MCP-1 INHIBITS DNA SYNTHESIS IN RAT PANCREATIC STELLATE CELLS

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Abstract

Activated pancreatic stellate cells (PSCs) synthesize various kinds of cytokines and chemokines including monocyte chemoattractant protein-1 (MCP-1) and play major roles in promoting inflammation and fibrogenesis in the pancreas.   MCP-1 is a potent chemotactic factor for leukocytes and it has recently been shown that the target is not restricted.   The aim of this study was to investigate whether MCP-1 exerts a biological effect on PSCs.   Cultured rat PSCs secreted MCP-1 independent of the concentration of transforming growth factor-β_1 (TGF-β_1) in the culture media.   Although PSCs lack the typical receptor system (C-C chemokine receptor 2 (CCR2)), MCP-1 inhibited DNA synthesis in PSCs without activation, suggesting the presence of CCR2-independent MCP-1 signaling pathway.   Further, MCP-1 inhibited the proliferation of PSCs in which TGF-β_1/Smad pathway was blocked by the dominant-negative Smad2/3 over-expression. MCP-1 did not affect the phosphorylation state of mitogen-activated protein kinase (MAPK), Akt, nor epidermal growth factor receptor (EGFR).   Taken together, MCP-1 inhibited DNA synthesis of cultured rat PSCs in an autocrine or paracrine manner without activation and this effect was exerted through CCR2-independent and TGF-β_1/Smad-independent pathway.   These data provide new insights to better understand MCP-1 participation in pancreatic inflammation and also to develop a new strategy for its treatment.

Key words : MCP-1, CCR2, Pancreatic stellate cells, DNA replication, Autocrine, Paracrine

Introduction

Pancreatic stellate cells (PSCs) were recently identified, isolated, and characterized[1,2].   In the normal pancreas, PSCs possess fat droplets containing vitamin A and are quiescently defined by positive desmin and negative α-smooth muscle actin (α-SMA) staining[3].   When cultured *in vitro*, PSCs are auto-activated and change their morphological and functional features[2].   PSCs commence losing vitamin A containing lipid droplets, highly proliferating, increasing the expression of α-SMA, and producing and secreting extracellular matrix components such as collagen and fibronectin.   Namely, PSCs are auto-transformed to myofibroblast-like cells.   *In vivo*, PSCs are found in the periacinar space and have long cytoplasmic processes that encircle the base of the acinus. Activated PSCs are seen in the fibrotic area of pancreatic tissue of both human chronic pancreatitis and rat experimental model[4].

It is well accepted that the pivotal role of leukocytes in inflammation is mainly mediated by secretion of cytokines.   PSCs synthesize various cytokines and chemokines including IL-1β, IL-6, and TGF-β_1[5-9].   Chemokines have a broad range of effects on the recruitment and function of specific populations of leukocytes at the site of inflammation, and also play an important role in

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the initiation and maintenance of the host inflammatory responses.　The chemokines secreted locally in the pancreas may mediate the rapid influx and accumulation of neutrophils, monocytes, and other inflammatory cells that play a significant role in tissue destruction and repair in acute and/or chronic pancreatitis[5,8,10].　Chronic pancreatitis is defined by the presence of chronic inflammation, destruction of acinar and ductal cells, intra- and perilobular fibrosis, and finally the irreversible scarring of the parenchyma[11].

Monocyte chemoattractant protein-1 (MCP-1) is a potent chemotactic factor for monocytes, macrophages, memory T lymphocytes, and natural killer cells[12]. MCP-1 is a member of the family of proinflammatory cytokines, all of which share high degree of amino acid sequence homology[13].　Chemokines are divided into the C, CC, CXC, CX3C groups according to the spacing of their first 2 cysteine residues.　MCP-1 is a basic protein consisting of 76 amino acids and is classified as part of the CC subfamily of chemokines.　MCP-1 binds to CCR2 physiologically, which is highly conserved among human, mouce, and rabbit[14].　MCP-1 is produced in the presence of serum or specific stimuli by a variety type of cells, including monocytes, smooth muscle cells, fibroblasts, endothelial cells, and epithelial cells[15].

