Cholecystokinin receptor antagonist, loxiglumide, inhibits invasiveness of human pancreatic cancer cell lines

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Abstract Recently, cholecystokinin has been reported to be important in regulating the growth of pancreatic cancer. We investigated the effect of loxiglumide (LXG), a cholecytskinin receptor antagonist, on the invasiveness of two human pancreatic cancer cell lines. Cells were treated with LXG for 24 h, and examined in the invasion assay. The expression and activity of MMP-9 in supernatants from cancer cells were analyzed by Western blotting and zymogram. Interestingly, the invasiveness of cancer cells and expression of MMP-9 were decreased by LXG in a dose-dependent manner. LXG may be a useful therapeutic agent against pancreatic cancer.

Key words: Cholecystokinin receptor; Gelatinase; Invasion; Loxiglumide; Matrix metalloproteinase-9

1. Introduction

Although many therapeutic agents have been tried in pancreatic cancer, its prognosis remains poor. Invasive diseases with liver metastasis, vascular or retroperitoneal invasions are already present in many patients with pancreatic cancer at the time of diagnosis. In tumour invasion and cancer cell metastasis, matrix metalloproteinases (MMPs) play important roles in the degradation of the basement membrane and extracellular matrix (ECM). The expression of MMP-9, whose substrate is gelatin, has been reported in several human carcinomas such as lung [1], breast [2] and several metastatic tumour models [3,4]. These observations suggest that MMP-9 may play an important role in tumour cell invasion [5–7].

Cholecystokinin (CCK) is a gut hormone that stimulates pancreatic enzyme secretion [8], and gallbladder contraction [9]. Recent studies have reported that CCK promotes pancreatic carcinogenesis in animals [10–12], and also stimulates the growth of two human pancreatic cancer cell lines (PANC-1 and MIA PaCa-2) via a low-affinity CCK receptor [13]. Loxiglumide (LXG) has been reported as a selective and specific peripheral CCK-A receptor antagonist [14]. In Japan, a clinical trial has reported that LXG may be a useful agent for pancreatitis [15]. A recent study has reported that LXG inhibits the growth of human pancreatic cancer cell lines [16]. However, no known study has reported the relationship between CCK receptors and the invasiveness of human pancreatic cancer cells.

The present study is the first to show that the CCK receptor antagonist, LXG, inhibits the invasiveness of human pancreatic cancer cell lines.

2. Materials and methods

2.1. Cell culture

Two human pancreatic cancer cell lines, PANC-1 and MIA PaCa-2, purchased from the American Type Culture Collection, were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco Lab., Grand Island, NY, USA), pH 7.4, containing 100 U/ml penicillin-streptomycin (Sigma Chemical Co., St. Louis, MO, USA) and 10% foetal bovine serum (FBS) (Gibco). The cells were grown in 25 cm^2 plastic flasks (Corning, NY, USA), and placed in 5% CO_2 at 37°C.

2.2. Cell treatment

LXG was kindly supplied by Kaken Pharmaceutical Co. Ltd., Chiba, Japan. It was dissolved with phosphate-buffered saline (PBS, Gibco, Grand Island, NY, USA), pH 8.0. CCK-8 (purchased from Peptide Institute, Inc., Osaka, Japan) was also dissolved with PBS. The solution of LXG (1, 5, 10 and 50 μM) and CCK-8 (10^{-9} and 10^{-8} M) were diluted in PBS just before examination. For in vitro experiments, 1×10^6 cells/flask were maintained in DMEM supplemented with 0.1% FBS. After washing twice with fresh serum-free medium, LXG or CCK-8 solutions were added in flasks and maintained in 5% CO_2 at 37°C for 24 h. Thereafter, each supernatant was harvested and centrifuged at 1500 rpm for 5 min to remove cell debris. The protein content of each supernatant was prepared at 25 μg/ml, and used as a sample. Each sample was analyzed by Western blotting and gelatin zymogram.

2.3. Invasion assay

Cells were harvested followed by exposure to trypsin (0.25%) at 37°C for 20 min. After centrifugation (1500 rpm for 5 min), cells were resuspended in medium, and then examined using a Boyden chamber. Cell viability was evaluated with the 0.1% trypan blue exclusion method. Transwell cell culture chambers (Costar no. 3403, Cambridge, MA, USA) with 12-mm diameter filters were used for this assay. Polyvinylpyrollidone-free polycarbonate filters (pore size 12 μm) were coated with basement membrane matrigel (200 μg/filter) (Collaborative Research Inc., Bedford, MA, USA). The coated filters were placed on Boyden chambers, and the cells (1×10^5) suspended in DMEM containing 0.1% FBS were placed in the upper chamber. DMEM containing 0.1% FBS was placed in the lower compartment of the Boyden chambers. The chambers were incubated in 5% CO_2 at 37°C, and the cell numbers of the lower compartment were counted hourly for 6 h [17]. The assays were performed in triplicate.

