Insulin-like growth factor-I receptor in proliferation and motility of pancreatic cancer

Minoru Tomizawa, Fuminobu Shinozaki, Takao Sugiyama, Shigenori Yamamoto, Makoto Sueishi, Takanobu Yoshida

AIM: To develop a molecular therapy for pancreatic cancer, the insulin-like growth factor-I (IGF-I) signaling pathway was analyzed.

METHODS: Pancreatic cancer cell lines (MIA-Paca2, NOR-P1, PAN-C1, PK-45H, PK-1, PK-59 and KP-4) were cultured in media with 10 mL/L fetal bovine serum. Western blotting analysis was performed to clarify the expression of IGF-I receptor (IGF-IR). Picropodophyllin (PPP), a specific inhibitor of IGF-IR, LY294002, a specific inhibitor of mitogen-activated protein kinase, and PD98059, a specific inhibitor of phosphatidylinositol 3 kinase (PI3K), and PD98059, a specific inhibitor of mitogen-activated protein kinase, were added to the media. After 72 h, a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) assay was performed to analyze cell proliferation. A wound assay was performed to analyze cell motility with hematoxylin and eosin (HE) staining 48 h after addition of each inhibitor.

RESULTS: All cell lines clearly expressed not only IGF-IR but also phosphorylated IGF-IR. PPP significantly suppressed proliferation of MIA-Paca2, NOR-P1, PAN-C1, PK-45H, PK-1, PK-59 and KP-4 cells to 36.9% ± 2.4% (mean ± SD), 30.9% ± 5.5%, 23.8% ± 3.9%, 37.1% ± 5.3%, 10.4% ± 4.5%, 52.5% ± 4.5% and 22.6% ± 0.4%, at 2 µmol/L, respectively (P < 0.05). LY294002 significantly suppressed proliferation of MIA-Paca2, NOR-P1, PAN-C1, PK-45H, PK-1, PK-59 and KP-4 cells to 44.4% ± 7.6%, 32.9% ± 8.2%, 53.9% ± 8.0%, 52.8% ± 4.0%, 32.3% ± 4.2%, 51.8% ± 4.5%, and 30.6% ± 9.4%, at 50 µmol/L, respectively (P < 0.05). PD98059 did not significantly suppress cell proliferation. PPP at 2 µmol/L suppressed motility of MIA-Paca2, NOR-P1, PAN-C1, PK-45H, PK-1, PK-59 and KP-4 cells to 3.0% ± 0.2%, 0%, 0%, 2.0% ± 0.1%, 5.0% ± 0.2%, 3.0% ± 0.1%, and 5.0% ± 0.2%, respectively (P < 0.05). PD98059 at 20 µmol/L did not suppress motility. Cells were observed by microscopy to analyze the morphological changes induced by the inhibitors. Cells in medium treated with 2 µmol/L PPP or 50 µmol/L LY294002 had pyknotic nuclei, whereas those in medium with 20 µmol/L PPP did not show apoptosis.

CONCLUSION: IGF-IR and PI3K are good candidates for molecular therapy of pancreatic cancer.

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INTRODUCTION

Molecular alterations of pancreatic cancer need to be clarified to develop molecular therapy. Growth factors transmit signals to stimulate tumor growth and enhance metastasis. Insulin-like growth factor (IGF)-I signaling plays an important role in the growth and development of many tissues[1]. IGF-I signaling is also thought to be involved in tumorigenesis. Upon ligand binding, the tyrosine kinase of IGF-I receptor (IGF-IR) is activated, and results in autophosphorylation of tyrosine[2]. This initiates a phosphorylation cascade to the phosphatidylinositol-3 kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways.

IGF-I is upregulated in human pancreatic cancer tissues but is not expressed in surrounding non-cancerous tissue[3]. Serum level of IGF-I is elevated in pancreatic cancer patients[4]. Histological analysis has shown that IGF-IR is positive in the membrane of cells of pancreatic cancer tissues but is not expressed in surrounding non-tumorous tissues[3,5]. These facts imply that IGF-I acts as a growth factor for pancreatic cancer. IGF-IR is phosphorylated solely in pancreatic cancer tissues, and not in surrounding non-tumorous tissues[6]. The present study suggests that IGF-IR transmits signals to downstream pathways. This hypothesis is supported by the facts that IGF-I stimulates DNA synthesis of PANC-1 and MIA Paca2 pancreatic cancer cell lines, and motility of HPAF-II, another pancreatic cancer cell line[3,5].