Activated PSCs play a pivotal role in the development of pancreatic fibrosis in chronic pancreatitis[2,3,11,16].　Activated PSCs produce cytokines and chemokines such as IL-6[9], IL-8[10] and MCP-1[10,16-19], and up-regulated MCP-1 expression has been found during acute and chronic pancreatitis both in animal models and in human tissues, suggesting the contribution of this chemokine in the pathogenesis of mononuclear infiltration[20-22].　High levels of MCP-1 expression were demonstrated in serum[23] and pancreatic acinar cells[24] in animal models of acute pancreatitis.　A mutant human MCP-1 (1, 9-76 ; mMCP-1), which lacks N-terminal amino acids 2-8, serves as a potent dominant-negative MCP-1 agonist[25] and the injection of mMCP-1 into rat thigh muscles suppressed the induction of pancreatic fibrosis by dibutyltin dichloride (DBTC)[26].　Treatment with bindarit (a blocker of MCP-1 synthesis) also protected mice from acute pancreatitis[27].

Although leukocytes were thought to be the only tar-

get for chemokines, recent evidences indicate that the action of these proteins is not restricted to these cell types.　MCP-1 stimulates procollagen I expression in pulmonary fibroblasts via an autocrine loop involving TGF-β_1[15].　In the present study, we evaluated the effect of MCP-1 on cultured rat PSCs to examine whether the CC chemokine system promotes PSCs.　MCP-1 inhibited the proliferation of PSCs through TGF-β_1/Smad-independent pathway and this effect was not mediated by the CCR2 receptor system.

Materials and Methods

Materials

Recombinant rat MCP-1, rat epidermal growth factor (EGF), human TGF-β_1, and the primary antibody of anti-mouse MCP-1 were purchased from R&D Systems (Abington, UK).　Pronase, Nycodenz, and anti-α-SMA antibody were from Sigma (St Louis, MO).　DNase-1 was from Roche (Basel, Switzerland).　Collagenase P was from Boehringer Mannheim (Mannheim, Germany). Anti-tubulin antibody was from Thermo Fisher Scientific (Fremont, CA) and anti-CCR2 antibody was from Santa Cruz (Santa Cruz, CA).　Anti-extracellular regulated kinases 1 and 2 (ERK1/2), anti-phospho-ERK1/2, anti-N-terminal c-Jun kinase (JNK), anti-phospho-JNK, anti-p38 MAPK, anti-phospho-p38 MAPK, anti-Akt, anti-phospho-Akt antibodies were from Cell Signaling Technology (Beverly, MA).　Anti-EGFR and anti-phospho-EGFR antibodies were purchased from Abcam (Cambridge, UK). Horseradish peroxidase (HRP)-conjugated donkey anti-mouse IgG, HRP-conjugated donkey anti-rabbit IgG, and HRP-conjugated donkey anti-goat IgG were obtained from Jackson Immuno Research (West Grove, PA).

Isolation and culture of rat pancreatic stellate cells

The rat PSCs were isolated as described previously[1]. Briefly, the rat pancreas was resected and digested in Gey's balanced salt solution with a mixture of 0.05% collagenase P, 0.02% pronase, and 0.1% DNase-I.　The cells were filtered through a nylon mesh and centrifuged in a 13.2% Nycodenz gradient at 1,400×g for 20 minutes. PSCs were collected in the band just above the interface

of the Nycodenz solution, washed, and resuspended in Iscove's modified Dulbecco's medium (IMDM) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. PSCs were cultured at 37°C in a humidified environment of 95% air and 5% CO_2 atmosphere. The purity of isolated cells was greater than 90% according to the observation of their cytoplasmic droplets with vitamin A autofluorescence. PSCs were used between passages two and four as culture-activated PSCs in the experiments. Medium was changed to IMDM containing 2% FBS after the cells attached to the plates to examine MCP-1 production, PSCs activation and PSCs proliferation. All rat experiments were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Akita University.

