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Cholecystokinin Receptor Antagonist Therapy Decreases Inflammation and Fibrosis in Chronic Pancreatitis

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Abstract

Background and Aims—Chronic pancreatitis is associated with recurrent inflammation, pain, fibrosis, and loss of exocrine and endocrine pancreatic function and risk of cancer. We hypothesized that activation of the CCK receptor contributes to pancreatitis and blockade of this pathway would improve chronic pancreatitis.

Methods—Two murine models were used to determine whether CCK receptor blockade with proglumide could prevent and reverse histologic and biochemical features of chronic pancreatitis: the 6-week repetitive chronic cerulein injection model and the modified 75% choline-deficient ethionine (CDE) diet. In the CDE-fed model, half the mice received water supplemented with proglumide, for 18 weeks. After chronic pancreatitis was established in the cerulein model, half the mice were treated with proglumide and half with water. Histology was scored in a blinded fashion for inflammation, fibrosis and acinar ductal metaplasia (ADM) and serum lipase levels were measured. RNA was extracted and examined for differentially expressed fibrosis genes.

Results—Proglumide therapy decreased pancreatic weight in the CDE diet study and the cerulein-induced chronic pancreatitis model. Fibrosis, inflammation, and ADM scores were significantly reduced in both models. Lipase values improved with proglumide but not in controls in both models. Proglumide decreased pancreas mRNA expression of amylase, collagen-4, and TGFβR2 gene expression by 44, 38, and 25%, respectively, compared to control mice.

Conclusion—New strategies are needed to decreased inflammation and reduce fibrosis in chronic pancreatitis. CCK receptor antagonist therapy may improve chronic pancreatitis by

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reversing fibrosis and inflammation. The decrease in ADM may reduce the risk of the development of pancreatic cancer.

Keywords

CCK; Cholecystokinin; Pancreatitis; Cerulein

Introduction

Fibrosis is the end result of years of chronic injury and inflammation. Chronic pancreatitis [1] may occur after chronic alcohol abuse, but also as a consequence of chronic relapsing idiopathic pancreatitis [2]. In chronic pancreatitis, the inflammation and fibrosis result in exocrine insufficiency and endocrine dysfunction with a decrease in a number of acinar and islet cells [3]. The most serious long-term complication related to chronic pancreatitis, however, is the risk of cancer [4, 5]. Pancreatic cancer is projected to become the second leading cause of cancer-related death by 2030 [6].

Pancreatic fibrosis results from the activation of tissue myofibroblasts or stellate cells of the pancreas that proliferate, migrate, and produce extracellular matrix components, such as type I collagen, and express cytokines and chemokines [7, 8]. The pancreatic stellate cells (PSC) [9] lose their lipid droplets upon activation and undergo morphological changes associated with function and secretion of soluble factors that potentiate the inflammatory process. Some of these functions and secretions include: α -SMA expression, proliferation, extracellular matrix protein (ECM) production (i.e., type I, III collagen), cytokine and chemokine production, adhesion molecule (ICSM-1) expression, migration, contractility, matrix metalloproteinase (MMP) expression, Toll-like receptor expression, and angiogenesis [7, 8]. Practice guidelines have been established to aid in the diagnosis of chronic pancreatitis [10]. Unfortunately, the current treatments only provide temporary pain relief and management of complications and do not arrest or slow the progression of this disabling disease [11].

Although it was thought for years that fibrosis was not reversible, studies have now shown that in other organs like the liver, treatment of hepatitis [12, 13] can reverse fibrosis. Anti-fibrotic strategies have also been attempted in chronic pancreatitis by utilizing agents that inhibit the activation of PSCs, agents that block PSC function, anti-cytokine or anti-growth factor therapies, and, of course, elimination of the insulting agent (such as alcohol) [14]. Some agents under investigation include peroxisome proliferation-activated receptor- γ ligands, angiotensin receptor blockers, angiotensin-converting enzyme inhibitors, antioxidants, serine-protease inhibitors, and NADPH oxidase inhibitors [15]. Unfortunately, many of these agents are not specific to the fibroblasts, and treatment results in deleterious effects on other organs. The stellate cells also play an important role in the normal function of pancreas and are thought to help with regeneration after injury and maintenance of cell function [16]. Therefore, agents that eliminate or injure the PSCs can be counterproductive.

