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Insulin-Like Growth Factor I Receptor Involvement in Proliferation of NOR-P1 Cells in Serum-Free Media

Minoru Tomizawa,[1*] Fuminobu Shinozaki,[2] Takao Sugiyama,[3] Shigenori Yamamoto,[4] Makoto Sueishi,[3] and Takanobu Yoshida[5]

[1]*Department of Gastroenterology, National Hospital Organization Shimoshizu Hospital, 934-5 Shikawatashi, Yotsukaido City, Chiba 284-0003, Japan*

[2]*Department of Radiology, National Hospital Organization Shimoshizu Hospital, 934-5 Shikawatashi, Yotsukaido City, Chiba 284-0003, Japan*

[3]*Department of Rheumatology, National Hospital Organization Shimoshizu Hospital, 934-5 Shikawatashi, Yotsukaido City, Chiba 284-0003, Japan*

[4]*Department of Pediatrics, National Hospital Organization Shimoshizu Hospital, 934-5 Shikawatashi, Yotsukaido City, Chiba 284-0003, Japan*

[5]*Department of Internal Medicine, National Hospital Organization Shimoshizu Hospital, 934-5 Shikawatashi, Yotsukaido City, Chiba 284-0003, Japan*

ABSTRACT

Insulin-like growth factor (IGF)-I is up-regulated in pancreatic cancer tissues. Pancreatic cancer cell lines were analyzed in serum-free media as a model of the fibrous tissues that these cells often invade. Pancreatic cancer surgical specimens were immunostained with anti-IGF-I receptor (IGF-IR)β antibody. The growth of pancreatic cancer cells in serum-free media was also analyzed. Cell lysates were analyzed for protein by western blot analysis. Cells cultured in the presence of picropodophyllin (PPP), LY294002, or PD98059, were subjected to cell proliferation and scratch assays. In addition, BrdU uptake and apoptosis were analyzed in these cells. IGF-IRβ was detected in pancreatic cancer cells invading fibrous tissues. NOR-P1 grew most rapidly in serum-free media. The concentrations of IGF-I and IGF-II in the media were higher in NOR-P1 than the other cell lines. Cell proliferation in NOR-P1 cells was enhanced by IGF-I or IGF-II treatment more than in MIA-Paca2 or PK-1 cells. PPP, LY294002, and PD98059 suppressed proliferation and motility of NOR-P1 cells and inhibited BrdU uptake, while PPP induced apoptosis. IGF-IRβ may be a potential therapeutic target to inhibit invasion of pancreatic cancer. J. Cell. Biochem. 113: 2714–2720, 2012.

KEY WORDS: 5-BROMO-2′-DEOXYURIDINE; APOPTOSIS; CELL PROLIFERATION; SCRATCH ASSAY

Pancreatic cancer is associated with metastasis even at an early stage [Mao et al., 1995]. Molecular events leading to the loss of cohesiveness of cancer cells, such as alterations in the expression or activity of E-cadherin, cause cell dissemination and are associated with poor prognosis [Vogel et al., 1999; Winter et al., 2008]. Inhibiting the dissemination of tumor cells would significantly improve the prognosis of pancreatic cancer patients.

Insulin-like growth factor (IGF)-I signaling plays an important role in the development and growth of cancers [LeRoith and Roberts, 2003]. Upon ligand binding, the IGF-I receptor (IGF-IR) tyrosine kinase is activated and the receptor is autophosphorylated [Samani et al., 2007]. This initiates a phosphorylation cascade that activates the phosphatidyl inositol-3 (PI3) kinase and mitogen activated protein (MAP) kinase pathways. IGF-I is up-regulated in human pancreatic cancer tissues, but it is not expressed in surrounding non-cancerous ones [Bergmann et al., 1995]. The serum level of IGF-I is elevated in pancreatic cancer patients [Karna et al., 2002]. Histological analyses have shown that IGF-IR is positive in the cell membrane in pancreatic cancer tissues while it is not expressed in surrounding non-tumor tissues [Bergmann et al., 1995; Ouban