IGF-IR is not involved in metabolism, therefore, it was expected that inhibition of IGF-IR and its downstream pathway would not cause any adverse effects[7]. We therefore focused on the IGF-IR signaling pathway. Pancreatic cancer tissues express growth factors other than IGF-IR[8]. We used fetal bovine serum (FBS) as a growth model of pancreatic cancer cells instead of addition of IGF-I in serum-free medium, because pancreatic cancer tissues and serum from patients contain various growth factors.

MATERIALS AND METHODS

Culture and reagents
Pancreatic cancer cell lines, PANC-1, NOR-P1, PK-45H, PK-1, PK-59, and MIA-Paca2 were purchased from RIK-EN Cell Bank (Tsukuba, Japan)[9]. NOR-P1 was cultured in Dulbecco's Minimum Essential Medium (DMEM): F12 medium and MIA-Paca2 was cultured in DMEM, and the others in Roswell Park Memorial Institute (RPMI)-1640 (Sigma, St. Louis, MO, USA). All the media were purchased from Sigma, and supplemented with 100 mL/L FBS (Trace Scientific, Melbourne, Australia). All the cell lines were cultured in 50 mL/L carbon dioxide at 37℃ in a humidified chamber. LY294002, a specific inhibitor of PI3K, and PD98059, a specific inhibitor of MAPK, were purchased from Wako Pure Chemicals (Osaka, Japan), and picropodophyllin (PPP), a specific inhibitor of IGF-IR, was from Calbiochem (Darmstadt, Germany)[10].

Western blotting analysis
Protein was isolated from cells after 72 h culture. Twenty micrograms of protein was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to a nylon filter. Primary antibodies were polyclonal rabbit anti-IGF-IR antibody, anti-phosphorylated IGF-IR (Cell Signaling Technology, Danvers, MA, USA), and mouse monoclonal anti-tubulin-α antibody (Lab Vision, Fremont, CA, USA). Second antibodies were horseradish peroxidase (HRP)-linked anti-rabbit antibody (Amersham Bioscience, Tokyo, Japan) and HRP-linked anti-mouse antibody (Amersham Bioscience). Dilutions were 1:500 for primary antibodies, and 1:1000 for second antibodies. The filter was reprobed with anti-tubulin-α antibody. The specific antigen-antibody complexes were visualized by enhanced chemiluminescence (GE Healthcare Bio-Sciences Corp, Piscataway, NJ, USA).

Cell proliferation assay
Cells were trypsinized, harvested, and spread onto 96-well flat-bottom plates (Asahi Techno Glass, Tokyo, Japan) at a density of 1000 cells per well. Following 24 h of culture under RPMI-1640, DMEM, or DMEM: F12 with 100 mL/L FBS, medium was changed to RPMI-1640, DMEM, or DMEM: F12 without FBS, respectively, to quench the FBS effects. After 24 h of culture, the medium was changed to RPMI-1640, DMEM, or DMEM:F12 with 100 mL/L FBS, in addition to LY294002, PD98059, or PPP. After 72 h, a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) assay was performed according to the manufacturer's instructions (Promega Corporation, Tokyo, Japan). MTS was bioreduced by cells into a colored formazan product that reduced absorbance at 490 nm. Absorbance was analyzed with a multiple plate reader at a wavelength of 490 nm with a BIO-RAD iMark microplate reader (Bio-RAD, Hercules, CA, USA).

Wound assay
Wound assays were performed according to a previously described procedure[11]. Briefly, cells were spread onto four-well chambers (Becton Dickinson, Franklin Lakes, NJ, USA). Cells were then cut with a sterile razor, and stained with hematoxylin and cosin (HE). Five images were taken by a light microscope (Olympus, Tokyo, Japan). For each
experiment, the number of cells that migrated more than 150 µm per 100 µm cut surface was counted.

**Statistical analysis**
Cell proliferation and wound assay were analyzed statistically by one-factor analysis of variance. Statistical analysis was performed with JMP5.0J (SAS Institute Japan, Tokyo, Japan). \( P < 0.05 \) was accepted as statistically significant.

## RESULTS

### Expression and phosphorylation of IGF-IR in pancreatic cancer
To reveal involvement of IGF-IR in proliferation of pancreatic cancer cell lines stimulated with FBS, protein was isolated and subjected to Western blotting analysis. All cell lines clearly expressed not only IGF-IR but also phosphorylated IGF-IR, which suggested that IGF-IR played a role in proliferation with FBS (Figure 1). This result prompted us to analyze the effects of IGF-IR inhibitors.

