INTRODUCTION

Autoimmune hepatitis is a progressive chronic disease that is prevalent across all age groups including children and adults, and is seen to affect women more frequently than men. Although genetic predisposition, viral infections and certain drugs are presumed to induce autoimmune hepatitis, the precise etiology of the disease is still unclear.\(^1\) In the standard immunosuppressive therapy of autoimmune hepatitis, administration of prednisolone alone or in combination with azathioprine, is found to have better prognosis; but some patients do not respond to these therapies.\(^2\) Therefore, new treatment strategies are necessary to restore tolerance to hepatic autoantigens and induce long-term remission.\(^3\)

Concanavalin A (Con A), a T cell mitogen, induces hepatitis in mice.\(^4\) This well-established model has been widely used to understand the pathology of autoimmune hepatitis or to evaluate the drug candidates for treating hepatitis.\(^5\) Autoimmune hepatitis has been known to be mediated by various types of cells such as CD4\(^+\) T cells, monocytes, macrophages including Kupffer cells, natural killer T cells, and neutrophils.\(^3\)–\(^5\) By utilizing knockout mice or neutralizing antibodies in the animal model, it has been shown that interferon (IFN)-\(\gamma\), interleukin (IL)-6, IL-12, and tumor necrosis factor (TNF)-\(\alpha\) are involved in the exacerbation of hepatitis, and in contrast, IL-10 has a protective role.\(^4\)–\(^6\)

G protein-coupled receptor 39 (GPR39) is a member of the ghrelin receptor family and is expressed in the gastrointestinal tract, pancreas, liver, kidney, adipose tissue, thyroid, heart, and lung.\(^7\) Zinc is known as the natural ligand of GPR39.\(^9\) Following various reports implicating the receptor in pathophysiology of diseases such as metabolic syndrome, depression, and colitis and in wound healing,\(^10\) a selective GPR39 agonist TC-G 1008 has been identified\(^10\) and used as a pharmacological tool to clarify the physiological roles of GPR39.\(^12\)–\(^14\) In our report regarding an anti-inflammatory role of GPR39, we found that oral administration of TC-G 1008 increased IL-10 mRNA in the liver in lipopolysaccharide (LPS)-induced murine model of sepsis. These data suggested that GPR39 agonists could be effective in liver-related diseases, such as hepatitis.

In this study, we investigated the protective role of GPR39 in Con A-induced hepatitis in mice and its potential mechanism of action. Our data suggests that GPR39 agonists are beneficial in the treatment of autoimmune hepatitis.

MATERIALS AND METHODS

Mice C57BL/6 mice (6–7 weeks old, male) were purchased from Charles River Japan and maintained in specific pathogen-free conditions. All animal experiments were approved by the Ethics Committee for Animal Experiments of Asubio Pharma Co., Ltd. (Approval: AEK-17-101).

Reagents TC-G 1008 and LY2784544 were purchased from Tocris Bioscience (Bristol, U.K.) and MedChem Express (Monmouth Junction, NJ, U.S.A.), respectively. Concanavalin A (C2010) and LPS were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.).

Con A-Induced Hepatitis in Mice Autoimmune hepatitis was elicited by the methods described previously\(^3\) with slight modification. Briefly, Con A was injected into C57BL/6 mice intravenously at a dose of 15 mg/kg, and TC-G 1008 suspended in 0.5% hydroxypropyl cellulose (Nippon Soda, Tokyo, Japan) was administered orally at doses of 3, 10 and 30 mg/kg 30 min before the elicitation. Serum samples were collected at 6 h (for cytokine measurement) and 24 h (for glutamic-pyruvic transaminase measurement). The protective effects of G protein-coupled receptor 39 (GPR39) on concanavalin A (Con A)-induced hepatitis in mice was examined. In a dose dependent manner and at 24 h after the elicitation with Con A, oral administration of TC-G 1008, a GPR39 agonist, reduced both, the glutamic-pyruvic transaminase levels (a marker for liver injury) and the necrosis area, as revealed by the histological analysis of tissues from mice with Con A-induced hepatitis. TC-G 1008 also suppressed serum interleukin (IL)-6 and tumor necrosis factor (TNF)-\(\alpha\) significantly at 6 h after the elicitation, suggesting that the cells producing IL-6 and/or TNF-\(\alpha\) are the targets of TC-G 1008. One potential target cell appears to be a monocyte-derived macrophage because TC-G 1008 treatment suppressed lipopolysaccharide-induced IL-6 production from U937 macrophages in vitro. Taken together, GPR39 agonist TC-G 1008 ameliorates liver injury in the Con A model by blocking pro-inflammatory cytokine production. Use of GPR39 agonists for monotherapy or in combination with immunosuppressants might prove to be beneficial in the treatment of autoimmune hepatitis.
transaminase (GPT) measurement) after the elicitation.

**Histological Analysis** The liver samples were collected 24 h after Con A administration and fixed in 10% (v/v) neutral buffered formalin, embedded in paraffin, sectioned at 4 µm thickness, and then stained with hematoxylin and eosin. The images of liver sections were captured by a digital camera (DS-Fi1 and Digital sight DS-L2, Nikon, Tokyo, Japan), and the whole section area and necrotic area were then calculated.