Conventional PCR and Real-time Quantitative RT-PCR

Total RNA was obtained from PSCs using RNeasy Mini kit with DNase treatment (QIAGEN, Valencia, CA). First-stranded cDNA was synthesized from total RNA using SuperscriptTM First-stranded Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Conventional RT-PCR for rat CCR2, CCR1 and CCR3 was performed with the following primers : (a) rat CCR2 (GenBank Accession No. : NM_021866) : sense 5′-CTCTTCCTGCTCA-CACTCCC-3′ (307-326), anti-sense 5′-CAAG-TAGGGGCAGGATCAAA-3′ (688-669), (b) rat CCR1 (GenBank Accession No. : NM_020542) : sense 5′-GTTGGGACCTTGAACCTTGA-3′ (200-219), anti-sense 5'-AGGGAAAACACTGCATGGAC-3′ (775-756), (c) rat CCR3 (GenBank Accession No. : NM_053958) : sense 5′-AAACTTGCAAAACCTGAGAAGC-3′ (49-70), anti-sense 5′-ATAGCGAGGACTGCAGGAAAG-3′ (787-767), (d) rat β-actin (GenBank Accession No. : NM_031144): sense 5′-TGAGAGGGAAATCGTGC-GTG-3′(693-712), anti-sense 5′-GATCCACATCT-GCTGGAAGGTG-3′ (1152-1131). The reactions were conducted as follows; denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds, for 35 cycles. Real-time quantita-

tive RT-PCR for rat MCP-1 and β-actin was carried out with the following primers ; (e) rat MCP-1 (GenBank Accession No. : NM_031530) : sense 5′-TATGCAG-GTCTCTGTCACGC-3′ (75-94), anti-sense 5′-TGCT-GCTGGTGATTCTCTTG-3′ (247-228) ; (f) rat β-actin: sense 5′-TGAGAGGGAAATCGTGCGTG-3′ (693-712), anti-sense 5′-TCATGGATGCCACAGGATTCC-3′ (908-888). The reactions were conducted using ABI PRISM 7900HT (Applied Biosystems) as follows: denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds.

Western blotting

Western blotting was performed as described previously[28]. For gel electrophoresis, 10 μg of protein were loaded onto each lane of a 10% sodium dodecyl sulphate-polyacrylamide gel. Enhanced chemiluminescence reagent (Amersham, Piscataway, NJ) was used to visualize the secondary antibody.

Measurement of DNA synthesis

PSCs were seeded at a density of $1\text{-}2\times10^4$/well in a plastic 96 well plate and cultured in IMDM containing 2% FBS. PSCs were treated with MCP-1 or TGF-β_1 at indicated concentrations for 48 h. BrdU was added to the culture medium and the cells were incubated for further 2 h and the BrdU incorporation was measured using a commercial kit from R&D Systems (Abington, UK) according to the manufacturer's instructions.

Measurement of MCP-1and TGF-β1 peptide secretion

PSCs were seeded at a density of $1\text{-}2\times10^5$/well in a plastic 24-well plate. PSCs were treated with TGF-β_1 or MCP-1 at indicated concentrations for 48 h. Cell culture supernatant was harvested and stored at -80°C until measurement. MCP-1 and TGF-β_1 levels in the conditioned media were measured by enzyme-linked immunosorbent assay (Immuno-Biological Laboratories, Takasaki, Japan, and R&D Systems, Abington, UK, respectively), according to the manufacturer's instructions.

Adenovirus infection

Recombinant adenovirus of Dominant-Negative

Smad2/3 (AdDNSmad2/3) was kindly provided by Dr. Kohei Miyazono (University of Tokyo, Tokyo, Japan). An adenovirus expressing ß-galactosidase (AdLacZ) as an infection control.　The cells were infected with a recombinant adenovirus at a dose of 10 plaque-forming units (pfu) per cell.

Statistics

Any differences between the groups were evaluated by analysis of variance (ANOVA), followed by post hoc analysis using Bonferroni's correction.　A p-value <0.05 was considered to be statistically significant.

Results

Cultured PSCs produced and secreted MCP-1

We first examined whether cultured PSCs produce and secrete MCP-1.　TGF-β_1 activates PSCs[3,5] and TGF-β_1 up-regulates the expression of MCP-1 in human microvessel endothelial cells and umbilical cord vein epithelial cells[29].　Then, the secretion of MCP-1 was determined in various concentrations of TGF-β_1 in the culture media.　As reported[10,16-19], PSCs produced and secreted MCP-1 and the amount of secretion was independent of the concentration of TGF-β_1 (Figure 1A).　Real-time quantitative PCR was used to verify the independence. MCP-1 expression was quantitated and normalized to β-actin.　The expression level was not altered in the presence of TGF-β_1 (Data not shown).

