Life Sciences 89 (2011) 603–608

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

CCK-2/gastrin receptor signaling pathway is significant for gemcitabine-induced gene expression of VEGF in pancreatic carcinoma cells

Hiroki Kato [a], Koichi Seto [b,*], Nobuyoshi Kobayashi [b], Koji Yoshinaga [b], Tim Meyer [c], Mineo Takei [b]

[a] Department of Clinical Research, Zeria Pharmaceutical Co., Ltd., Tokyo, Japan
[b] Central Research Laboratories, Zeria Pharmaceutical Co., Ltd., Saitama, Japan
[c] UCL Cancer Institute, University College London, UK

ARTICLE INFO

Article history:
Received 19 January 2011
Accepted 21 July 2011

Keywords:
Z-360
CCK-2/gastrin receptor
VEGF
Pancreatic carcinoma
Gemcitabine

ABSTRACT

Aims: As activation and overexpression of the cholecystokinin-2 (CCK-2)/gastrin receptor can lead to carcinogenesis, it has been explored as a therapeutic target in pancreatic cancer. We demonstrated that Z-360, a CCK-2/gastrin receptor antagonist, combined with gemcitabine prolonged survival and reduced gemcitabine-induced vascular endothelial growth factor (VEGF) expression in a pancreatic carcinoma orthotopic xenograft mouse. In this study, we investigated the role of the CCK-2/gastrin signaling pathway on gemcitabine-induced VEGF expression in PANC-1 human pancreatic carcinoma cells.
Main methods: In PANC-1 cells treated with Z-360, anti-gastrin IgG or kinase inhibitors, the gene expression levels were analyzed by quantitative real-time RT-PCR, and the protein levels of Akt and phosphorylated Akt (p-Akt) in cellular extracts were measured by ELISA.
Key findings: Gemcitabine-induced expression of VEGF and hypoxia-inducible factor-1 alpha (HIF-1 alpha) were suppressed by the treatment with an anti-gastrin antibody. In addition, VEGF and HIF-1 alpha gene expression was inhibited by treatment with an inhibitor of phosphatidylinositol 3-kinase (PI3K), which is involved in the downstream signaling pathway of the CCK-2/gastrin receptor, and was also suppressed by treatment with Z-360. Moreover, although Akt phosphorylation was increased by treatment with gemcitabine, this elevation was partially, but significantly, inhibited by an exposure of Z-360.
Significance: Gemcitabine might induce gene expression of VEGF via the PI3K/Akt signaling pathway in the downstream of the CCK-2/gastrin receptor. The suppression of the CCK-2/gastrin signaling pathway by treatment with Z-360 could be a useful approach for potentiating prolonged survival of pancreatic cancer patients receiving gemcitabine therapy.

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Introduction

The cholecystokinin-2 (CCK-2)/gastrin receptor has been proposed as a novel therapeutic target in pancreatic cancer (Wong and Lemoine, 2009). It is known that gastrin stimulates the cell survival and proliferation of gastric, colorectal (Watson et al., 1988), and pancreatic cancer cells (Smith et al., 1995, 1996). A recent study revealed that the intracellular signaling pathway involved in the activation of the CCK-2/gastrin receptor can lead to carcinogenesis (Grabowska and Watoson, 2007). Moreover, studies on transgenic mice overexpressing gastrin or CCK-2/gastrin receptor demonstrated that gastrin is involved in the development of gastric and pancreatic tumors (Clerc et al., 2002; Mathieu et al., 2005). Additionally, down-regulation of gastrin gene expression using short hairpin RNA (shRNA) can inhibit the growth of human pancreatic cancer (Matters et al., 2009). In previous studies, we showed that Z-360, a novel orally active CCK-2/gastrin receptor antagonist, significantly inhibited the growth of subcutaneous xenografts of human pancreatic tumor cells in mice (Grabowska et al., 2008; Kawasaki et al., 2008), and that Z-360 combined with gemcitabine prolonged survival in a pancreatic carcinoma orthotopic xenograft mice (Kawasaki et al., 2008). Together, these various findings indicate that the CCK-2/gastrin receptor or gastrin may be closely correlated with the progression of pancreatic cancer; however, its role in pancreatic cancer is not precisely known.

