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Original article

A sustained prostacyclin analog, ONO-1301, attenuates pancreatic fibrosis in experimental chronic pancreatitis induced by dibutyltin dichloride in rats

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ABSTRACT

Background: ONO-1301, a novel sustained-release prostacyclin agonist, has an anti-fibrotic effect on the lungs, heart, and kidneys that is partly associated with the induction of hepatocyte growth factor (HGF). This study examined the anti-fibrotic effect of ONO-1301 on chronic pancreatitis (CP) progression.
Methods: CP was induced in rats *in vivo* by dibutyltin dichloride (DBTC). Seven days after DBTC injection (day 7), a slow-release form of ONO-1301 (10 mg/kg; ONO-1301–treated group) or vehicle (DBTC-treated group) was injected. On days 14 and 28, we evaluated the histopathological CP score and mRNA expressions of HGF, cytokines, and collagen in the pancreas by real-time RT-PCR. *In vitro*, monocytes and pancreatic stellate cells (PSCs) were isolated from normal rat spleen and pancreas, respectively. The cytokine and collagen expressions of monocytes and PSCs were detected by real-time RT-PCR, and PSCs proliferation was examined by BrdU assay.
Results: Histopathological CP scores *in vivo* improved in the ONO-1301–treated group compared to the DBTC-treated group, particularly inflammatory cell infiltration on day 14 and interstitial fibrosis on day 28. HGF mRNA increased significantly after ONO-1301 administration, whereas IL-1β, TNF-α, TGF-β, MCP-1, and collagen mRNA decreased significantly. Cytokine expression in monocytes was suppressed *in vitro* not only by HGF, but also ONO-1301 alone. However, neither ONO-1301 nor HGF affected the proliferation, or cytokine or collagen expression of PSCs.
Conclusions: ONO-1301 suppresses pancreatic fibrosis in the DBTC-induced CP model by inhibiting monocyte activity not only with induction of HGF but also by ONO-1301 itself.

1. Introduction

ONO-1301 is a synthetic nonprostanoid prostacyclin agonist possessing potent inhibitory activity against thromboxane A_2 (TXA_2) synthase [1,2]. Prostacyclin and its analogs are not stable *in vivo*, because 15-hydroxy PG dehydrogenase metabolizes their prostanoid structures. Unlike prostacyclin, ONO-1301 lacks typical prostanoid structures including a 5-member ring and allylic alcohol, thus leading to the improved biological and chemical stability *in vivo* [3]. A novel sustained-release form of ONO-1301 was recently developed [1,3]. A single subcutaneous injection of sustained-release ONO-1301 results in sustained elevation of its circulating levels for 3 weeks [1,3]. There are some studies which reported the therapeutic effects of ONO-1301 in experimental models of pulmonary fibrosis [4], dilated cardiomyopathy [5,6], ischemic heart disease [7], and obstructive nephropathy [3]. These beneficial effects of ONO-1301 in various models are partly mediated via the upregulation of hepatocyte growth factor (HGF). However, the anti-fibrotic effect of ONO-1301 on chronic pancreatitis (CP) is not clear.

In 1997, Sparmann et al. [8] reported a rat model of pancreatic fibrosis induced by dibutyltin dichloride (DBTC). We subsequently made some minor improvements to the DBTC-induced pancreatitis model and demonstrated the model is considered to be an appropriate model of CP both histologically and enzymatically [9].

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Furthermore, we previously reported that monocyte/macrophage recruitment via MCP-1 plays an important role in the development of pancreatic fibrosis using the modified DBTC-induced pancreatitis model [9–12]. On the other hand, ever since pancreatic stellate cells (PSCs) were identified and characterized by Apte et al., in 1998 [13,14], increasing evidence from recent studies indicates PSCs play an important role in pancreatic fibrogenesis [15]. When PSCs are exposed to the cytokines PDGF and TGF-β, PSC proliferation and collagen synthesis increase [13]. Therefore, PSCs and monocytes/macrophages are thought to be very important in the progression of pancreatic fibrosis.

Therefore, in the present study, we investigated whether ONO-1301 inhibits the progression of pancreatic fibrosis and whether its inhibitory mechanism is mediated via HGF.

2. Materials and methods

2.1. Preparation of ONO-1301 and its slow-release form

ONO-1301 was synthesized by ONO Pharmaceutical Co., Ltd. (Osaka, Japan). A slow-release form of ONO-1301 was generated by polymerizing ONO-1301 with poly-lactic and glycolic acid (ONO-1301 PLGA) as described previously [1,2]. Polyvinyl alcohol (Nacalai Tesque Ltd., Kyoto, Japan) and Tween 80 (Nacalai Tesque Ltd.) were used as dispersants in the production of PLGA microspheres.