### Suppression of proliferation of pancreatic cancer with inhibitors
The MTS assay was performed to clarify whether PPP, LY294002, or PD98059 suppressed proliferation of pancreatic cancer cell lines. PPP suppressed proliferation of all the cell lines examined (Figure 2A). At 2 µmol/L, MIA-Paca2, NOR-P1, PANC-1, PK-45H, PK-1, PK-59 and KP-4 proliferation was reduced to 36.9% ± 2.4% (mean ± SD), 30.9% ± 5.5%, 23.8% ± 3.9%, 37.1% ± 5.3%, 10.4% ± 4.5%, 52.5% ± 4.5% and 22.6% ± 0.4%, respectively (\( P < 0.05, n = 3 \)). Next, we analyzed the downstream pathway of IGF-IR. LY294002 suppressed proliferation of all the cell lines examined (Figure 2B). At 50 µmol/L, proliferation of MIA-Paca2, NOR-P1, PANC-1, PK-45H, PK-1, PK-59 and KP-4 cells was reduced significantly to 44.4% ± 7.6%, 32.9% ± 8.2%, 53.9% ± 8.0%, 52.8% ± 4.0%, 32.3% ± 4.2%, 51.8% ± 4.5%, and 30.6% ± 9.4%, respectively (\( P < 0.05, n = 3 \)). PD98059 did not suppress cell proliferation (Figure 2C). Although NOR-P1 and KP-4 cells showed a marginal decrease in proliferation in the presence of 20 µmol/L PD98059, we could not analyze higher concentrations because PD98059 precipitated in the medium. Analysis with 50 µmol/L PD98059 failed since PD98059 crystallized and precipitated. The other inhibitor of MAPK was not analyzed.

### Suppression of motility of pancreatic cancer with inhibitors
Suppression of motility is the initial step in the inhibition of metastasis. We analyzed changes in motility with inhibitors, by the wound assay (Figure 3). PPP at 2 µmol/L suppressed motility of MIA-Paca2, NOR-P1, PANC-1, PK-45H, PK-1, PK-59 and KP-4 cells to 3.0% ± 0.2%, 0%, 0%, 2.0% ± 0.1%, 5.0% ± 0.2%, 3.0% ± 0.1%, and 5.0% ± 0.2%, respectively (\( P < 0.05, n = 3 \)). LY294002 at 50 µmol/L suppressed motility of MIA-Paca2, NOR-P1, PANC-1, PK-45H, PK-1, PK-59 and KP-4 cells to 3.0% ± 0.2%, 0%, 3.0% ± 0.2%, 0%, 0%, and 3% ± 0.1%, respectively (\( P < 0.05, n = 3 \)). PD98059 at 20 µmol/L did not suppress motility, although NOR-P1 cells showed a marginal, non-significant decrease.

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**Figure 1** Western blotting analysis of pancreatic cell lines. Protein was isolated from cultured pancreatic cancer cell lines in 100 mL/L fetal bovine serum (FBS), and subjected to western blot analysis. It was shown clearly that all cell lines expressed IGF-IR as well as phosphorylated IGF-IR (pIGF-IR). Tubulin-α was used as an internal control. 1: MIA-Paca2; 2: NOR-P1; 3: PANC-1; 4: PK-45H; 5: PK-1; 6: PK-59; 7: KP-4.

**Figure 2** MTS assay of cells cultured with inhibitors. Pancreatic cancer cell lines were cultured in 100 mL/L FBS with picropodophyllin (PPP) (A), LY294002 (B), or PD98059 (C). After 72 h, MTS assay was performed to analyze the change in cell numbers. PPP and LY294002 significantly suppressed cell numbers, whereas PD98059 did not (\( P < 0.05, n = 3 \)).
Wound assay was performed to analyze cell motility. Almost no cells cultured with PPP or LY294002 migrated > 150 µm, whereas those with PD98059 showed motility of > 150 µm (P < 0.05, n = 3). Solid line: Edge of scratch; Dotted line: 150 µm from a solid line. Original magnification: 4 ×; Scale bar: 100 µm. FBS: Medium with 100 mL/L FBS; PPP: Medium with 2 µmol/L PPP; LY: Medium with 50 µmol/L LY294002; and PD: Medium with 20 µmol/L PD98059. Original magnification: 20 ×; Scale bar: 25 µm, the arrows indicate cells in apoptosis.

**Microscopic features of pancreatic cancer with inhibitors**

Cells were observed under the microscope to analyze the morphological changes induced by inhibitors. Cells in medium with 2 µmol/L PPP or 50 µmol/L LY294002 had pyknotic nuclei, which suggested that they were apoptotic (Figure 4). Cells in medium with 20 µmol/L PD98059 did not show signs of apoptosis (Figure 4).

