Original Paper

The Role of Interstitial Cells of Cajal in Acute Cholecystitis in Guinea Pig Gallbladder

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Key Words

Interstitial Cajal cells • Acute cholecystitis • Stem cell factor • C-kit

Abstract

Background/Aims: Acute cholecystitis is common in gallbladder motility disorder. Interstitial cells of Cajal (ICCs) in the gallbladder are involved in the regulation of gallbladder motility. The aim of this study was to explore the change of gallbladder ICCs in acute cholecystitis. **Methods:** Thirty adult guinea pigs were randomly divided into 3 groups: a sham-operated group (healthy controls) and 2 study groups. The animals in the study group were subjected to bile duct ligation and then to laparotomy and cholecystectomy at 24 and 48 hours after surgery. Immunohistochemistry, immunohistochemistry, and laser confocal microscopy were performed to observe the shape, size, morphology, and density of gallbladder ICCs. Western blot and real-time PCR were performed to detect stem cell factor and c-kit protein and mRNA expression, respectively. **Results:** There were no differences in the shape, size, and morphology of the gallbladder ICCs in the control and the two acute cholecystitis groups. Density of gallbladder ICCs, SCF level, and c-kit protein and mRNA expression all decreased in the acute cholecystitis groups. Further, SCF level and c-kit protein and mRNA expression decreased with progress of acute cholecystitis (all $P < 0.05$). **Conclusion:** Acute cholecystitis can decrease ICCs through repression of SCF and c-kit expression and that ICCs loss play a role in acute cholecystitis.

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Introduction

Acute cholecystitis is a frequent reason for a visit to the emergency department. It is caused by many factors, such as gallstones, motility disorders, and direct chemical injury [1, 2]. Most cases of acute cholecystitis occur with gallbladder motility disorder [2, 3].

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Gallbladder motility is regulated by various mechanisms, and interstitial cells of Cajal (ICCs) in the gallbladder are involved in generating and spreading the spontaneous contractions of the gallbladder, promoting gastrointestinal electrical activity, and mediating and regulating neurotransmitters of the gastrointestinal tract [4-9]. Gallbladder ICCs have also been linked to various biliary system diseases, such as cholelithiasis, and malignant tumors of the biliary system [10, 11]. However, it remains unclear whether decrease in the density of gallbladder ICCs would induce acute cholecystitis and whether abnormalities in gallbladder ICCs and the SCF/c-kit pathway can lead to acute cholecystitis. The present study was designed to explore the cellular and molecular mechanisms of gallbladder ICCs in acute cholecystitis in a guinea pig model. We used immunohistochemistry, immunohistochemistry, and laser confocal microscopy to observe the shape, size, morphology, and density of gallbladder ICCs, and western blot and real-time PCR (RT-PCR) were used to detect stem cell factor (SCF) and c-kit protein and mRNA expression, respectively.

Materials and Methods

Animals and Animal experiments

Thirty adult guinea pigs (males and females; weight, 250 - 350 g) were obtained from Wuhan Institute of Biological Products Company with Limited Liability. The guinea pigs were maintained in standard laboratory conditions (22 ± 2°C with a 12 h light/dark cycle and a relative humidity of 40-60%). The guinea pigs were given access to diet and water freely. All animal experiments were approved by the Institutional Animal Care and Use Committee of Wuhan University, and the protocol was designed to minimize pain and discomfort to the animals.

For experiments involving bile duct ligation (BDL), guinea pigs were randomly divided into 3 groups (10 animals per group). The sham-operated group constituted the healthy control group. The animals in the study groups were subjected to BDL. The animals in the BDL 24 h group and the BDL 48 h group were subjected to laparotomy and cholecystectomy at 24 and 48 h after surgery, respectively [12-15].

Hematoxylin-eosin staining was performed for 1 gallbladder specimen from each group for detecting the degree of acute cholecystitis using a light microscope (Olympus BX53, Tokyo, Japan).

