Pharmacological Characterization of a Novel Mouse Model of Cholestatic Pruritus

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Patients with cholestatic liver diseases, such as primary biliary cirrhosis, usually suffer from pruritus. However, the pathogenesis of cholestatic pruritus is unclear, and there is no current effective treatment for it. In order to find a treatment for the condition, an appropriate mouse model should be developed. Therefore, here, we established a surgically-induced mouse model of cholestatic pruritus. The bile duct was ligated in order to block bile secretion from the anterior, right, and left lobes, with the exception of the caudate lobe. Serum levels of total bile acid increased after bile duct ligation (BDL). The spontaneous hind paw scratching was also increased in BDL mice. Spontaneous scratching was reduced in BDL mice by naloxone (μ-opioid receptor antagonist), U-50,488H (κ-opioid receptor agonist), and clonidine (α2-adrenoceptor agonist). Azelastine (H1 receptor antagonist with membrane-stabilizing activity) slightly reduced scratching. However, terfenadine (H1 receptor antagonist), methysergide (serotonin (5-HT2 receptor antagonist), ondansetron (5-HT1 receptor antagonist), proteinase-activated receptor 2-neutralizing antibody, fluvoxamine (selective serotonin reuptake inhibitor), milnaapran (serotonin-noradrenaline reuptake inhibitor), and cypromeptadine (H1 and 5-HT1 receptor antagonist) did not affect scratching. These results suggested that partial obstruction of bile secretion in mice induced anti-histamine-resistant itching and that central opioid system is involved in cholestatic itching.

Key words cholestasis; itching; opioid; bile duct ligation; scratching

INTRODUCTION

The progression of cholestasis, which affects bile flow from the liver to the duodenum, causes liver failure, such as in primary biliary cholangitis (PBC). Pruritus is one of the primary symptoms in PBC, and it occurs in about 70% of the patients.1,2 Pruritus is an intrusive and distressing condition that leads to impaired QOL in patients with PBC.3 Therefore, the control of pruritus is important for the patients.

Therapeutic recommendations for the management of pruritus in the guidelines of PBC were updated by American Association for the Study of Liver Diseases and European Association for the Study of the Liver (EASL).3 Both associations recommend the administration of bile acid sequestrants as the preferred initial therapy to treat pruritus, followed by ursooxycholic acid, rifampicin, opioid antagonists, and selective serotonin reuptake inhibitors (SSRIs). However, since these treatments are not based on the mechanism of the pruritus, the outcome is generally poor.

The events during the development of cholestatic pruritus consisted of accumulation of bile acid in the skin of patients,4 increase in serum opioid peptide levels (Met-enkephalin) related to the release of histamine from mast cells,5 decrease in central μ-opioid receptors,6 activation of autotxin, increase in serum lysophosphatidic acid (LPA) levels,7 and activation of TGR5 receptor by bile salts.8 However, the detailed mechanism of cholestatic pruritus still remains unclear.

To investigate the mechanism of pruritus in the presence of cholestasis and thus to evaluate the effectiveness of therapeutic drugs, animal models are needed. Several rodent models of cholestasis have been established by drug treatment,9,10 surgery,11,12 and genetic manipulation.13 Among them, mice with surgically-induced cholestasis more closely mimicked the human disease.9,10,13 Recently, a mouse model of cholestatic pruritus induced by common bile duct ligation (BDL) was reported,11 although its life time was short.11,12 Since the severity of cholestatic pruritus is associated with chronic progression of cholestasis, long-term survival of the mouse model is required in order to develop the most effective treatment. Therefore, in this study, we developed a new cholestatic pruritus mouse model induced by BDL and evaluated its treatment with several anti-pruritic drugs.

MATERIALS AND METHODS

Animals Male ICR mice (Japan SLC, Ltd., Shizuoka, Japan) were used. The mice were 5 weeks old at giving surgery. They were kept under controlled temperature (23 ± 1°C), humidity (60 ± 5%), and lighting (light on 8:00–20:00 h). Food and water were freely available. Experimental procedures in the animals were approved by the Committee for Animal Experiments of the University of Toyama and was conducted in accordance with the ethical guidelines of the Japanese Pharmacological Society.