We previously reported that gemcitabine increased the expression of vascular endothelial growth factor (VEGF), a potent angiogenic peptide, in both an orthotopic xenograft mice and cultured pancreatic cells (Kobayashi et al., 2010). VEGF gene expression is also induced in breast cancer cells upon treatment with gemcitabine (Hernández-Vargas et al., 2007). During hypoxia, which often occurs in tumor microenvironments, the expression of VEGF is up-regulated by the transcription factor hypoxia-inducible factor (HIF-1). Activation of HIF-1alpha is mediated by several signaling pathways, including the phosphatidylinositol 3-kinase (PI3K)/Akt kinase pathway, a major

* Corresponding author at: Central Research Laboratories, Zeria Pharmaceutical Co., Ltd., 2512-1 Oshikiri, Kumagaya, Saitama, 360-0111, Japan. Tel.: +81 48 536 3456; fax: +81 48 539 1072.
E-mail address: kouichi-seto@zeria.co.jp (K. Seto).

0024-3205/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.lfs.2011.07.019

signaling pathway downstream of the CCK-2/gastrin receptor (Kowalski-Chauvel et al., 1996; Vatinel et al., 2006; Arsham et al., 2002), and the Src kinase pathway (Chao et al., 2007).

It was also previously reported that the i4sv variant of the CCK-2/gastrin receptor, which represents a spliced form with intron 4 retention, is constitutively activated and strongly activates Src and FAK kinases compared with the normal form of the receptor (Olszewska-Pazdrak et al., 2002). Other significant effects of the i4sv variant have been noted; for example, CCK-2/gastrin i4sv receptor-expressing cells have elevated expression of HIF-1alpha and VEGF and their injection into mice increased the weight of tumors compared with that of wild-type CCK-2/gastrin receptor (Chao et al., 2007). Although the CCK-2/gastrin receptor participates in carcinogenesis, such as the pancreatic carcinoma described above, little is known about the correlation between the CCK-2/gastrin signaling pathway and the anti-tumor activity of gemcitabine.

Here, to clarify the efficacy of combined CCK-2/gastrin receptor antagonist Z-360 and gemcitabine therapy in cancer, we examined the role of CCK-2/gastrin receptor signaling on the gemcitabine-induced gene expression of VEGFA. We also investigated the induction of the splice variant of the CCK-2/gastrin receptor by gemcitabine.

Materials and methods

Materials

Z-360 was synthesized at the Central Research Laboratories of Zeria Pharmaceutical Co., Ltd (Saitama, Japan). PP2 and wortmannin were purchased from Merck (Darmstadt, Germany). Anti-gastrin antibody (an affinity purified goat polyclonal antibody against C-terminus of human gastrin) and a goat IgG affinity purified were purchased from Santa Cruz Biotechnology (California, USA).

Cell lines

The human pancreatic carcinoma cell line PANC-1 was purchased from the European Collection of Cell Cultures (Wiltshire, UK). PANC-1 cells were grown as adherent cultures in Dulbecco's modified Eagle's medium (DMEM) (Wako, Tokyo, Japan) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; Thermofisher Scientific, Waltham, MA, USA), 100 U/ml of penicillin, and 100 μg/ml of streptomycin (Invitrogen, Carlsbad, CA, USA) in a humidified 5% CO_2 atmosphere at 37 °C.

Gene expression analysis of PANC-1 cells

PANC-1 cells were cultured on collagen-coated 6-well-plates (Becton Dickinson, Franklin Lakes, NJ, USA) for 24 h under the above described conditions and were then treated with either Z-360, gemcitabine, or DMSO (vehicle) at the concentrations indicated in the figures/legends. After 24 h of treatment with the compounds, cells were harvested with lysis buffer from an RNeasy Mini Kit (QIAGEN, Hilden, Germany), and were homogenized using a syringe for the preparation of total RNA.

To examine the effect of kinase inhibitors on gemcitabine-induced gene expression, PANC-1 cells were cultured for 24 h and then incubated with medium containing 10 μM gemcitabine (f.c.). After 21 h, PP2 or wortmannin (1 μM) was added in cultured medium in presence of gemcitabine, and then cells were further incubated for 3 h. Cells were then harvested using lysis buffer from an RNeasy Mini Kit, and were homogenized using a syringe for preparation of total RNA.