An animal model to study acute and chronic pancreatitis has been well established [17]. This model involves the repeated injection of a cholecystokinin (CCK) analog, cerulein [18]. Partial or complete pancreatic duct obstruction alone [19] or in combination with ethanol

[20] can also induce chronic pancreatitis. Other models have been developed and include genetic alterations that induce pancreatitis with KRAS [21] or CFTR [22] mutations. A diet that is choline-deficient and supplemented with ethionine (a derivative of methionine) was found to induce severe necrotizing pancreatitis in mice [23]. Recently a modified version of this CDE diet has been described [24] as a model of chronic pancreatitis.

Under physiologic conditions during normal digestion, CCK regulates growth of the pancreas and release of digestive enzymes from the pancreas [25]. CCK exerts its effects through a G-protein coupled receptor called the CCK receptor (CCK-R) [26]. CCK receptors have also been identified on tissue fibroblasts [27] and pancreatic stellate [28] cells, and when these receptors are stimulated, the cells become activated to produce the desmoplastic stroma characteristic of the microenvironment of chronic pancreatitis and pancreatic cancer [29, 30]. Evidence that CCK receptors play a role in the fibrosis of the pancreas cancer microenvironment was demonstrated when CCK receptor blockade inhibited fibrosis in the KRAS murine model for pancreatic carcinogenesis [31] and of pancreatic cancer [32]. Since blockade of CCK receptors on fibroblasts and stellate cells halts fibrosis, we hypothesized that treatment with CCK receptor antagonist could prevent and perhaps reverse chronic pancreatitis.

Materials and Methods

Animal Models of Chronic Pancreatitis

All mouse studies were performed under protocol # 2016-1193 in an ethical fashion and approved by the IACUC committee on 10/05/2017 for animal research by the Georgetown University Comparative Medicine Department.

Chronic Cerulein Injection

In the cerulein model, C57BL/6 female mice ($N=30$) were treated with the high-dose cerulein (50 μg ip, qhr $\times 6$, TIW $\times 6$ weeks, in 100 μl volume). At 6 weeks, after chronic pancreatitis had been induced, some mice ($N=10$) were ethically euthanized and served as baseline controls. The remainder of the mice ($N=20$) entered a 2-week “recovery phase” during which 10 mice were treated with the CCK receptor antagonist proglumide (Tocris Bioscience, Bristol, UK) and 10 mice received H_2O . Proglumide was added to the drinking water at a concentration of 0.1 mg/ml and the estimated amount ingested per mouse was 30 mg/kg/days, a dose we previously showed blocked the CCK receptor [31]. At 1 and 2 weeks after initiation of proglumide treatment, the experiment was terminated.

Modified Choline-Deficient Ethionine-Supplemented Diet

In the CDE pancreatitis model, we used a 75% CDE diet as described by Passman [33] to induce chronic pancreatitis (rather than 100%) since this modified diet has less toxicity and does not cause necrotizing pancreatitis. C57BL/6 (10-week-old) female mice ($N=60$) were treated with the 75% CDE diet with or without proglumide supplemented water for 18 weeks to determine whether CCK receptor blockade could prevent pancreatitis. In a reversal study, after 12 weeks of the CDE diet, mice ($N=30$) were then randomized to untreated water ($N=15$) or proglumide-treated water ($N=15$) for 6 subsequent weeks

while continuing the CDE diet until week 18 to determine whether proglumide could reverse pancreatitis.