PSCs do not express the typical receptor (CCR2) of MCP-1

We assessed whether PSCs have a receptor system for MCP-1.　CCR2, a heterotrimeric G-coupled receptor, is the only known receptor that functions at physiologic concentrations of MCP-1.　No signals for CCR2 were detected by Western blotting (Data not shown) and we examined the mRNA expression by reverse transcriptase-PCR using total RNA from rat spleen as a positive control.　As shown in Figure 1B, rat cultured PSCs did not express CCR2 mRNA.　It is notable that this finding is identical to the result of analogous experiment in human hepatic stellate cells (HSCs)[30].　The expressions of
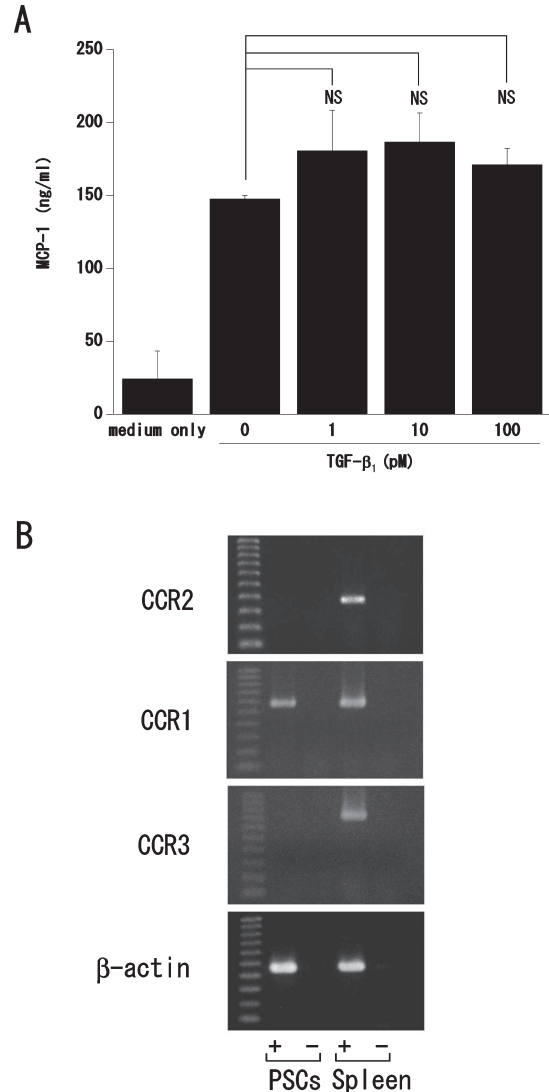
Fig. 1.　Secretion of MCP-1 and expressions of chemokine receptors in PSCs.
(A) Cultured rat PSCs (passage three) were seeded at 1-2×10^5 cells/well in 24-well plates and cultured for 48 h in the presence of 0, 1, 10 or 100 pM TGF-β_1. The conditioned media was collected and MCP-1 concentration in the supernatant was measured using a rat MCP-1 ELISA kit.　Values are expressed as the means±SD.　The experiments were repeated three times independently and the representative figure was shown.　(B) The expressions of CCR2, CCR1, and CCR3 were determined by RT-PCR using total RNA from cultured PSCs and rat spleen as a positive control. Samples with (+) or without (−) reverse transcriptase treatment were loaded.　The left-end lane indicates a 100-bp ladder size marker.

CCR1 and CCR3 were also determined. PSCs expressed only CCR1 mRNA.

MCP-1 inhibited the proliferation of PSCs without the activation

MCP-1 has been shown to modulate the biology of myofibroblastic cells via CCR2 dependent and independent mechanisms, suggesting the existence of an alternative receptor in this cell type[30,31]. We then examined whether MCP-1 activates PSCs by measuring the expression of α-SMA. Recombinant MCP-1 was added to the culture medium for 48 h and Western blotting was subsequently performed. As shown in Figure 2A, MCP-1 did not affect the α-SMA expression level.

