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Original Article

Simvastatin attenuates macrophage-mediated gemcitabine resistance of pancreatic ductal adenocarcinoma by regulating the TGF-β1/Gfi-1 axis

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ABSTRACT

Pancreatic ductal adenocarcinoma (PDAC) is a highly lethal malignancy with an intrinsic resistance to almost all chemotherapeutic drugs, including gemcitabine. An abundance of tumor-associated macrophages (TAMs), which can promote the resistance of PDAC to gemcitabine, has been observed in the microenvironments of several tumors. In this study, we confirmed that incubation in TAM-conditioned medium (TAM-CM) reduces the gemcitabine-induced apoptosis of PDAC cells. Simvastatin attenuated the TAM-mediated resistance of PDAC cells to gemcitabine. Further investigation found that simvastatin reversed the TAM-mediated down-regulation of Gfi-1 and up-regulation of CTGF and HMGB1. Simvastatin induced Gfi-1 expression, which increased the sensitivity of PDAC cells to gemcitabine by decreasing TGF-β1 secretion by TAMs. A luciferase reporter assay and ChIP assay revealed that Gfi-1 directly repressed the transcription of CTGF and HMGB1. Simvastatin also reversed the effects of gemcitabine on the expression of TGF-β1 and Gfi-1 and reduced the resistance of PDAC to gemcitabine in vivo. These results provide the first evidence that simvastatin attenuates the TAM-mediated gemcitabine resistance of PDAC by blocking the TGF-β1/Gfi-1 axis. These findings suggest the TGF-β1/Gfi-1 axis as a novel therapeutic target for treating PDAC.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a highly lethal malignancy with an estimated 5-year survival rate below 5% [1]. Most patients with PDAC are diagnosed in an advanced stage when the tumors are deemed unresectable and exhibit resistance to chemotherapeutic drugs, including gemcitabine and 5-fluorouracil [2]. Thus, it is urgent to explore the mechanism of this resistance and identify novel therapeutic strategies for treating PDAC.

Increasing evidence has identified a key role of tumor microenvironments (TMEs) in the progression of cancer, especially in the stroma-rich PDAC microenvironment [3–5]. Current standard-of-care therapies provide few consistent responses in PDAC, largely due to the heterogeneous TMEs [6]. The cellular component of the TME contains pancreatic stellate cells (PSCs), cancer-associated fibroblasts, various immune cells, endothelial cells and pericytes, in addition to cancer cells [6]. The immune cell population is composed of tumor-associated macrophages (TAMs), monocytic myeloid-derived suppressor cells (Mo-MDSCs), and granulocytic MDSCs (G-MDSCs) [7]. TAMs are the most abundant immune cells in the PDAC stroma [8]. Tumor cells secrete specific cytokines that stimulate TAMs, which in turn promote tumor vascularization, accelerate metastases, and confer resistance to chemotherapeutics [9]. Gemcitabine, the most frequently employed chemotherapeutic agent for treating PDAC, is metabolized to active forms by deoxycytidine kinase. Cytidine deaminase serves as a key enzyme that catalyzes the conversion of gemcitabine to inactive metabolites, and TAMs have been found to enhance the chemoresistance of PDAC by up-regulating cytidine deaminase [10]. In accordance with these findings, gemcitabine is more effective in macrophage-depleted mice than in their wild-type counterparts [11]. Thus, it seems that TAMs attenuate the responses of tumors to gemcitabine.

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However, the exact mechanism by which TAMs contribute to the gemcitabine resistance of PDAC remains largely unknown.

Statins, inhibitors of HMG-CoA, the enzyme that catalyzes cholesterol synthesis, exhibit chemopreventive effects beyond their cholesterol-lowing function. An epidemiological study showed that the use of statins for over 6 months caused a 67% reduction in the risk of pancreatic cancer [12]. A recent clinical case-control study suggested that the reduction in the risk of pancreatic cancer in statin users mainly appears in men and long-term users [13]. A population-based cohort study also implied that statin use significantly reduces the risk of pancreatic cancer in patients with type 2 diabetes [14]. However, two meta-analyses found no significant reduction in pancreatic cancer risks [15,16]. Although a series of studies focused on the association between statin use and the risk of pancreatic cancer have been performed, little attention has been paid to the effect of statins on the resistance of PDAC to gemcitabine.