Materials Naloxone hydrochloride (Sigma-Aldrich, St. Louis, MO, U.S.A.), fluvoxamine maleate (Sigma-Aldrich),
and clonidine hydrochloride (Sigma-Aldrich) were dissolved in physiological saline solution. However, the first one was subcutaneously injected into mice 15 min before observation; meanwhile, the latter two drugs were intraperitoneally injected 30 min before observation. On the other hand, U-50,488H (Research Biochemicals Inc. [RBI], Natick, MA, U.S.A.), azelastine hydrochloride (Sigma-Aldrich), methysergide maleate (Sigma-Aldrich), ondansetron hydrochloride (Sigma-Aldrich), milnacipran hydrochloride (Sigma-Aldrich), and cyproheptadine hydrochloride (Sigma-Aldrich) were dissolved in tap water and orally administered 60, 30, 60, 60, and 60 min before observation, respectively. Additionally, terfenadine (Sigma-Aldrich) was dissolved in 0.5% sodium carboxymethyl cellulose (Wako Pure Chemical Corporation, Osaka, Japan) and orally administered 30 min before observation. Proteinase-activated receptor 2 (PAR2) neutralizing antibody (SAM-11, which has the epitope of the tethered ligand sequence (amino acids 37–50) of human PAR2 and binds to human and murine PAR2; Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.) or nonspecific-immunoglobulin G (IgG) was intravenously injected 2 h before observation.

BDL Five-weeks-old mice were anesthetized by intraperitoneal administration of sodium pentobarbital (80 mg/kg, Sigma-Aldrich). After midline laparotomy (2 cm), the common bile duct was ligated with 4-0 silk sutures between the right and caudate lobes (Fig. 1A). Sham operation was performed by gently touching the bile duct. The abdomen was closed in layers.

Blood Testing Six weeks after BDL, mice were anesthetized by intraperitoneal administration of sodium pentobarbital (80 mg/kg, Sigma-Aldrich). Blood was collected from the heart, and after centrifugation, the serum was isolated. The levels of the following compounds were analyzed by FUJIFILM Monolith Co., Ltd. (Tokyo, Japan): total bile acid, total bilirubin, alkaline phosphatase, total protein, albumin, glutamic oxaloacetic transaminase, glutamic pyruvic transaminase, urea nitrogen, total cholesterol, triglyceride, glucose.

Hematoxylin and Eosin Staining Six weeks after BDL, mice were anesthetized by intraperitoneal administration of sodium pentobarbital (80 mg/kg, Sigma-Aldrich), and transcardially perfused with phosphate-buffered saline and then with 4% paraformaldehyde. Liver samples were embedded in paraffin. The paraffin blocks were cut with a microtome and the sections were placed on standard slides. After deparaffinization, these sections were stained with eosin followed by hematoxylin (Wako Pure Chemical Corporation). After rehydrating the slides with water, they were sequentially immersed in 60, 80, 90, and 95% ethanol for 1 min each, and then in 100% ethanol and xylene for 10 min each. The preparations were mounted in Canada balsam and observed using a light microscope (BX-61, Olympus, Tokyo, Japan).

Behavioral Observation During acclimation, mice were individually placed in an acrylic cage composed of four cells (13 × 9 × 35 cm) for at least 1 h. Later, their behaviors were recorded using a digital video camera (HDC-TM25, Panasonic, Osaka, Japan) for 1 h. Scratching toward any region of the body by the hind paw was observed during video playback. The mice stretched either the left or the right hind paw, leaned their head towards the paws, rapidly scratched themselves, and brought their toenails to their mouth. A series of these movements was counted as one bout of scratching. Grouping of BDL mice was performed in a way that the average of the number of scratching events was the same in order to evaluate the effects of several drugs. To determine the effects of several drugs or antibodies on spontaneous scratching in BDL mice, the ratio of the number of scratch bouts following agent administration to that at the grouping was calculated.

Statistical Analysis Statistical analysis was performed using the SigmaPlot Software version 12 (Systat Software, Ltd., Chicago, IL, U.S.A.). All data are presented as the mean ± standard error of the mean (S.E.M.). Statistical significance was evaluated by the Student’s t-test or two-way repeated measures ANOVA, followed by a post-hoc Holm–Šidák test. A p-value <0.05 was considered statistically significant.

RESULTS BDL and Survival Rate BDL procedure elicited jaundice in all treated mice as similarity observed in human patients. The survival rate of mice decreased for 2 weeks following BDL treatment (Fig. 1B), but after that, it was maintained at 67% for at least 9 weeks (Fig. 1B).