**DISCUSSION**

IGF-I stimulates thymidine incorporation of pancreatic cancer cell lines, such as MIA-Paca2, as strongly as FBS, which suggests that it is an autocrine growth factor[13]. It was expected that inhibition of IGF-IR activity would lead to pancreatic cancer regression. Antisense oligonucleotide to IGF-IR suppresses proliferation of ASPC-1, a pancreatic cancer cell line[14]. Antisense oligonucleotide to K-ras synergistically enhances the suppression of pancreatic cancer cell lines by antisense oligonucleotide to IGF-IR[15]. Antisense oligonucleotide is unstable in blood, therefore, small molecules that inhibit the tyrosine kinase of IGF-IR are desirable. NVP-AEW541, an IGF-IR inhibitor, suppresses growth of HPAF-II cells, a pancreatic cancer cell line[15]. However, it is possible that NVP-AEW541 does not affect other cell lines. PPP is the first inhibitor to discriminate IGF-IR and insulin receptor[16]. In our experiments, PPP suppressed proliferation of all pancreatic cancer cell lines. Previous studies and the present one indicate that IGF-IR is a good candidate for molecular therapy of pancreatic cancer.

The downstream pathway of the IGF-I receptor is intriguing since shutting down the pathway could reduce cancer. Antibody to IGF-IR suppresses phosphorylation of Akt, a downstream molecule of PI3K and MAPK[13]. LY294002 significantly suppresses proliferation of PAN-1 and BxPC3 cells, and viability of KP-4 and PAN-1 cells[13]. Our results showed that LY294002 suppressed proliferation of all the pancreatic cancer cell lines examined. In addition to proliferation, our data clearly demonstrated that LY294002 suppressed cell migration. We conclude that PI3K is a suitable target for molecular therapy of pancreatic cancer.
MAPK is another downstream pathway of IGF-1R. MIA-PaCa2 treated with 20 µmol/L PD98059 in 100 mL/L FBS showed downregulation of phosphorylated extracellular signal-regulated kinase 1/2[18]. Although it was expected that PD98059 would suppress proliferation of pancreatic cancer cells, 20 µmol/L PD98059 did not suppress proliferation and motility of pancreatic cancer cells in our experiments. PD98059 at 20 µmol/L does not suppress DNA synthesis stimulated by IGF-1 in the culture medium, although phosphorylation of MAPK is suppressed[19]. Curiously, PD98059 stimulates cell growth at concentrations of 0.1 µmol/L to 0.1 pmol/L.[20] SB203580, another inhibitor of MAPK, stimulates growth of PAN-C1 cells in serum-free conditions[21]. In our experiments, PD98059 marginally stimulated growth of PAN-C1 cells with 10 mL/L FBS. These results suggest that inhibition of MAPK does not always suppress growth of pancreatic cancer cell lines with unknown mechanisms. It may be concluded that IGF-1R and PI3K are good candidates for molecular therapy of pancreatic cancer.

COMMENTS

Background
Insulin-like growth factor-I receptor (IGF-IR) is expressed in pancreatic cancer tissues but not in surrounding non-cancerous tissues. Although it was speculated that IGF-I might play a role in proliferation of pancreatic cancer, the detailed mechanism is not known. Research frontiers
IGF-IR is not involved in metabolism, therefore, it is expected that inhibition of IGF-IR and its downstream pathway should not cause adverse effects when used as a target for molecular therapy of pancreatic cancer. Innovations and breakthroughs
IGF-IR was phosphorylated in cultured pancreatic cancer cell lines, which suggested that IGF-IR and its downstream pathway were activated. Each inhibitor of IGF-IR or phosphatidylinositol-3-kinase (PI3K) suppressed not only cell proliferation but also motility. Applications
By revealing that IGF-IR was activated, this study indicated that IGF-IR might be a good candidate for molecular therapy of pancreatic cancer. Terminology
IGF-1 is a growth factor that is involved in cell proliferation and differentiation. Stimulation of IGF-I is transmitted via IGF-I to downstream pathways, such as PI3K and mitogen-activated protein kinase. Peer review
The authors have demonstrated that IGF-IR, phosphorylated and total, is involved in the proliferation of pancreatic cell lines. In Figure 1, we see that all cell lines expressed phosphorylated and total IGF-IR. Their experiments have shown that inhibition of IGF-IR activity results in a decrease in proliferation and motility of pancreatic cancer cell lines. These findings, along with the inhibition of PI3K, are interesting and show promise.

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