We next examined the effect on the proliferation of PSCs. Recombinant MCP-1, ranging from 1.5 to 150 ng/ml, inhibited DNA synthesis in a dose-dependent manner (Figure 2B). TGF-β_1 inhibits the proliferation of PSCs. The growth inhibitory effect of MCP-1 may have been mediated by the induction of TGF-β_1. However, when TGF-β_1 secreted into culture media was measured with or without the incubation of MCP-1, the concentration of TGF-β_1 did not change (Data not shown). To confirm the growth inhibitory effect of MCP-1 directly, we used a dominant-negative adenovirus system (AdDNSmad2/3)[32]. Transfection efficacy was more than 98% and we could sufficiently inhibit the TGF-β_1/Smad signaling pathway with this system[32]. When the TGF-β_1/Smad signaling was blocked, DNA synthesis of PSCs was increased to about 140% and when incubated with MCP-1, its growth inhibitory effect was not attenuated compared with the control vector (AdLacZ)-transfected cells (Figure 2C). These results suggest that this effect was independent of TGF-β_1/Smad signaling. As MCP-1 activates MAPK pathway ; ERK1/2, JNK, and p38 MAPK ; in leukocytes and vascular endothelial cells which express CCR2[33,34], and MCP-1 induces a time- and concentration-dependent phosphorylation of ERK1/2 in CCR2$-$/$-$ aortic smooth muscle cells[31], we evaluated whether MCP-1 affect the MAPK signaling cascade. We also examined the phosphorylation states of Akt and EGFR. However, MCP-1 did not alter the phosphorylation of ERK, JNK, p38 MAPK, Akt, nor EGFR (data not shown).
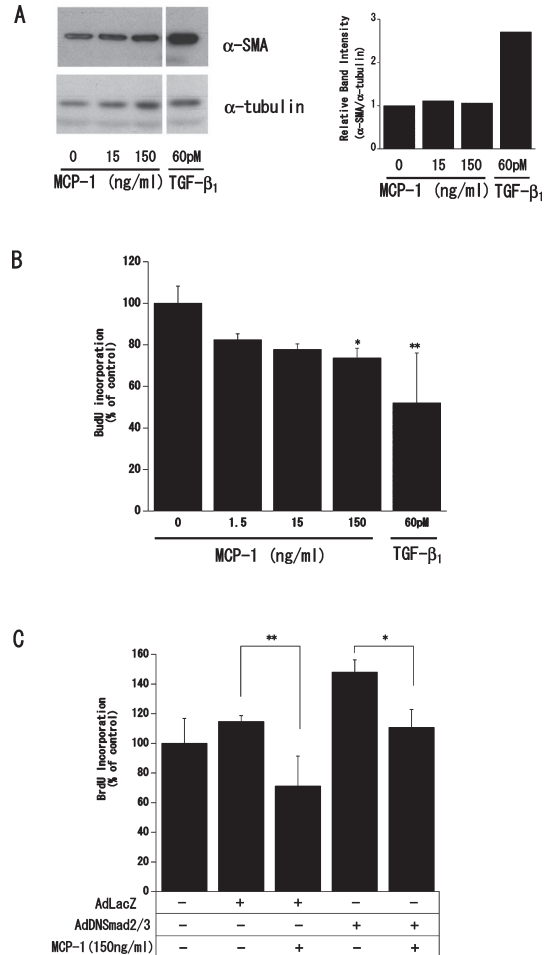

Fig. 2.　The effect of MCP-1 on the activation and proliferation of PSCs.
(A) PSCs were incubated with the indicated amount of MCP-1 or TGF-β_1 for 48 h. Total cellular homogenates were prepared and aliquots of 10 μg of protein were subjected to electrophoresis. Western blotting analysis was carried out using anti-α-SMA and anti-tubulin monoclonal antibodies. Right panel shows the relative band intensity compared with the control (MCP-10 ng/ml) by using Image J software. (B) Cultured PSCs (passage three) were seeded at 1-2×10^4 cells/well in 96-well plates and incubated with the indicated amounts of MCP-1 for 48 h, followed by the determination of DNA synthesis using a BrdU incorporation assay kit. (C) PSCs were infected with AdLacZ or AdDNSmad2/3. After 48 h of incubation, DNA synthesis was determined. Values are expressed as the means ± SD. The experiments were repeated three times independently and the representative figures were shown. *, $P<0.05$; **, $P<0.01$ vs. control, by analysis of variances.