The Serum Concentration of Indicators, and the Pathological Condition of the BDL Mouse Liver The serum concentrations of total bile acid, alkaline phosphatase, total protein, glutamic oxaloacetic transaminase, and glutamic pyruvic transaminase were significantly increased 1 week after BDL, when compared to those of sham-operated mice. After 6 weeks of BDL, only the serum concentration of total bile acid was still higher than that of sham-operated mice (Table 1). On

![Fig. 1. Bile Duct Ligation (BDL) and Survival Rate in Mice](Image)
the other hand, the serum concentration of glucose in BDL mice significantly decreased 1 week, but not 6 weeks, after BDL, compared with that in sham-operated mice (Table 1).

Fibrosis and the neogenesis of small bile ducts were observed in the right lobes but not in the caudate lobes 6 weeks after ligation, compared with sham-operated mice without the obstruction of bile secretion (Fig. 2).

Spontaneous Scratching in BDL Mice Compared with sham-operated mice, the number of spontaneous scratching events by hind paw was significantly increased in BDL mice from week 5 after BDL procedure (Fig. 3) and was maintained at the same level at least up to week 9 (Fig. 3).

Effects of Various Anti-pruritic Agents on Spontaneous Scratching in BDL Mice The μ-opioid receptor antagonist naloxone (1 mg/kg, Fig. 4A), α-opioid agonist U-50,448H (3 mg/kg, Fig. 4B), and α2 adrenalin receptor agonist clonidine (10 µg/kg) (Fig. 4J) significantly inhibited spontaneous scratching in BDL mice compared with that in vehicle-treated BDL mice. On the other hand, administration of H1 histamine receptor antagonist terfenadine (30 mg/kg) or H2 histamine receptor and 5-HT2 serotonin receptor antagonist cyproheptadine (1 mg/kg) did not inhibit spontaneous scratching in BDL mice (Figs. 4C, K). Azelastine (10 mg/kg) inhibited the frequency of spontaneous scratching in BDL mice compared with that in vehicle-treated mice (p = 0.065) (Fig. 4D). Methysergide, 5-HT2 receptor antagonist (1 mg/kg, Fig. 4E), 5-HT1 receptor antagonist ondansetron (1 mg/kg, Fig. 4F), PAR2-neutralizing antibody (0.3 mg/kg, Fig. 4G), SSRI fluvoxamine (10 mg/kg, Fig. 4H) and serotonin-noradrenalin reuptake inhibitor milnacipran (30 mg/kg, Fig. 4I) did not affect spontaneous scratching in BDL mice.

DISCUSSION

In this study, BDL mice, which maintained intact bile secretion from the caudate lobe, showed long-term survival (> 9 weeks after the surgery) and experienced spontaneous scratching. In our preliminary experiments, mice with the common BDL died within 1 week after the surgery (data not shown). In another study, blockade of the bile secretion from the left lobe after performing BDL experienced showed long-term survival, but without inducing spontaneous scratching. The mouse model established in this study showed several symptoms similar to those experienced in patients, such as jaundice, fibrosis, neogenesis of small bile ducts in the cholestatic liver, and increase in the serum concentrations of total bile acid, alkaline phosphatase, total protein, glutamic oxaloacetic transaminases, and glutamic pyruvic transaminase. Taken together, we considered that this mouse model was a more valid model of cholestatic pruritus.

In BDL mice, spontaneous scratching was inhibited by administration of μ-opioid receptor antagonist, which also reduced itching and scratching in other animal models, and in patients with several pruritic diseases (e.g., chronic renal failure, chronic urticaria, cholestasis, and atopic dermatitis). In addition, μ-opioid receptor antagonists suppressed itch-, but not pain-, related behaviors through the receptors in the central nervous system, especially in the lower brain-
stem.\textsuperscript{28} Taken together, it was suggested that the spontaneous scratching in BDL mice was an itch-related response. Furthermore, an intracisternal injection of endogenous endomorphin,\textsuperscript{29} morphine,\textsuperscript{30} and $\mu$-opioid receptor agonist [D-Ala(2), N-Me-Phe(4), Gly(5)-ol] enkephalin\textsuperscript{30} elicit the scratching behavior. However, $\delta$-opioid receptor agonist [D-Pen(2,5)] enkephalin or $\kappa$-opioid receptor agonist U-50,488H does not.\textsuperscript{30} Therefore, it was suggested that endogenous $\mu$-opioid receptor ligands were involved in the scratching behavior and onset of cholestatic pruritus in BDL mice.