Biochemical Blood Test Assessment

Mice were ethically euthanized by CO₂ asphyxiation and cervical dislocation at the termination of each experiment. Blood was collected by cardiac puncture, centrifuged and serum-collected, and analyzed by VRL Laboratories, Gaithersburg, MD. In both animal models, blood was collected for pancreatic lipase.

Histologic Assessment of Pancreas Tissue

Pancreata were dissected, weighed, fixed in 4% paraformaldehyde, paraffin-embedded, and sectioned at 5 μ m. Mounted sections were stained with hematoxylin and eosin. The tissues were scored in a blinded fashion by a pathologist for inflammation, fibrosis, edema, and ADM (Table 1). Pancreatic fibrosis was analyzed by Masson's trichrome staining and α -SMA immunoreactivity (Ab124964, Abcam; 1:3500) and scored by quantitative morphometry.

Assessment of Differentially Expressed Genes by qRT-PCR

RNA was extracted (Qiagen) from pancreas tissues from C57BL/6 mice. CDNA was generated and subjected to real-time PCR (qRT-PCR) using SYBR® Green (Life Technologies) in an Applied Biosystems 7300 thermal cycler with the following conditions: initial incubation for 10 min at 95 °C followed by 40 cycles of 95 °C $\times$ 30 s, 60 °C $\times$ 1 min, and 72 °C for 30 s, using primers with the oligonucleotide primers in Table 2 for amylase, collagen-4, and TGF β R2.

Statistics

Mean values for each treatment group were compared to controls or baseline groups using computer software by Prism© and Minitab©. Student's *t* tests were used to evaluate statistical significance with a *p* < 0.05 considered to be statistically significant. Where multiple comparisons were made to a control a Bonferroni adjustment was made. RT-PCR results were expressed as a pairwise Student's *t* tests on the normalized mean Δ CT (the difference between the cycle count of the gene of interest minus the count of an endogenous control) values for each group according to the method of Livak and Schmittgen [34].

Results

Pancreatic Weight Recovers Faster with Proglumide

Mouse pancreatic weight typically increases with pancreatitis due to edema and inflammation. After cerulein administration, pancreatic weight decreased more rapidly with proglumide treatment than controls (Fig. 1a), but this difference did not reach statistical significance (*p* = 0.07). In the CDE-fed mice, proglumide concomitant therapy for 18 weeks prevented increased pancreatic weight (Fig. 1b). However, in the CDE-recovery study, proglumide did not reverse the increased pancreatic weight when given for only 6 weeks after pancreatitis had been established.

Proglumide Therapy Decreases Serum Lipase

Pancreatic serum lipase levels were collected from mice after 6 weeks of cerulein injections and again during proglumide or untreated water recovery after 1 and 2 weeks. One week after stopping the cerulein therapy proglumide-treated mice had lower lipase values than the water-treated controls (Fig. 2a), yet this difference was not significant ($p = 0.07$). When proglumide was administered in the water of CDE-fed mice for 18 weeks, lipase serum levels were significantly lower than mice receiving untreated drinking water (Fig. 2b). Furthermore, when proglumide was administered for a 6-week course after induction of chronic pancreatitis with 12 weeks of CDE diet, serum lipase values also normalized in spite of continuing the CDE diet (Fig. 2b).

CCK-R Blockade Decreases Pancreatic Inflammation and Acinar Ductal Metaplasia

Histology scores of the pancreata for edema did not change significantly with proglumide therapy (Fig. 3a). Scores for ADM improved upon withdrawal of cerulein (Fig. 3b) and after 2 weeks proglumide tissues had lower ADM scores than water controls. Inflammation decreased at 2 weeks after stopping cerulein in both controls and proglumide-treated mice (Fig. 3c). Inflammation scores also significantly improved in the 18 weeks CDE-fed mice that were treated with proglumide for the last 6 weeks of the 18 weeks while continuing the CDE diet (See supplementary material, Fig. 1).