Immunohistochemistry

Immunohistochemistry was performed on paraffin-embedded sections by using a microwave-based antigen retrieval technique. Gallbladder specimens fixed in 4% polyformaldehyde solution and embedded in paraffin. The paraffin-embedded sections were cut to 5 µm thickness and mounted on positively charged slides. First, the sections were identified by incubation with rat monoclonal antibody raised against CD117/c-kit (eBioscience, San Diego, USA) at room temperature for 24 hours, followed by incubation with appropriate secondary antibodies. Subsequently, the sections were stained with diaminobenzidine and counterstained with hematoxylin. The stained samples were observed under a light microscope (Olympus BX53, Tokyo, Japan). Images of CD117/c-kit-positive cells were taken for 5 randomly chosen fields (×400 magnification) per section by fluorescence microscope. The CD117/C-kit-positive cell density was assessed with the Image-Pro plus 6.0 software (Media Cybernetics, Bethesda, MD, United States).

Immunohistofluorescence with laser confocal microscopy observation

Gallbladder specimen was fixed in 4% polyformaldehyde solution and stored at 4°C for 30 min. Subsequently, the gallbladder specimen was washed with phosphate-buffered saline (PBS) for 10 min 3 times. After fixing, the gallbladder specimen was cut. Fixed samples were dipped in PBS with 0.3% Triton X-100 followed by blocking with normal goat serum for 30 min at room temperature. Next, the samples were incubated with primary rat monoclonal anti-c-kit antibody (eBioscience, San Diego, USA) at 4°C overnight. Then, gallbladder tissues were washed for 10 min 3 times with tris-buffered saline and tween-20 solution (TBST) and were incubated with a CY3-conjugated rabbit anti-rat secondary antibody in PBS for 1 h at room temperature. After removing the unbound secondary antibody by washing with TBST for 5 min 3 times, they were mounted on a slide with a coverslip sealed with glycerol. Images were captured by laser confocal microscope (Olympus FV1000, Tokyo, Japan).

Protein extraction and Western blot analysis

Gallbladder muscular layer tissues from 1 guinea pig (weight 150 mg) were used for protein extraction with RIPA lysis buffer. Electrophoresis in 10% SDS polyacrylamide gels was performed and the bands were transferred to a nitrocellulose membrane (Pierce Biotechnology, Inc., Rockford, USA). Then, the membrane was incubated with 5% skim milk at room temperature to block nonspecific binding sites for 2 h. The samples were incubated with primary antibody against c-kit (eBioscience, San Diego, USA) or SCF (Abcam, Cambridge, UK) overnight at 4°C. After washing with TBST for 10 min times, the secondary antibody of matching conjugated horse radish peroxidase was applied for 1 h at room temperature. Specific protein bands were visualized in X-ray film (Kadok China Investment Co. Ltd., Xiamen, China) by using the chemiluminescence detection kit (ECL; Amersham, Pittsburgh, USA). Optical density of the bands was analyzed with Alpha Innotech (Alpha Innotech Co., California, USA).

RNA Extraction

For RNA extraction, 150 mg gallbladder muscular layer from 1 guinea pig was used. Total RNA was extracted with the TRIzol reagent (Invitrogen) following the manufacturer's instructions.

RT-PCR Analysis

cdNA was reverse-transcribed from 4.885 µg of total RNA and amplified by 40 cycles of denaturation (2 min at 50°C, 10 min at 95°C), annealing (30 sec at 95°C) and synthesis (30 sec at 60°C). The primers for GAPDH were 5'-ATC ACT GCC ACC CAG AAG ACT-3' (forward) and 5'-CAG ATC CAC AAC CGA CAC ATTA-3' (reverse). The generated amplicon was 195 bp. The primers for SCF were 5'-GAA AGA TTC CAG AGT CAG TGTCA-3' (forward) and 5'-AAG CAA AGC CAA TCA CAA GAG-3' (reverse), and generated an amplified product of 179 bp. The primers for c-kit were 5'-TAT CCT CCT TAC TCA TGG TCGAA-3' (forward) and 5'-CGG GCA TTT CCT TTA ACC ACATA-3' (reverse), and generated amplicons of 99 bp. Subsequently, the PCR product was separated by electrophoresis with size markers on a 1.5% agarose gel stained with ethidium bromide. The GAPDH gene was used as an internal control. The mRNA values were expressed as relative units, and the ratio of density between the SCF/c-kit amplification product and the GAPDH amplification product was calculated.

Statistical Analysis

All statistical analyses were performed using SPSS for Windows version 17.0 (SPSS, Chicago, USA). Continuous variables were presented as mean ± standard deviation (SD). Continuous variables were compared using the *t* test. Categorical variables were compared using ANOVA. A two-sided *P* value < 0.05 was regarded as statistically significant.