In this study, U-50,488H inhibited the scratching behavior in BDL mice. Sumi et al.\textsuperscript{31} also reported that nalfurafine, a $\kappa$-opioid receptor agonist, was effective for the treatment of cholestatic pruritus. In addition, nalfurafine was also effective

Table 1. Several Factors in Serum of Mice with Bile Duct Ligation (BDL) and Sham Operation

| Serum level | Sham 1 week later ($n=6$) | BDL 1 week later ($n=11$) | Sham 6 week later ($n=11$) | BDL 6 week later ($n=11$) |
|-------------|--------------------------|---------------------------|---------------------------|---------------------------|
| Total bile acid (TBA) mmol/L | 1.4 ± 0.1 | 35.5 ± 10.0* | 1.1 ± 0.1 | 2.6 ± 0.7* |
| Total bilirubin (T-BIL) mg/dL | 0.1 ± 0.0 | 0.2 ± 0.0 | 0.1 ± 0.0 | 0.1 ± 0.0 |
| Alkaline phosphatase (ALP) IU/L | 510.8 ± 33.8 | 1921.0 ± 461.8* | 234.1 ± 25.9 | 257.8 ± 37.3 |
| Total protein (TP) g/dL | 4.7 ± 0.1 | 5.0 ± 0.1* | 4.7 ± 0.1 | 4.6 ± 0.1 |
| Alubmin (ALB) g/dL | 2.1 ± 0.0 | 2.1 ± 0.0 | 2.0 ± 0.0 | 1.9 ± 0.0 |
| Glutamic oxaloacetic transaminas (GOT) IU/L | 73.8 ± 2.5 | 393.0 ± 114.0* | 122.2 ± 15.9 | 122.4 ± 17.0 |
| Glutamic pyruvic transaminase (GPT) IU/L | 27.7 ± 1.8 | 351.8 ± 64.6* | 61.8 ± 10.0 | 59.1 ± 11.6 |
| Urea nitrogen (BUN) mg/dL | 25.7 ± 1.2 | 22.5 ± 0.4 | 28.5 ± 1.2 | 30.0 ± 1.7 |
| Total cholesterol (TCHO) mg/dL | 119.8 ± 1.4 | 120.3 ± 4.2 | 101.4 ± 5.5 | 106.0 ± 5.6 |
| Triglyceride (TG) mg/dL | 56.7 ± 7.5 | 59.2 ± 6.8 | 50.4 ± 7.5 | 42.7 ± 4.5 |
| Glucose (GLU) mg/dL | 218.2 ± 9.2 | 159.8 ± 9.9* | 112.2 ± 5.2 | 115.3 ± 6.5 |

* $p < 0.05$ vs. sham.

Fig. 4. Effects of Various Anti-pruritic Agents on Spontaneous Scratching in Bile Duct Ligated (BDL) Mice

Varied drugs with their corresponding vehicle controls were administered through several routes, and at different times prior observing mouse scratching behavior. (A) Naloxone (NAL, 1 mg/kg) (subcutaneously, 15 min). (B) U-50448H (U-50488, 3 mg/kg) (orally, 60 min). (C) Terfenadine (TEF, 30 mg/kg) (orally, 60 min). (D) Azelastine (AZL, 10 mg/kg) (orally, 60 min). (E) Methysergide (MSG, 1 mg/kg) (orally, 60 min). (F) Ondansetron (ODS, 1 mg/kg) (orally, 60 min). (G) Proteinase-activated receptor 2-neutralizing antibody (SAM-11, PAR2ab, 0.3 mg/kg) (intravenously, 2 h). (H) Fluvoxamine (FXM, 10 mg/kg) (intraperitoneally, 30 min). (I) Milnacipran (MNP, 30 mg/kg) (orally, 60 min). (J) Clonidine (CLN, 10 $\mu$g/kg) (intraperitoneally, 30 min). (K) Cyproheptadine (CPH, 1 mg/kg) (orally, 60 min). Mice were subjected to drug evaluation from 6 to 9 weeks after BDL. Vehicles used were: VH1 (saline), VH2 (tap water), VH3 (0.5% carboxymethyl cellulose), nonspecific-IgG (nIgG, 0.3 mg/kg). Data are presented as the mean ± S.E.M. (A–C, n = 5; D–K, n = 6). * $p < 0.05$ vs. VH- or nIgG treated mice (Student’s t-test).
for treating uremic pruritus in dialysis patients and atopic-like dermatitis mice. It was demonstrated that dynorphin released by the activation of spinal inhibitory interneurons (B5-I neurons) regulated the neuronal transmission of itch signal through κ-opioid receptor. However, a recent study showed that topical nalfurafine attenuated scratching in an atopic-like dermatitis mouse model. This finding suggested that κ-opioid receptor agonist may mediate pruritus through the central and peripheral systems.