Pancreatic fibrosis Is Improved with CCK-R Blockade

Evaluation of the fibrosis of the pancreata using Masson's trichrome staining revealed increased inter- and intralobular fibrosis in baseline tissues (Fig. 4a). There was no change in the pancreatic fibrosis scores after 1 or 2 weeks in water-treated mice in the cerulein recovery model compared to baseline fibrosis scores obtained on the pancreata of the 6-week cerulein-treated mice (Fig. 4b). After 2 weeks of therapy, proglumide-treated mice had significantly less fibrosis compared to baseline cerulein-treated controls (Fig. 4c). Quantitative morphometric analysis of fibrosis demonstrated that only proglumide-treated mice had a statistically significant reversal of fibrosis compared to baseline (Fig. 4d; $p = 0.0293$). α SMA immunoreactivity in the pancreas of mice at baseline shows increased fibrosis (Fig. 5a). The α SMA immunoreactivity was less in water-treated mice during recovery (Fig. 5b) but this was not significant. Only mice treated with proglumide therapy showed statistically less pancreatic fibrosis by α SMA immunoreactivity (Fig. 5c, d).

Differentially Expressed Genes

In addition to finding changes in serum lipase in proglumide-treated mice, mRNA expression of amylase was also decreased in the pancreas of CDE-fed mice, substantiating the decreased pancreatic inflammation (Fig. 6a). One of the major genes involved in collagen formation and fibrosis in chronic pancreatitis is collagen-4. The gene expression of collagen-4 was decreased in the pancreas tissue of CDE-fed mice that concomitantly received proglumide (Fig. 6b). TGF β signaling is an important pathway leading to increased extracellular matrix and is associated with increased expression of the TGF β -receptor-2 (TGF β R2). CDE-fed mice treated with proglumide had decreased mRNA expression of TGF β R2 (Fig. 6c).

Discussion

In this investigation, we utilized two murine models of chronic pancreatitis in order to test the hypothesis that activation of the CCK receptors contributes to the pathological and histologic changes in this disorder. In the chronic cerulein injection model, we could only evaluate whether proglumide could reverse biochemical and histologic changes of chronic pancreatitis, because concomitant administration of the CCK receptor antagonist proglumide would have prevented the actions of cerulein. In the CDE chronic pancreatitis model, both prevention and reversal proglumide studies were performed. We used a 75% CDE model over an extended period of time to induce chronic inflammation and fibrosis, in contrast to hemorrhagic pancreatitis that occurs with the 100% CDE-fed diet [33]. It is possible that higher percentage CDE diet could have induced greater fibrosis and inflammation, but it has a higher mortality. Elevation of serum lipase in both models was comparable and confirmed biochemical evidence of pancreatic inflammation, and therapy with proglumide lowered serum lipase in the prevention and reversal studies. These findings have clinical relevance in that treatment with proglumide may be useful for preventing ERCP-induced pancreatitis or hastening recovery and shortening hospital stays for bouts of acute or chronic relapsing pancreatitis.

Cholecystokinin and its analog cerulein have been known to produce pancreatic injury in a time and dose-dependent manner in mice and rats [18, 35–37]. The mechanism of human chronic pancreatitis is speculated to be due to repeated bouts of pancreatic injury [38]. Although the details of the mechanism of acute pancreatitis in humans have been elusive, it is speculated that it involves premature activation of zymogens. In rodents, CCK plays a major role in meal-induced pancreatic acinar cell secretion [18]. This response is in contrast to human acinar cells, which respond poorly to CCK, but is regulated by cholinergic pathways that involve neurogenic CCK release [39]. Basal plasma levels of CCK are elevated in patients with chronic pancreatitis compared to controls, suggesting that CCK plays a pathophysiologic role in human chronic pancreatitis. In the present study, we used two different modalities of induction of chronic pancreatitis but achieved similar reductions in inflammation and fibrosis using a CCK antagonist. This result suggests that at least in mice, despite the inciting event in chronic pancreatitis, CCK plays a key role in the inflammation and fibrosis of chronic pancreatitis. Interestingly, in a porcine model of direct bile salt perfusion induced acute pancreatitis, CCK-8 reduced acinar necrosis and edema. Since CCK receptor antagonists have previously been used in human trials, it should be straightforward to test the effects of these agents in patients with chronic pancreatitis.