MCP-1 inhibited the proliferation of PSCs in an autocrine fashion

Next, we abolished the effect of MCP-1 by using a neutralizing antibody in order to examine whether MCP-1 exerts its effect in an autocrine or paracrine fashion, although the intracellular mechanism of growth inhibitory effect is not clear.　As shown in Figure 3, the proliferation of PSCs was stimulated to almost the same extent in the presence of a neutralizing antibody as that with 10% FBS.　The addition of preimmune mouse serum did not affect the BrdU incorporation and the addition of TGF-β_1 (60 pM) decreased the DNA synthesis as reported[5].

Discussion

Chemokines and chemokine receptors play a central role in the regulation of cell migration and local inflammation[35].　Therefore, chemokines and their receptors could represent potential targets for antifibrotic therapies.　A variety of inflammatory mediators play key roles in the pathogenesis and progression of inflammation.　The influx and the accumulation of inflammatory cells into the pancreas play an important role in acute and/or chronic pancreatitis.　Activated PSCs produce various inflammatory cytokines and chemokines, including IL-6[9], IL-8[10], TGF-β_1[3,5,32], and MCP-1[10,16-19].　An over-expression of MCP-1 is observed in the lesions of inflammatory processes including idiopathic pulmonary fibrosis[36], crescentic glomerulonephritis[37], systemic sclerosis[38], inflammatory bowel diseases[39] and chronic pancreatitis[21].　Although leukocytes have been considered the only target for chemokines, recent evidences indicate that the action of these proteins is not restricted to leukocytes.　MCP-1 stimulates procollagen I expression via an autocrine loop involving TGF-β_1 in pulmonary fibroblasts[15] and MCP-1 contributes to fibrogenesis via up-regulation of TGF-β_1 in renal glomerular cells[40,41].　TGF-β_1 up-regulates the expression of MCP-1 in human microvessel endothelial cells and umbilical cord vein epithelial cells[29].　The current study evaluated the effect of MCP-1 on cultured rat PSCs.

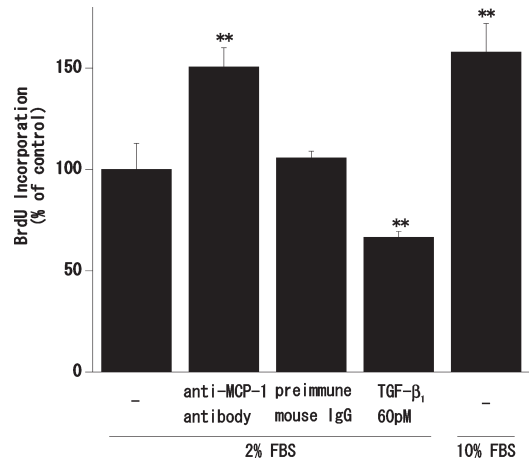The amount of MCP-1 produced and secreted from cultured PSCs was not affected by TGF-β1 in the culture

Fig. 3.　MCP-1 inhibited the proliferation of PSCs in an autocrine fashion.
Cultured rat PSCs (passage three) were seeded at 1–2×10^4 cells/well in 96-well plates and incubated with a neutralizing antibody (1 μg/well) or preimmune mouse IgG (1 μg/well) in the presence of 2% FBS for 48 h, followed by the determination of the DNA synthesis using a BrdU incorporation assay kit. Values are expressed as the means±SD.　The experiments were repeated three times independently and the representative figure was shown.　**, $P<0.01$ vs. control, by analysis of variances.

media (Figure 1A) and vice versa (Data not shown). The concentration of secreted MCP-1 was about ten times higher in these experiments, in comparison to the previous reports[10,16-19].　This discrepancy may be due to the higher density of the cells (1–2×10^5/well in 24-well plates), the presence of 2% FBS in culture media, and the longer incubation time (48 h).　MCP-1 inhibited the proliferation of PSCs without the activation and this inhibitory effect was independent of the TGF-β_1/Smad signaling (Figure 2).　The MAPK pathway is a major cell signaling pathway mediating ethanol induced and growth factor induced PSCs activation[17,42] and MCP-1 induced activation of MAPK in human endothelial cells[34].　However, MCP-1 did not alter the phosphorylation state of MAPK, Akt, nor EGFR.　A neutralizing antibody of MCP-1 stimulated the proliferation of PSCs (Figure 3).