2.2. Animals and experimental protocol

Male Lewis rats weighing 150–200 g were purchased from KBT Oriental (Saga, Japan) and maintained in accordance with the Guidelines of the Committee on Animal Care of Kyushu University. The experimental CP model was induced by DBTC (Schering AG, Berlin, Germany) as described previously [9]. On day 7 after DBTC administration, the rats were randomly divided into 2 groups: one group received a subcutaneous administration of vehicle (DBTC-treated group), and the other received a subcutaneous administration of ONO-1301 PLGA at 10 mg/kg body weight (ONO-1301–treated group) on day 7. The rats were sacrificed on days 14 and 28 after DBTC administration (Fig. 1A). The pancreas was quickly removed for histopathological analysis and reverse-transcription polymerase chain reaction (RT-PCR). Normal Lewis rats without DBTC administration were used as controls.

2.3. Histopathology and immunohistochemistry

Histological assessment was performed on all tissue specimens by hematoxylin–eosin (HE) and Azan staining. Morphological changes were evaluated in a blinded manner in each microscopic field. The results of the microscopic findings were concordant with the previous report by Kaku et al. [10]. In preparation for immunostaining, paraffin sections of the pancreas were rehydrated. Sections were incubated 0.3% H_2O_2 for 30 min to block endogenous peroxidases and washed. For ED-1 immunostaining, mouse anti-mouse/rat ED-1 antibody (abcam, Tokyo, Japan) diluted to 1:200 in PBS in a humid chamber at 4 °C overnight. After washes, sections were incubated with goat anti-mouse IgG Fc (HRP) (abcam, Tokyo, Japan) diluted to 1:1000 in PBS in a humid chamber for 1 h. After washes, Histofine Simple Stain DAB (Nichirei, Tokyo, Japan) was applied according to the manufacturer's instructions. Sections were then counterstained in hematoxylin and mounted in nonaqueous mounting solution. The number of ED-1 positive cells was counted by 2 blinded observers in each section (5 high-power fields), and the average of positive cells per section was reported.

2.4. Real-time RT-PCR

Total RNA was extracted using ISOGEN (Nippon Gene, Tokyo, Japan) as described previously [16]. Total RNA was reverse transcribed into first-strand complementary DNA (cDNA) using River Tra Ace-α (Toyobo, Tokyo, Japan). The sequences of primers used in this study and expected sizes of the PCR products are shown in Table 1. Real-time RT-PCR was performed on a LightCycler Real-time PCR system (Roche, Switzerland) using the SYBR Green II (Takara Bio, Otsu, Japan). The reaction conditions were as follows: denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 60 s. To control for variations in the reactions, all PCR data were normalized against that of GAPDH expression.

2.5. Isolation of rat monocytes and cell culture

Monocytes were isolated from the spleen of a normal rat by the density-gradient centrifugation method as described previously [11,17]. The isolated cells were washed and resuspended in RPMI 1640 (Invitrogen Corp., Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; Invitrogen Corp.). The cells were counted, and viability was assessed by trypan blue. After cell density was adjusted to 1×10^6 mononuclear cells/mL, the cells were seeded in 6-well plates and maintained in RPMI 1640 with 10% FBS at 37 °C in a humidified 5% CO_2 environment for 2 h.

Mononuclear cells, which were mainly monocytes, adhered during this incubation period. After 2 h, non-adherent cells were removed and adhered monocytes were washed with serum-free RPMI 1640. Thereafter, the cells were exposed to lipopolysaccharide (LPS; Sigma-Aldrich, St Louis, MO, USA) and ONO-1301 or recombinant human HGF (PEPROTECH, Rocky Hill, USA) dissolved in RPMI 1640 with 10% FBS. After incubation for 6 h at 37 °C, supernatants were removed and adherent cells were used for RT-PCR assay.

2.6. PSC isolation and cell culture

PSCs were isolated from the pancreas of rat by density-gradient centrifugation as described previously [18,19]. The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM)/Ham's F-12 (F-12) nutrient mixture (Invitrogen Corp.) supplemented with 10% FBS, 50 U/mL penicillin, and 50 mg/mL streptomycin (Invitrogen Corp.). All experiments using PSCs were performed with cells passage 3. The PSCs were incubated in serum-free medium for 24 h before experimental reagents were added. Thereafter, PSCs were used for cell proliferation and RT-PCR assays.

2.7. Quantification of HGF by enzyme immunoassay (EIA)

The plasma HGF levels of rats were measured by EIA (Rat HGF EIA, Institute of Immunology Co., Ltd., Tokyo, Japan) according to the manufacturer's instructions. Plasma HGF was quantified according to the differences in absorbance at 450 and 620 nm. The mean absorbance of each set of duplicate standards, controls, and samples was calculated, and the average zero standard optical density was subtracted.

2.8. PSC proliferation assay

Cultured rat PSCs were adjusted to 1×10^3 cells/well in 96-well plates and incubated with resting DMEM/F-12 for 24 h. Thereafter, the cultured media were changed to freshly prepared media containing 10 ng/mL recombinant rat platelet-derived growth factor-BB (PDGF-BB; Sigma-Aldrich), which was coincident with ONO-1301 or HGF. After 48 h of incubation, cell proliferation was