There are several potent CCK receptor antagonists available for animal experimentation [40]. Some of these antagonists have high potency at the CCK-A receptor [41], while others show high specificity for the CCK-B receptor [42, 43]. However, since the presence of both CCK-A and CCK-B receptors has been described on pancreatic stellate cells and tissue fibroblasts [27, 28], we decided that a nonselective CCK receptor antagonist, proglumide, would be best for this pilot investigation. Furthermore, more potent CCK-A antagonists impair gall bladder emptying [44] which may result in acute cholecystitis. Proglumide is an older drug that was originally developed for peptic ulcer disease [45] but is no longer marketed in the USA. Proglumide is a nonselective, orally bioavailable, CCK-A and CCK-B

receptor antagonist that is well tolerated and has a good safety profile in human subjects. CCK-A receptors have also been associated with sensory nerves [46], and administration of a CCK-A receptor antagonist has been shown to effectively decrease pain in patients with chronic pancreatitis [47]. CCK-B receptors are over-expressed on precancerous pancreatic intraepithelial neoplasia (PanINs) [31] and on pancreatic cancer epithelial cells [48, 49] where stimulation promotes cancer growth and metastasis. Therefore, using a CCK receptor antagonist with affinity to both the CCK-A and CCK-B receptor could be beneficial to those with chronic pancreatitis.

Patients with chronic pancreatitis have increased fibrosis histologically suggesting overactivity of the stellate cells. Of interest, patients with chronic pancreatitis also have been found to have elevated endogenous CCK plasma levels [50]; therefore, CCK antagonist therapy may have a role in preventing fibrosis in patients with chronic pancreatitis. We clearly demonstrated in this investigation that proglumide treatment decreased pancreatic fibrosis histologically and also lowered selective fibrosis-associated gene expression. Pancreatic stellate cells release α SMA when activated [30, 51] in part due to stimulation of the CCK receptors on stellate cells [28]. Pancreas tissues of mice treated with proglumide had significantly less α SMA immunoreactivity than control mice in our study. Collagen-4 has been shown to be elevated in patients with chronic pancreatitis and may be a predictive biomarker for disease severity [52]. Gene expression of this important collagen in the pancreas tissue microenvironment was significantly decreased in mice treated with proglumide suggesting a reversal of the chronic inflammatory state. TGF β is an important cytokine involved in fibrogenesis and inhibition of TGF β interaction with its receptor, TGF β R2, decreases pancreatic fibrosis [53, 54]. Decreasing the expression of TGF β R2 with proglumide may have resulted in less activation of the TGF β signaling pathway and fibrosis in the proglumide-treated mice.

Another very important role of CCK receptor blockade may be to decrease the risk for the development of pancreatic cancer associated with chronic pancreatitis. In mice, acinar ductal metaplasia is a well-defined precursor to pancreatic intraepithelial neoplasia (PanIN), and eventually, to pancreatic ductal adenocarcinoma [55, 56]. Our study revealed that proglumide treatment decreased ADM in the mouse pancreas.

Novel strategies are needed to prevent and reverse histologic, functional, and biochemical damage in chronic pancreatitis. Our study shows that the CCK receptor pathway plays an important role in inflammation, fibrosis and precancerous state of chronic pancreatitis. Treatment with the orally available nonselective CCK receptor antagonist proglumide has the potential to decrease neurogenic pain, risk of cancerous transformation, and fibrosis of those with chronic pancreatitis

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ADM	Acinar ductal metaplasia
CCK	Cholecystokinin
CDE	Choline-deficient ethionine
MMP	Matrix metalloproteinase
PSC	Pancreatic stellate cells

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Fig. 1.