2.4. Western blot analysis

A volume of 15 μl from each sample as described above was prepared for electrophoresis on a 10% SDS-polyacrylamide gel. The resolved proteins were transferred to a nitrocellulose membrane with 20% methanol, 25 mM Tris-HCl, pH 8.3 and 192 mM glycine at 150 mA and 4°C for 3 h. The membrane blocked with the horse serum and incubated with a mouse monoclonal anti-MMP-9 antibody (2 μg/ml), (IM10, Oncogene Science Inc., Cambridge, MA, USA). After incubating with biotinylated antimouse secondary antibody (Vectastain ABC kit, Vector Labs, Burlingame, California, USA), and a streptavidin-horseradish complex (Vectastain ABC kit), the colour was developed using diaminobenzidine-hydrogen peroxide (Vectastain DAB kit).

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Abbreviations: CCK, cholecystokinin; DMEM, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; FBS, foetal bovine serum; LXG, loxiglumide; MMP, matrix metalloproteinase.

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2.5. Gelatin zymogram

A volume of 15 μl from the same samples as described above was applied to 10% polyacrylamide gel containing 2 mg/ml gelatin. After electrophoresis, the gel was washed with washing buffer (50 mM Tris-HCl, pH 7.4 and 2.5% Triton X-100) at room temperature for 30 min, and then placed in incubation buffer (30 mM Tris-HCl, pH 7.4, 0.2 M NaCl, 10 mM $CaCl_2$ and 0.02% NaN_3) at 37°C overnight. The gel was stained with 0.02% Coomassie brilliant blue in 20% methanol and 10% acetic acid, and destained with 20% methanol and 10% acetic acid. Molecular weights of the gelatinolytic bands were estimated using molecular weight markers (Daiichi Pure Chemicals, Tokyo, Japan).

2.6. Statistical analysis

Invasion assays were performed in triplicate, and results were expressed as mean plus or minus the standard deviation (mean ± SD). Statistical analysis was performed using regression analysis. Statistical significance was assumed with a P value < 0.01.

3. Results

3.1. Invasion assay

This assay can simply evaluate the ability of the cells to attach and degrade the matrix [17]. These events are considered to be important in the invasion of tumour cells through the basement membrane. Benign or normal cells except neutrophils did not penetrate through the basement membrane matrigel [17]. Figs. 1A and 2A show the penetrating cell numbers treated with LXG at 6 h. The generation time of PANC-1 and MIA PaCa-2 cells ranging from 10.5 to 11 h did not affect the cell number in the lower compartment (data not shown). CCK-8, at a dose of 10^{-8} M, increased the numbers of penetrating cells by 50% in PANC-1 and 40% in MIA PaCa-2 compared to untreated cells. In contrast, LXG, at a dose of 50 μM, decreased the number of penetrating cells by 83.1% in PANC-1, and 82.9% in MIA PaCa-2 compared to untreated cells, respectively. The results of regression analysis in PANC-1 and MIA PaCa-2 treated with CCK-8 are shown in Figs. 1B and 2B, and those with LXG are displayed in Figs. 1D and 2D. The numbers of penetrating cells were significantly increased with CCK-8, and decreased with LXG in a dose-dependent manner ($P<0.01$).