In this study, we found that TAMs enhanced the resistance of PDAC cells to gemcitabine and that this effect was attenuated by simvastatin treatment. Further investigation demonstrated that simvastatin treatment reduced TGF-β1 levels in TAM-conditioned medium (TAM-CM), leading to increased Gfi-1 expression in PDAC cells. Gfi-1 negatively regulated the expression of CTGF and HMGB1, contributing to the gemcitabine resistance of PDAC cells. Our data provide a novel mechanism for TAM-mediated gemcitabine resistance and suggest simvastatin as a candidate for overcoming the gemcitabine resistance of PDAC.

Materials and methods

Antibodies and reagents

The following monoclonal antibodies (mAbs) and polyclonal antibodies (pAbs) were used: anti-Gfi-1 (pAb, sc-8558) was purchased from Santa Cruz Biotechnology and anti-CTGF (pAb, ab6992), anti-HMGB1 (pAb, ab79823), anti-TGF-β1 (pAb, ab64715) and anti-β-actin (pAb, ab8227) were purchased from Abcam (Cambridge, MA). Cell Counting Kit-8 was purchased from Dojindo Laboratories (Tokyo, Japan). Simvastatin was purchased from Sigma Chemical Co. (USA). Gemcitabine was purchased from Eli Lilly (Indianapolis, IN, USA).

Cell culture

The human PDAC cell lines Panc-1 and BxPC-3 were purchased from American Type Culture Collection (Manassas, VA). Cells were cultured in RPMI 1640 medium (HyClone, USA) supplemented with 10% fetal bovine serum (HyClone, USA), 100 U/mL penicillin, 100 U/mL streptomycin and 0.03% L-glutamine at 37 °C in 5% CO_2.

Preparation of conditioned medium

Macrophage generation was performed as described by Cavel O et al. [17]. Mononuclear cells were isolated from human peripheral blood via Ficoll-Hypaque density gradient centrifugation (Sigma Chemical Co., St. Louis, MO, USA). The obtained mononuclear cells were placed in 6-well microplates for 2 h at 37 °C. After removing the suspended cells, the adhered cells were then cultured for 7 days in RPMI 1640 medium containing 50 ng/ml of M-CSF to generate resting macrophages. Resting macrophages were incubated in serum-free RPMI 1640 medium for 48 h to generate macrophage-conditioned medium (CM). Panc-1 cells were incubated in serum-free RPMI 1640 medium for 48 h to generate tumor-conditioned medium. Resting macrophages were incubated in tumor-conditioned medium to generate TAMs. TAMs were incubated in serum-free RPMI 1640 medium for 48 h to generate TAM-CM. In some experiments, CM or TAM-CM was produced by culturing macrophages or TAMs in serum-free RPMI 1640 medium containing the indicated concentration of simvastatin to examine the effect of the drug.

Cell viability assay

PDAC cells were seeded at a density of 1.0×10^4 cells/well in a 96-well microplate. After the cells reached approximately 80% confluence, the medium was replaced with CM or TAM-CM in the presence or absence of gemcitabine. After incubation for 72 h, the cells were co-cultured with 10 μl of WST-8 dye for 2 h at 37 °C. Absorbance was detected at 450 nm using a SpectraMax M2 plate reader.

Flow cytometric analysis

PDAC cells were seeded at a density of 2×10^5 cells/well in a 6-well microplate. After treatment, cell apoptosis was determined via flow cytometric analysis. Briefly, the cells were digested using trypsin without EDTA and phenol red, followed by incubation with a FITC-conjugated annexin-V reagent (2.5 mg mL^{-1}) and PI (5 mg mL^{-1}). Labeled cells were detected using flow cytometry.