CCR2, which is mainly expressed on the surface of monocytes and macrophages, is a functional receptor for MCP1.　No expression of CCR2 was detected at the

mRNA level (Figure 1b) and the protein level (Data not shown), whereas CCR1 was expressed in cultured PSCs. Although several other CC chemokine receptors bind MCP-1, none appear to function at physiologic concentrations of the ligand.　For example, CCR1 binds MCP-1, but the concentration of MCP-1 required to induce Ca^{2+} flux is 10-100 times higher than that needed to activate CCR2[43].　Hansen *et al.* reported that subnanomolar concentrations of MCP-1 specifically induced mouse astrocyte chemotaxis, whereas RT-PCR study failed to detect CCR2 mRNA in the astrocytes[44].　Similarly, Schecter *et al.* reported that MCP-1 induced tissue factor activity and phosphorylation of ERK in smooth muscle cells derived from CCR2−/− mice[31].　These findings suggest the presence of CCR2-independent MCP-1 signaling pathway.

In this study, we showed that MCP-1 inhibited the growth of PSCs in an autocrine or paracrine fashion without activation.　Antichemokine gene therapy using dominant-negative MCP-1 resulted in a dramatic amelioration of pancreas pathology, preservation of exocrine secretory function and reduction of inflammation and fibrosis[26].　Treatment with a blocker of MCP-1 synthesis also protected mice from acute pancreatitis[27].　Therefore, MCP-1 undoubtedly contributes to the inflammation and fibrogenesis in pancreas and may play some roles on PSC itself.　A subset of endothelial progenitor cells acquire the ability to adhere to injured epithelium in a MCP-1 dependent manner, leading to re-endothelialization associated with the inhibition of intimal hyperplasia[45].　MCP-1 mediates TGF-β_1-induced angiogenesis by stimulating vascular smooth muscle cell migration toward endothelial cells[29,46] and MCP-1 also acts as a chemoattractant in human HSCs[30].　Recently PSCs have been shown to have phagocytic activity *in vivo* and *in vitro* and therefore might also function as resident phagocytic cells during pancreatitis[47].　Phagocytosis is essential to limit the extent of inflammation and phagocytic activity may inhibit inflammatory mediator production and facilitate fibrogenesis.　Although TGF-β_1 significantly stimulates PSCs to synthesize and secrete matrix proteins such as type I collagen, fibronectin and laminine, TGF-β_1 still has a growth inhibitory effect through TGF-β_1/Smad3-dependent pathway that may lead to calm

the inflammation down[32].　Even though MCP-1 has a potent chemotactic property for monocytes and macrophages, its growth inhibitory effect on PSCs may provide a negative feedback on the control of an excessive inflammatory reaction and this effect was totally independent of TGF-β_1/Smad-signaling.　Further experimental evaluations of MCP-1 are needed to clarify its biological aspects on PSCs.

In conclusion, our results demonstrate that MCP-1 inhibited the proliferation of PSCs in an autocrine or paracrine manner through CCR2-independent and TGF-β_1/Smad-independent pathway.　This finding provides a new insight to better understand MCP-1 participation in pancreatic inflammation and fibrogenesis and also to develop a new strategy for its treatment.

Abbreviations

α-SMA, α-smooth muscle actin ; CCR2, C-C chemokine receptor 2 ; EGF, epidermal growth factor ; EGFR, epidermal growth factor receptor ; ERK1/2, extracellular regulated kinases 1 and 2 ; ELISA, Enzyme-Linked ImmunoSorbent Assay ; HRP, horse radish peroxidase ; HSC, hepatic stellate cell ; Ig, Immunoglobulin ; IL, Interleukin; MAPK, mitogen activated protein kinase ; JNK, N-terminal c-Jun kinase ; MCP-1, monocyte chemoattractant protein-1 ; NF-κB, nuclear factor-kappaB ; PI3K, phosphatidylinositol 3-kinase ; PSC, pancreatic stellate cell ; TGF-β_1, transforming growth factor-β_1

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