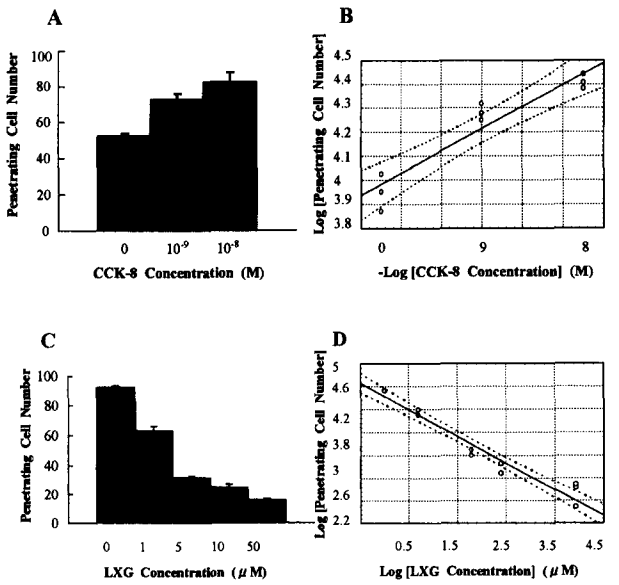

Fig. 1. Invasion assay using a Boyden chamber in PANC-1. (A) Penetrating cell number treated with CCK-8 at 4 h and (B) statistical analysis using regression analysis. The cell numbers penetrating the matrigel are significantly increased in a dose-dependent manner (regression coefficient = 0.94720, $p < 0.01$). (C) Penetrating cell number treated with LXG at 6 h and (D) statistical analysis using regression analysis. The cell numbers penetrating the matrigel are significantly decreased in a dose-dependent manner (regression coefficient = −0.97434, $p < 0.01$).

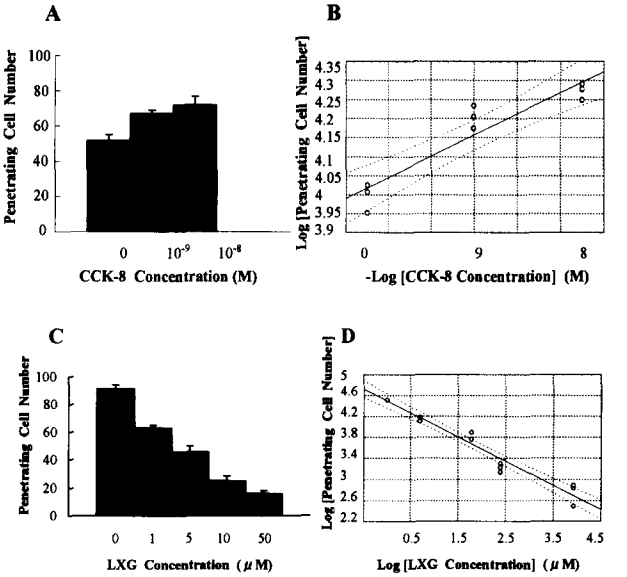

Fig. 2. Invasion assay using a Boyden chamber in MIA PaCa-2. (A) Penetrating cell number treated with CCK-8 at 4 h and (B) statistical analysis using regression analysis. The cell numbers penetrating the matrigel are significantly increased in a dose-dependent manner (regression coefficient = 0.93777, $P < 0.01$). (C) Penetrating cell number treated with LXG at 6 h and (D) statistical analysis using a regression analysis. The cell numbers penetrating the matrigel are significantly decreased in a dose-dependent manner (regression coefficient = −0.92608, $P < 0.01$).

3.2. Western blot analysis

The results of Western blotting to detect the expression of MMP-9 in PANC-1 and MIA PaCa-2 cells are shown in Fig. 3A–D. The expression of MMP-9 tended to increase with CCK-8, and to decrease with LXG in a dose-dependent manner in both cells.

3.3. Gelatin zymogram

A gelatin zymogram assay was performed on each supernatant from PANC-1 and MIA PaCa-2 to examine the effect of CCK-8 and LXG on the gelatinase activity of MMP-9. In this assay, clear zones against the blue background indicated the presence of gelatinolytic activity. Fig. 4A and B demonstrates that the gelatinase activities of MMP-9 in PANC-1 and MIA PaCa-2 tended to increase with CCK-8 in a dose-dependent manner. In contrast, Fig. 4C and D demonstrate that the gelatinase activities of MMP-9 in PANC-1 and MIA PaCa-2 tended to decrease with LXG in a dose-dependent manner. CCK-8 or LXG were added to the media from untreated cells, and incubated at 37°C for 24 h. However, no effect on the gelatinase activities of MMP-9 was observed in the media (data not shown). Additionally, CCK-8 and LXG exert no growth effect in PANC-1 and MIA PaCa-2 for 24 h (data not shown).

4. Discussion

It has been reported that cancer cells may overexpress some receptors which are activated by specific growth factors, and cause the activation of growth signal pathways [18]. Human pancreatic cancer cells overexpress epidermal growth factor (EGF), tumour necrosis factor (TNF-α) and the receptors for EGF [13,19,20]. Some growth factors, such as EGF, transforming growth factor-beta and TNF-α, are considered to be induced and activated MMP-9 [21,22].