Western blot analysis

Cells were lysed in RIPA lysis buffer. Total protein was quantified using the bicinchoninic acid method. Western blot analyses were performed as previously described [18]. Equal amounts of protein were loaded on a 10% sodium dodecyl sulfate polyacrylamide gel, separated via electrophoresis, and transferred onto polyvinylidene fluoride membranes. Membranes were blocked with 5% BSA and probed with primary antibodies overnight at 4 °C, followed by incubation with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. The signals were detected using an enhanced chemiluminescence method and quantified using an Alpha Imager 2200 system (Alpha, USA).

Immunohistochemistry

Tumor sections were fixed and embedded in paraffin, deparaffinized in xylene and hydrated with ethanol. Antigen retrieval was performed via microwave treatment in a 10 mmol/L sodium citrate buffer for 10 min, followed by treatment with 3% hydrogen peroxide for 15 min and blocking with 10% goat serum for 20 min. Subsequently, the sections were incubated with primary antibodies at 4 °C overnight and then visualized using biotinylated secondary antibodies. The images were captured using a fluorescence microscope (Leica DM IRE2).

ELISA

After the indicated treatments, supernatants were collected and analyzed using a TGF-β ELISA kit (R&D Systems Wiesbaden, Germany) according to the manufacturer's protocol.

Gene transfection

Panc-1 and BxPC-3 cells were seeded at a density of 2×10^5 cells/ml in 6-well microplates. After growing overnight, the cells were transfected with retroviruses carrying shRNAs against Gfi-1 (GeneChem, Shanghai, China), CTGF and HMGB1 (Santa Cruz Biotechnology). For Gfi-1 overexpression, the cells were transfected with Gfi-1-pcDNA3.1 using Lipofectamine 3000.

Luciferase reporter assay

Panc-1 cells were co-transfected with 1 μg of Gfi-1-pcDNA3.1 and 0.3 μg of CTGF or HMGB1 promoter luciferase reporter plasmids using Lipofectamine 3000 (Roche) in TAM-CM; the pRL-SV40 plasmid was transfected for normalization (Promega). After incubation for 24 h, cellular lysates were obtained for use in dual luciferase activity assays performed according to the manufacturer's instructions.

Chromatin immunoprecipitation assay (ChIP)

ChIPs were performed using a ChIP assay kit (Millipore) as described by Geng C et al. [19]. In brief, Panc-1 cells were crosslinked with fresh 1% formaldehyde and then lysed in SDS lysis buffer containing 1% protease inhibitors. The harvested cell lysate was sonicated to shear crosslinked DNA into segments 200–1000 base pairs in length. Protein/DNA complexes were immunoprecipitated with 3 μg of anti-Gfi-1 antibody or control IgG. After being eluted from the antibodies, the complexes were dissociated with 5 M NaCl. ChIP samples were analyzed via real-time quantitative PCR using primers specific for the human CTGF or HMGB1 promoter. The primers are listed in Supplementary Table 1.

Quantitative RT-PCR

Total RNA was extracted using Trizol reagent (Invitrogen). Reverse transcription was performed using the RevertAid First Strand cDNA Synthesis Kit (Fermentas) according to the manufacturer's protocol. cDNA was used as a PCR template to determine the expression of CTGF and HMGB1 using the following primers: CTGF forward (5′-AATGCTGCGAGGAGTGGGT-3′), reverse (5′-CGGCTCTAATC ATAGTTGGGTCT-3′) [20]; HMGB1 forward (5′-TCAAAGGA-GAACATCCTGGC CTGT-3′), reverse (5′-CTGCTTGTCATCTGCAGCAGTGTT-3′) [21]; GADPH forward (5′-GGTGAAGGTCGGAGTCAACGG-3′), reverse (5′-GGTCATG AGTC CTTCCACGATACC-3′) [21]. Real-time PCR reactions were performed using a SYBR Premix ExTaq kit (Takara) and a LightCycler 480 System. GAPDH was used as an internal control.

Mice xenograft models

The orthotopic PDAC nude mouse model was established as previously described [22], with a minor modification. In brief, 4- to 6-week-old BALB/c nude mice of both sexes were purchased from Weitonglihua Animal Center (Beijing, China) and maintained under specific pathogen-free conditions in the animal facility. Panc-1 cells (2×10^6 in 100 μl of PBS) were injected into both flanks of the