Results

Evaluation of animal model

No guinea pigs from the sham-operated group and the BDL 24 h group died, whereas 3 guinea pigs died in the BDL 48 h group (mortality rate, 30%) due to cholecystic duct obstruction and gallbladder rupture.

In histopathological analysis, gallbladder tissue from the sham-operated group was found to be intact and without obvious inflammatory cell infiltration. In contrast, gallbladder tissue from the BDL 24 hour group showed edema, blood-expanded hyperemia, and inflammatory cell infiltration. Gallbladder tissue from the BDL 48 h group, showed edema and hyperplasia of fibroblasts, blood-expanded hyperemia and hemorrhage, and a large number of inflammatory cell infiltration (mainly neutrophils) in the mucosa and muscularis (Fig.1).

These findings indicate that BDL may induce acute cholecystitis in guinea pigs.

Immunohistochemical analysis

Gallbladder ICCs were predominantly fusiform in shape with several slender lateral branches in all groups. ICCs were predominantly located in the muscular layer, mostly within the muscularis propria, and were primarily located parallel to smooth muscle cells. The ICCs

Fig. 1. Histopathological analysis on animal model (200X). (A) Gallbladder tissue from the sham-operated group was found to be intact and without obvious inflammatory cell infiltration. (B) Gallbladder tissue from the BDL 24 hour group showed edema, blood-expanded hyperemia, and inflammatory cell infiltration. (C) Gallbladder tissue from the BDL 48 h group, showed edema and hyperplasia of fibroblasts, blood-expanded hyperemia and hemorrhage, and a large number of inflammatory cell infiltration (mainly neutrophils) in the mucosa and muscularis.

Fig. 2. Immunohistochemical observation (400X). Gallbladder tissues were from the sham-operated group (A), the BDL 24 hour group (B) and the BDL 48 h group (C). Gallbladder ICCs (green arrows) were predominantly fusiform in shape with several slender lateral branches in all groups. ICCs were predominantly located in the muscular layer, mostly within the muscularis propria, and were primarily located parallel to smooth muscle cells. The ICCs typically appeared singly or in small clusters of two to three cells. Besides, mast cells (red arrows) were also immunolabeled positively for Kit, and were observed in the mucosa of the gallbladders.

typically appeared singly or in small clusters of two to three cells. Besides, mast cells were also immunolabeled positively for Kit, and were observed in the mucosa of the gallbladders (Fig. 2).

Fig. 3. Density of ICCs in the sham-operated group and the BDL groups. With immunohistochemical analysis, images of CD117/c-kit-positive cells were taken for 5 randomly chosen fields ($\times 400$ magnification) per section by fluorescence microscope. Density of ICCs was decreased in the BDL groups, density of ICCs in the BDL 48h group was lower than the BDL 24h group (42.0000 ± 2.00000 vs. 26.3333 ± 1.52753 vs. 20.0000 ± 1.00000 , $F = 157.409$, $P < 0.05$).

Fig. 4. Immunohistofluorescence with laser confocal microscopy observation (630X). Gallbladder tissues came from the sham-operated group (A), the BDL 24 hour group (B) and the BDL 48 h group (C). Gallbladder ICCs (white arrows) were predominantly fusiform in shape with several slender lateral branches in all groups. ICCs were predominantly located in the muscular layer, mostly within the muscularis propria, and were primarily located parallel to smooth muscle cells. The ICCs typically appeared singly or in small clusters of two to three cells. Mast cells (green arrows) were observed in the mucosa of the gallbladders. However, there were no differences in the shape, size, and morphology of the ICCs in all groups.

The number of ICCs in the sham-operated group was significantly higher than that in the two BDL groups. However, there were no differences in the shape, size, and morphology of the ICCs in all groups. ICC density in the BDL groups was significantly lower than that in the sham-operated group. Furthermore, the density of ICCs in the BDL 48 h group was lower than that in the BDL 24 h group (42.0000 ± 2.00000 vs. 26.3333 ± 1.52753 vs. 20.0000 ± 1.00000 , $F = 157.409$, $P < 0.05$). In acute cholecystitis guinea pigs, gallbladder ICCs density was decreased (Fig. 3).