A recent study has reported that CCK receptors overexpress in human pancreatic cancer [23]. CCK receptors activated by CCK may stimulate the growth of human pancreatic cancer cell lines [13]. LXG, a specific CCK receptor antagonist, has been reported to inhibit DNA synthesis and the growth of human pancreatic cancer [16]. Many previous reports have provided evidence in support of CCK receptors playing as growth factor receptors. However, little is known as to whether CCK receptors have any potentials in the invasiveness of cancer cells. This is the first study to evaluate the relationship between the CCK receptor and the invasiveness of human pancreatic cancer cell lines. Surprisingly, this study has demonstrated that CCK receptors may play a role not only as growth factor receptors but also to regulate the signal pathways of invasion associated with MMP-9 in human pancreatic cancer. It is unclear how the CCK receptor antagonist can inhibit the invasiveness in these cell lines. Protein kinase C (PKC) is thought to be an important signal transducer of MMP-9 in rat embryo fibroblasts [24]. From this point of view, we are investigating the relationship between the CCK receptor and the signal pathways of MMP-9 mediated by PKC.

In conclusion, our data suggest that LXG may be a useful therapeutic agent against pancreatic cancer. Further studies will be needed to clarify the signal pathways of MMP-9, and to evaluate LXG as a useful therapeutic agent against the invasion of pancreatic cancer in vivo.

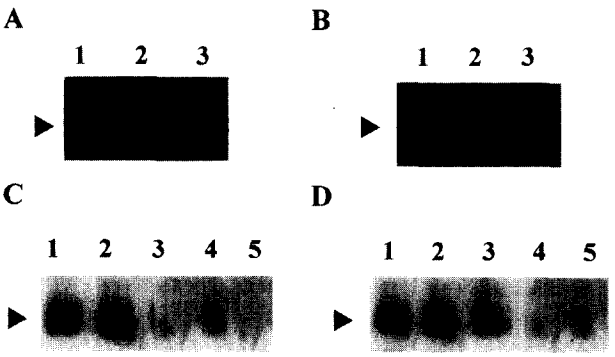

Fig. 3. Western blot analysis. Culture media were collected and subjected to immunoblot analysis with a mouse monoclonal anti-MMP-9 antibody (IM 10). (A) PANC-1 treated with CCK-8. The concentrations of CCK-8 were as follows: 0 (lane 1), 10^{-9} (lane 2) and 10^{-8} M (lane 3). (B) MIA PaCa-2 treated with CCK-8. The concentrations of CCK-8 were as follows: 0 (lane 1), 10^{-9} (lane 2) and 10^{-8} M (lane 3). (C) PANC-1 treated with LXG. The concentrations of LXG were as follows: 0 (lane 1), 1 (lane 2), 5 (lane 3), 10 (lane 4), and 50 μM (lane 5). (D) MIA PaCa-2 treated with LXG. The concentrations of LXG were as follows: 0 (lane 1), 1 (lane 2), 5 (lane 3), 10 (lane 4), and 50 μM (lane 5). The arrowhead indicates the position of MMP-9.

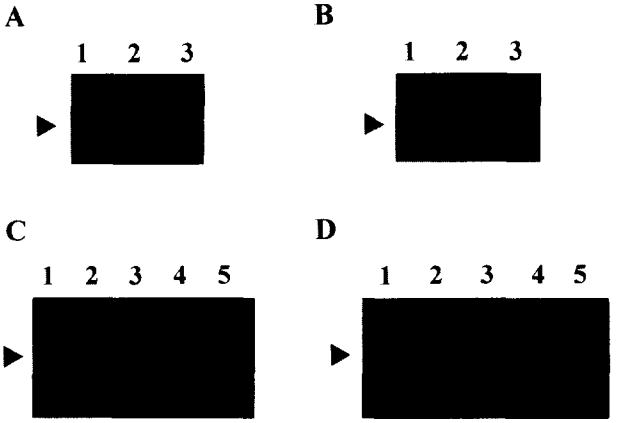

Fig. 4. Gelatin zymogram. The media were collected and subjected to zymography. (A) PANC-1 treated with CCK-8. The concentrations of CCK-8 were as follows: 0 (lane 1), 10^{-9} (lane 2) and 10^{-8} M (lane 3). (B) MIA PaCa-2 treated with CCK-8. The concentrations of CCK-8 were as follows: 0 (lane 1), 10^{-9} (lane 2) and 10^{-8} M (lane 3). (C) PANC-1 treated with LXG. The concentrations of LXG were as follows: 0 (lane 1), 1 (lane 2), 5 (lane 3), 10 (lane 4), and 50 μM (lane 5). (D) MIA PaCa-2 treated with LXG. The concentrations of LXG were as follows: 0 (lane 1), 1 (lane 2), 5 (lane 3), 10 (lane 4), and 50 μM (lane 5). The arrowhead indicates the position of MMP-9.

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