Mouse pancreatic weight mg/g of body weight. **a** Cerulein-treated mice show slightly less pancreatic weight after 1 week of proglumide, ($p = 0.07$) compared to water-treated mice. **b** CDE-fed mice that concomitantly received proglumide for 18 weeks had significantly less pancreatic weights ($p = 0.0062$). There was no difference in the pancreatic weight of mice that were on the CDE diet for 12 weeks before receiving proglumide for 6 weeks of the 18-week study

Fig. 2.

a Serum lipase levels in the cerulein chronic pancreatitis were decreased in mice treated with proglumide after 1 week. **b** In the CDE-fed mice, serum lipase levels were significantly lower with proglumide therapy in both the prevention and recovery study compared to CDE-fed mice treated with regular water, * $p < 0.05$

Fig. 3.

Histology scores of pancreas mean $\pm$ SD in cerulein study. **a** Edema scores did not significantly decrease with proglumide. **b** Acinar ductal metaplasia (ADM) scores decreased in the cerulein recovery phase and were less after 2 weeks with proglumide compared to control water-treated mice. $*p = 0.013$; $**p = 0.0009$; $***p = 0.0002$. **c** Histologic inflammation scores only improved 2 weeks after withdrawing cerulein and were less in proglumide-treated mice. $*p < 0.0001$

Fig. 4. Masson's trichrome staining of various cerulein-treated of mice **a** Baseline pancreas histology after 6 weeks of induction of chronic pancreatitis with cerulein. **b** Pancreas from water-treated control group 2 weeks post-cerulein therapy. **c** Proglumide-treated group 2 weeks post-induction of chronic pancreatitis. **d** Quantitative morphometry analysis shows significantly decreased fibrosis scores after 2 weeks of proglumide therapy ($p = 0.0293$)

Fig. 5.

Alpha-SMA staining of pancreas tissues from various groups of cerulein-treated mice **a**. Baseline α SMA staining after 6 weeks of induction of chronic pancreatitis. **b** α SMA immunoreactivity in pancreas of mice treated with water 2 weeks post-induction of chronic pancreatitis. **c** α SMA immunoreactivity in pancreas of mice treated with proglumide 2 weeks post-induction. **d** Mean fibrosis scores $\pm$ SD shows only significant reduction in fibrosis after 2 weeks of proglumide therapy, $p = 0.045$

Fig. 6.

Differentially expressed genes for fibrosis from CDE-fed mice. **a** Amylase mRNA expression decreased by 44% in CDE-fed mice that received proglumide compared to those that had untreated water. **b** Collagen-4 gene expression was decreased by 38% in CDE-fed mice that received proglumide. **c** TGF β R2 gene expression was decreased by 25% in CDE-fed proglumide-treated mice compared to water-treated controls

Table 1**Histopathological scoring system**

ADM	0 (No ADM)	1 (< 1%)	2 (1–5%)	3 (6–10%)	4 (> 10%)
Inflammation	0: absence of inflammatory infiltrates	1: inflammatory infiltration in ducts	2: inflammatory infiltration in the parenchyma < 50%	3: inflammatory infiltration in the parenchyma > 50%	
Edema	0: none	1: < 10%	2: 10–50%	3: > 50%	
Fibrosis	0: No fibrosis	1: only periductal	2: intralobular < 10%	3: intralobular 10–50%	4: intralobular > 50%

ADM acinar ductal metaplasia

Table 2

Primers used for qRT-PCR

Gene	Forward primer 5' to 3'	Reverse primer 5' to 3'
Amylase	CAAAATGGTTCTCCCAAGGA	ACATCTTCTCGCCATTCCAC
Collagen-4	GATGGGCTATCCTGGGAACCACT	TTCTCTCCTCGTTGCGCCTTTGG
TGFβR2	TTTCGGAAGAATACACCAC	GACACGGTAGCAGTAGAA