**In Vitro Culture of Cell Lines** Mice Kupffer cell line (SCC-I19, Merck Millipore, Billerica, MA, U.S.A.) and human monocyte U937 cells (American Type Culture Collection) were cultured in RPMI 1640 supplemented with 10% fetal bovine serum and in a humidified incubator at 37°C and 5% CO₂. To obtain monocyte-derived macrophage cells, U937 cells were cultured with phorbol myristate acetate (100 ng/mL) for 2–3 d and differentiation was confirmed with elevated expression of CD11b. The cells were exposed with TC-G 1008 (10 µM) or LY2784544 (1 µM) 30 min before stimulation. The cells were stimulated with LPS (1 µg/mL) for 6 h, and the culture supernatants were collected.

**Measurement of Serum Transaminase Activity and Cytokines** Levels of GPT activity were quantified using Transaminase CII-test Wako (Wako Pure Chemical Industries, Ltd., Osaka, Japan), according to manufacturers’ instructions. Concentrations of human and mouse IL-6, IL-10 and TNF-α in the serum samples or in the culture supernatants were quantified using AlphaLISA immunoassay kit (PerkinElmer, Inc., Waltham, MA, U.S.A.) according to manufacturer’s instructions.

**Statistical Analysis** The statistical analysis was performed with Student’s *t*-test or Dunnett’s multiple comparison test using JMP13.0 (SAS Institute Japan). *p*-Values less than 0.05 were considered as statistically significant.

**RESULTS AND DISCUSSION**

The hepatoprotective effects of TC-G 1008 were examined...
in Con A-induced hepatitis model. In agreement with previous reports, an intravenous injection of Con A into mice evoked severe liver injury indicated by a marked elevation in serum GPT activity and histopathological findings such as hepatocellular necrosis and cellular infiltration. We found that an oral administration of TC-G 1008, in a dose-dependent manner, inhibited the increase of the serum GPT activity and reduced necrosis area in the liver tissues sampled at 24 h after the elicitation. Significant efficacy was observed at doses of 10 and 30 mg/kg as compared with Con A-treated control group. The serum GPT level was well correlated with the necrosis area, in each mouse (Figs. 1 and 2). These results demonstrated that TC-G 1008 mitigated the liver injury in the hepatitis model. We next focused on the effect of TC-G 1008 on production of IL-6, IL-10 and TNF-α, which have been implicated in pathophysiology of hepatitis in the used model. As shown in Fig. 3, intravenous injection of Con A (15 mg/kg) in mice significantly increased serum levels of IL-6, IL-10, and TNF-α at 6 h after the elicitation. Oral administration of TC-G 1008 (30 mg/kg) significantly suppressed serum levels of IL-6 and TNF-α, while there was no difference in serum IL-10 levels between vehicle-treated and TC-G 1008-treated mice (Fig. 3). These results suggested that IL-6 and/or TNF-α were the key factor of the observed protective effect and cells producing these cytokines were the targets of GPR39 agonists.

A variety of cells are reported to mediate Con A-induced hepatitis. In this study, we investigated whether GPR39 agonists targeted macrophages. Quantitative PCR revealed that U937 macrophage expressed GPR39, while Kupffer cells SCC-119 did not (data not shown). LPS stimulation significantly increased IL-6 production by U937 macrophage, and TC-G 1008 treatment suppressed IL-6 production significantly. This effect was considered to be dependent on GPR39 because another chemically distinct GPR39 agonist LY2784544 also showed the same results (Fig. 4). These results suggested that one of the target cells of the GPR39 agonists with hepatoprotective effects was not liver resident macrophages but monocyte-derived macrophages. These data were focused on cell lines of macrophages, detailed study of endogenous macrophages must be investigated in future. Also, the involvement of other cells and the precise mechanism how GPR39 signaling interacts with the target cells must be elucidated. Moreover, therapeutic effects of GPR39 agonists should be carefully examined further in animal models and clinical trials.

It is reported that long-term supplementation of zinc, the natural ligand of GPR39, improves transaminase levels in hepatitis C patients and by analysis of public microarray data (ArrayExpress accession number: E-MTAB-950) we found that GPR39 expression was significantly elevated (around 2.5-fold) in hepatitis C patients as compared to healthy subjects, suggesting that GPR39 contributes to the efficacy of treatment and therefore GPR39 agonists could be effective in alleviating other hepatitis.

In conclusion, TC-G 1008, an orally active GPR39 agonist, ameliorated Con A-induced hepatitis in mice with significant suppression of serum IL-6 and TNF-α, suggesting that suppression of IL-6 and/or TNF-α production was involved to some extent in the efficacy of TC-G 1008 in mitigating Con A-induced hepatitis model.
A-induced hepatitis. This study indicates that GPR39 agonist could be beneficial in the treatment of autoimmune hepatitis.

Acknowledgments We are grateful to Dr. Maki Terakawa for her valuable comments on this study, and Rumiko Sho and Naomi Nakamura for their technical support. We also appreciate DBCLS TogoTV for providing the images used in graphical abstract. This work was funded by Asubio Pharma Co., Ltd.

Conflict of Interest The authors declare no conflict of interest. During the period of this study, all the authors were employed at the Asubio Pharma Co., Ltd.

REFERENCES