An old study first suggested that the mechanism of cholestatic pruritus involved the deposition of bile acids in the skin of patients. Bile acid was shown to elicit a scratching reaction in mice, although it has not been proved that it was itch-related. In our study, levels of serum bile acid increased within 1 week after the surgery in mice. However, the spontaneous scratching was not detected at least 4 weeks after the surgery. Therefore, we concluded that although bile acids elicited scratching in mice, they may not have the main role in inducing directly spontaneous scratching in this BDL mouse model. However, we do not deny the involvement of bile acids on the development of spontaneous scratching. Bile acids may influence plastic changes tissue and nervous system on the development of itching. It is necessary to conduct further analysis using this animal model in the future.

It is well known that histamine is a major pruritogen, which could be also involved in the onset of cholestatic pruritus. Even though anti-histamines are prescribed for cholestatic pruritus, it is unclear whether the administration of antihistamine decreases the sensation of itch itself from its sedative effect. Interestingly, it has also been reported that a non-sedative anti-histamine did not treat cholestatic pruritus.

In this study, terfenadine and cyproheptadine did not inhibit spontaneous scratching in BDL mice. Therefore, the role of histamine on scratching in BDL mice may be small. On the contrary, azelastine (anti-allergic agent with H₂ receptor antagonistic activity) attenuated the scratching. Azelastine has not only H₂ receptor antagonistic activity but may also have other actions, such as the ability to block the BLT1 leukotriene B₄ receptor and inhibit leukotriene B₄ production.

Serotonin is an itch mediator in humans and mice. In our study, cyproheptadine and methysgeride did not affect the scratching behavior in BDL mice. On the other hand, ondansetron slightly suppressed the scratching, compared with vehicle-treated control. It has been reported that ondansetron is effective for the treatment of cholestatic pruritus in patients. However, recent more rigorous trials have shown that the anti-pruritic effect of ondansetron is no better than that of the placebo. Therefore, serotonin may not have the main role in the development of cholestatic pruritus either.

PAR2 is activated by serine protease, such as tryptase and kallikrein and is involved in several pruritus conditions, such as atopic dermatitis. In this study, PAR2-neutralizing antibody did not inhibit the scratching behavior in BDL mice. Furthermore, tryptase, a PAR2-activating factor, is released from mast cells with histamine. In this study, H₂ receptor antagonist did not affect the scratching in BDL mice. Taken together, neither PAR2 nor mast cells may be involved in the onset of this condition in BDL mice.

Kremer suggested that LPA produced by autotaxin are key mediators of cholestatic pruritus. However, these antagonists and inhibitors have not been evaluated in clinical studies. In this study, we did not investigate the role of LPA in BDL mice. Therefore, in the future, the role of LPA needs to be investigated.

SSRI fluvoxamine suppresses chronic pruritus in several skin diseases, such as atopic dermatitis, systemic lymphoma, and solid carcinoma. Mayo et al. reported that SSRI sertraline relieved cholestatic pruritus in patients. Fluvoxamine and sertraline are SSRI with sigma-1 receptor agonist and antagonist, respectively. Sigma-1 receptor agonist attenuates pain sensation induced by capsaicin, which is a transient receptor potential vanilloid 1 (TRPV1) agonist. TRPV1 also is involved in itching; therefore, sigma-1 receptor agonist, such as fluvoxamine, may regulate itching. However, fluvoxamine did not attenuate the spontaneous scratching in BDL mice. As a future study, we are planning to investigate the anti-pruritic effect of sertraline in BDL mice. Since the relationship between sigma-1 receptors and pruritus remains unclear, further studies should be performed. In this investigation, milnacipran slightly suppressed spontaneous scratching in BDL (p = 0.078). In addition, clonidine, σ₂ receptor agonist, significantly inhibited the scratching in BDL mice, compared with that in sham-operated mice. Recently we showed that milnacipran and clonidine inhibited itch-related responses at the level of the spinal cord. Taken together, our results suggested that the increase in spinal noradrenaline, but not serotonin, may be involved in regulating the scratching in BDL mice.

CONCLUSION

This mouse model of BDL, which maintained bile secretion from the caudate lobe, was useful for studying cholestatic pruritus. Although the detailed mechanism of pruritus will be investigated in a future study using this mouse model, our results suggested that the central mechanism, such as opioid and descending itch inhibitory systems, rather than the periphery system, may be involved in this pruritus.

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Conflict of Interest The authors declare no conflict of interest.

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