. Histopathological and molecular biological detection all suggested that ethanol could induce pyroptosis, as characterized by an exaggerated inflammatory response, caspase-1 activation and pro-inflammatory factor release. Remarkably, caspase-1 inhibitor could significantly block pyroptosis, alleviate the inflammatory responses and ameliorate the gastric injury induced by ethanol accumulation. Previous studies have shown that the caspase-1 inhibitor Ac-yvad-cmk protects against acute gastric injury in mice by affecting the NLRP3 inflammasome and attenuating inflammatory processes and apoptosis [34]. Ac-yvad-cmk is an efficient caspase-1 inhibitor and is involved in many other diseases [61–65]. This finding is consistent with our results. Importantly, there was no adverse effect of this inhibitor on the effective dosage *in vivo* and *in vitro*. Furthermore, we clarified that pyroptosis was also involved in the gastric cancer progression, which suggested that the caspase-1 inhibitor, Ac-yvad-cmk, may be a potential agent for the treatment of gastric-related diseases.

In summary, three important findings are presented in the current study. First, caspase-1-induced pyroptosis was found to be involved in the pathogenesis of alcoholic gastritis. Second, treatment with a caspase-1 inhibitor alleviated ethanol-induced gastric injury. Lastly, the caspase-1 inhibitor, Ac-yvad-cmk, might be an effective agent for the protection against alcoholic gastritis. These findings will improve our understanding of the role of pyroptosis in alcoholic gastritis and provide a new therapeutic approach for the management of alcoholic gastritis and other diseases associated with pyroptosis.

Conclusion

In summary, the present study provides convincing evidence that pyroptosis was involved in the pathogenesis of ethanol induced gastritis. The caspase-1 inhibitor could inhibit ethanol-induced gastritis.

Disclosure Statement

The authors declare to have no conflict of interests.

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