Fig. 5. SCF and c-kit protein expression in different groups. SCF and c-kit protein expression in BDL groups were decreased. SCF (A) and c-kit (B) protein expression levels were evaluated by western bolt analysis. Lane 1 was represented the sham-operated group, lane 2 came from the BDL 24h group animals, lane 3 was from the BDL 48h group guinea pigs. β -actin mean loading control. (C) The optical mean density of SCF and c-kit protein in all groups, each vertical line stood by the mean $\pm$ standard deviation. The ratio

of SCF and β -actin in the sham-operated, BDL 24 h, and BDL 48 h groups was 0.5533 ± 0.07234 , 0.4100 ± 0.13000 , and 0.1267 ± 0.05859 ($F = 16.596$, $P < 0.05$), respectively. The ratio of c-kit and β -actin was 0.2233 ± 0.08083 , 0.1767 ± 0.05774 , 0.0367 ± 0.02887 (F -values, respectively, were 16.596 and 7.938, all $P < 0.05$).

Immunohistofluorescence analysis

In fluorescence and laser confocal microscopic examination, gallbladder ICCs mainly appeared in the muscularis and were in the astrocyte or fusiform shapes. ICCs that stained positive for c-kit had large, orbicular, or ovate nuclei; reduced cytoplasm; and scattered chromatin; each ICC also had 2-5 long synapses. ICCs were distributed throughout the wall of the gallbladder and were connected with each other to form a net-like structure.

Immunohistofluorescence analysis using laser confocal microscopy supported the findings of immunohistochemistry (Fig. 4).

SCF level and c-kit protein expression in the gallbladder of guinea pigs with acute cholecystitis

In western blot analysis, the protein levels of both c-kit and SCF were significantly lower in the BDL groups than in the sham-operated group. SCF and c-kit protein levels were lower in the BDL 48 h group than in the BDL 24 h group. The ratio of SCF and β -actin in the sham-operated, BDL 24 h, and BDL 48 h groups was 0.5533 ± 0.07234 , 0.4100 ± 0.13000 , and 0.1267 ± 0.05859 . The ratio of c-kit and β -actin was 0.2233 ± 0.08083 , 0.1767 ± 0.05774 , 0.0367 ± 0.02887 (F -values, respectively, were 16.596 and 7.938, all $P < 0.05$).

SCF and c-kit protein expression was decreased in the two BDL groups, and gallbladder ICCs were loss in acute cholecystitis (Fig. 5).

mRNA expression of SCF and c-kit in the gallbladder of guinea pigs with acute cholecystitis

RT-PCR analysis revealed that the level of SCF and c-kit mRNA decreased in the BDL groups. SCF and c-kit mRNA level was much lower in the BDL 48 h group than in the BDL

Fig. 6. mRNA expression of SCF and c-kit in different groups. The expression level of SCF and c-kit mRNA in the BDL groups were decreased. GAPDH (A), SCF (B) and c-kit (C) mRNA expression levels were detected by RT-PCR. Lane 1 was marker; lane 2 came from the sham-operated group guinea pigs, lane 3 was from the BDL 24h group animals, lane 4 was represented from the BDL 48h group guinea pigs. (D) The mean optical density of SCF and c-kit mRNA/GAPDH in all groups, each vertical line stood by the mean $\pm$ standard deviation. The sham-operated group, the BDL 24 h and BDL 48 h groups, the SCF and c-kit mRNA levels were 1.0000 ± 0.00000 vs. 0.4400 ± 0.09539 vs. 0.2000 ± 0.08544 and 1.0000 ± 0.0000 vs. 0.5967 ± 0.05132 vs. 0.2833 ± 0.08622 , in respectively (F -values respectively were 92.488 and 115.401, all $P < 0.05$).

24 h group. Compared with the sham-operated group, the SCF and c-kit mRNA levels in the BDL 24 h and BDL 48 h groups, respectively, were 1.0000 ± 0.00000 vs. 0.4400 ± 0.09539 vs. 0.2000 ± 0.08544 and 1.0000 ± 0.0000 vs. 0.5967 ± 0.05132 vs. 0.2833 ± 0.08622 (F -values respectively were 92.488 and 115.401, all $P < 0.05$).

The RT-PCR results supported the findings of immunohistofluorescence and western blot. Reduced expression of SCF and c-kit mRNA might affect the gallbladder ICCs function in acute cholecystitis (Fig. 6).