To examine the effect of anti-gastrin IgG on gemcitabine-induced gene expression, PANC-1 cells were cultured for 24 h and then incubated for an additional 24 h in the presence of with either 1.32 μg/ml normal IgG, 1.32 μg/ml anti-gastrin IgG, 10 μM gemcitabine at (f.c.). Cells were harvested using lysis buffer from an RNeasy Mini Kit, and were homogenized using a syringe for the preparation of total RNA. Gene expression analysis was then performed by quantitative real-time RT-PCR.

Real-time quantitative RT-PCR

Total RNA was prepared from tumor tissue or cell lysates with an RNeasy Mini Kit (QIAGEN) as described above. After purification, the amount of RNA was measured by spectrophotometry (OD260). Total RNA (2 μg) was converted into first-strand cDNA using Moloney Murine Leukemia Virus Reverse Transcriptase (RNase H free) (Promega, Madison, WI, USA) using oligo (dT) primer (Promega). The first-strand cDNAs were subjected to real time RT-PCR with ABsoluteTM QPCR ROX Mix (Thermofisher Scientific, Waltham, MA, USA) and TaqMan probes for vascular endothelial growth factor A (VEGFA) (Hs00900058_m1) and HIF-1alpha (Hs00153153_m1) using an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The program involved 15 min at 95 °C and then 45 cycles of 15 s at 95 °C and 1 min at 60 °C. Obtained data were normalized for the expression of peptidylprolyl isomerase A (PPIA, 4326316E) and were analyzed by the comparative CT method (Applied Biosystems).

Nested PCR analysis for the CCK-2/gastrin receptor

Nested PCR analysis for the CCK-2/gastrin receptor was performed using a procedure described previously, with minor modifications (Ding et al., 2002). For the initial PCR, briefly, 2 μl aliquots of first-strand cDNA solutions synthesized as described above were mixed with 10 μl Premix Ex *Taq* DNA polymerase and the oligonucleotide primers forward-1, 5′-ATGGAGCTGCTGAAG,CTG,AACC-3′, and reverse-1, 5′-CCTCTACTCCTCAGCCAG-3′, a 20 μl reaction volume. For the initial PCR, the reaction mixture with cDNA was denatured at 94 °C for 2 min and then 35 cycles of 94 °C for 45 s, 56 °C for 45 s, and 72 °C for 3 min were performed. For nested PCR, 2 μl of the initial PCR solution was mixed with 10 μl Premix Ex *Taq* DNA polymerase and the oligonucleotide primers forward-2, 5′-CGGACTACTCATGGTGCCCTA-3′, and reverse-1, 5′-GCCAACCGCGCCAGTCTCAG-3′ in a 20 μl reaction volume. For nested PCR, the reaction mixture was denatured at 94 °C for 2 min and then 35 cycles of 94 °C for 45 s, 60 °C for 45 s, and 72 °C for 1 min were performed. A part of the resulting PCR products were examined by electrophoresis on a 1.8% agarose gel and were visualized under UV light after staining with ethidium bromide.

ELISA for phosphorylated and total Akt

PANC-1 cells were cultured on collagen-coated flasks (Becton Dickinson, Franklin Lakes, NJ, USA) for 24 h under the above described conditions. Cells were then treated with gemcitabine (10 μM) and/or Z-360 (0.1 μM) for 24 h. After treatment with these compounds, cells were harvested using lysis buffer (Cell Signaling Technology, Beverly, MA, USA) containing 1 mM PMSF. Harvested cells were sonicated and centrifuged to obtain cell lysates, which were then used for the quantification of phosphorylated Akt 1 and total Akt 1 using a Pathscan Phospho-Akt 1 (Ser473) Sandwich ELISA kit (Cell Signaling Technology) and a Pathscan Total Akt 1 Sandwich ELISA kit (Cell Signaling Technology).

Western blot analysis for phosphorylated and total Akt

PANC-1 cells were cultured on collagen-coated flasks (Becton Dickinson, Franklin Lakes, NJ, USA) for 24 h under the above described conditions. Cells were then treated with gemcitabine (10 μM) and/or Z-360 (0.1 μM) for 24 h. After treatment with these compounds, cells were homogenized in solubilization buffer containing 50 mM Tris–

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