Discussion

Acute cholecystitis is an acute inflammatory disease of the gallbladder, influenced by many factors such as gallstones, motility disorders, and direct chemical injury [2]. It is a common diagnosis in both internal and surgery departments, and surgery is usually indicated for treatment. Furthermore, the heterogeneity of clinical presentation makes diagnosis and management difficult [1]. Moreover, most acute cholecystitis is accompanied by gallbladder motility disorder [2, 3].

Gallbladder motility is regulated by various mechanisms, such as the activity of the gallbladder smooth muscle and nervous circuit, which includes gallbladder ICCs. ICCs

were first described in the gastrointestinal tract by Santiago Ramo'n y Cajal in 1893 [16, 17]. More recently, studies have found out ICCs are also distributed in the gallbladder and extrahepaticbiliary duct of both guinea-pigs and human beings [18-20]. ICCs in the biliary system may function as pacemakers for smooth muscle to generate and spread the spontaneous contractions of the gallbladder, promoting gastrointestinal electrical activity and mediating and regulating the neurotransmitters in the gastrointestinal tract [4-9]. Further, gallbladder ICCs have been linked to various biliary system diseases such as acute cholecystitis and cholelithiasis [10]. ICC damage and/or loss has been observed in almost every gastrointestinal motility disorder, and abnormalities in motor activity would impair regional transit and symptoms [21].

ICCs express a specific marker, c-kit [22]. In this study, the gallbladder ICCs were positive for c-kit in immunofluorescent staining and were distributed throughout the muscular layer of the gallbladder. Further, most were found within the muscularis propria and parallel to smooth muscle cells. They typically appeared singly or in small clusters of two to three cells, and were connected with each other to form a network, consistent with previous reports [10]. Recent studies have confirmed that ICCs in biliary system play an important role in initiating pacemaker activity to adjust gallbladder movement, and their dysfunction is closely linked to a variety of gallbladder motility disorders [22-24].

Pervious studies have also shown that ICCs would undergo apoptosis over time and that regeneration was required for maintenance of normal ICC networks in healthy tissue [25]. Various factors have been implicated in ICC loss, such as regional obstruction and subsequent proximal dilation and injury to the nervous system and immune system [21]. Studies on animal models have found that ICC viability and function are compromised in bowel obstruction and that ICCs nearly disappear when Kit receptors are blocked [21, 26, 27]. During acute cholecystitis, biliary tract obstruction would injure ICCs in the gallbladder and block Kit receptors, leading to decreased in the density of gallbladder ICCs, thereby impairing the regeneration and maintenance of ICC networks. Reduced density of gallbladder ICCs might have a substantial effect on acute cholecystitis with gallbladder motility disorder. However, further studies are needed to determine the mechanism of gallbladder ICC loss during acute cholecystitis.

ICC not only act to initiate pacemaker activity for modulating gallbladder motility, but also mediate neurotransmission from enteric motor neurons [28]. Recent studies have demonstrated that LPA1/3 receptor, opioid receptor, the tyrosine kinase receptor c-kit and its ligand-SCF play an important part in the normal development, maturation, and phenotype maintenance of ICCs [29-32]. Studies have also shown that the kit receptor modulates the generation and rhythmicity of the electrical activity that regulates the excitability of gallbladder smooth muscle [23]. However, during acute cholecystitis, biliary tract obstruction would injure ICCs in the gallbladder and block the tyrosine kinase receptor c-kit and its ligand-SCF, the expression both of c-kit and SCF mRNA and protein were significantly decreased, the SCF/c-kit pathway was repressed, and ICC numbers were reduced [33]. The mechanism through which the SCF/c-kit pathway is repressed remains unknown. In this study, we observed that SCF/c-kit protein and mRNA were expressed in the gallbladder of guinea pigs with acute cholecystitis. This suggests that acute cholecystitis affecting the development of ICCs. Repression of the SCF/c-kit pathway might affect gallbladder ICCs function in acute cholecystitis.

In summary, our findings indicate that acute cholecystitis can decrease ICCs through repression of SCF and c-kit expression and that ICCs loss play a role in acute cholecystitis, and it may have relationships with gallbladder motility disorder.

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Disclosure Statement

No conflict of interest.

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