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*Correspondence to: Minoru Tomizawa, MD, PhD, Department of Gastroenterology, National Hospital Organization Shimoshizu Hospital, 934-5 Shikawatashi, Yotsukaido City, Chiba 284-0003, Japan
E-mail: nihminor-cib@umin.ac.jp

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et al., 2003]. These facts imply that IGF-I acts as a growth factor in pancreatic cancer. Interestingly, IGF-IR is phosphorylated solely in pancreatic cancer tissues [Stoeltzing et al., 2003]. Inhibition of IGF-I signaling could potentially suppress the progression of pancreatic cancer.

IGF-IR knockdown mediated by short hairpin (sh) RNA suppressed cell proliferation in the pancreatic cancer cell line BxPC3 [Wang et al., 2010]. We previously reported that picropodophyllin (PPP) and LY294002 successfully suppressed the proliferation of several pancreatic cancer cell lines [Tomizawa et al., 2010]. Serum-free media has been used as a model system to reproduce the microenvironment of fibrous tissues [Boraldi et al., 2007]. In the present study, IGF-IR was assessed as a candidate target for molecular therapy for the inhibition of tumor cell invasion into fibrous tissues using serum-free media as a model system. Based on the different responses of cell lines to specific inhibitors, IGF-IR, PI3 kinase, or MAP kinase inhibitors were tested to identify the most effective agent.

MATERIALS AND METHODS

IMMUNOSTAINING

Serial sections of human pancreatic cancer tissue from an adenocarcinoma of a 62-year-old man (BioChain, Hayward, CA) were deparaffinized, autoclaved, and incubated with hydrogen peroxide, followed by a 30-min incubation with 2% normal goat serum in phosphate buffered saline (PBS) (washing buffer). After overnight incubation with rabbit polyclonal anti-IGF-IRβ antibody (1:500) (Cell Signaling Technology Japan, K.K., Tokyo, Japan), specimens were rinsed with PBS, and incubated with horseradish peroxidase (HRP) labeled anti-rabbit antibody (1:500) (GE Healthcare Japan, Tokyo, Japan). Diaminobenzidine (DAKO Japan, Tokyo, Japan) was applied and nuclei were stained with hematoxylin (Muto Pure Chemicals Co., Ltd., Tokyo, Japan) for 15 s. One specimen was incubated without primary antibody as a negative control. Another specimen was stained with hematoxylin and eosin (Muto Pure Chemicals Co., Ltd.). Specimens were observed and photographed with a Provis AX80 microscope (Olympus).

CULTURE, CELL NUMBER COUNTING, AND CONCENTRATION OF IGF-I OR IGF-II

The pancreatic cancer cell lines MIA-Paca2, PANC-1, NOR-P1, PK-45H, PK-1, PK-59, and KP-4 were purchased from RIKEN Cell Bank (Tsukuba, Japan) [Sato et al., 2000]. NOR-P1 was cultured in Dulbecco's Minimum Essential Medium (DMEM): F12 and MIA-Paca2 was cultured in DMEM. The remaining cell lines were cultured in RPMI-1640 (Sigma, St. Louis, MO). Media were purchased from Sigma and supplemented with 10% fetal bovine serum (FBS) (Life Technologies Japan Ltd., Tokyo, Japan). All the cell lines were cultured in 5% carbon dioxide at 37°C in a humidified chamber. Cells were spread onto 6-well plates at a density of 10,000 cells per well and incubated in serum-free media with or without IGF-I (Wako Pure Chemicals, Osaka, Japan) or IGF-II (Wako Pure Chemicals). On days 1, 4, and 7, cells were trypsinized and counted by Trypan blue exclusion. Two days after plating, the media were collected and the concentration of IGF-I or IGF-II was analyzed with Human IGF-I Quantikine ELISA Kit (R&D systems, Minneapolis, MN) or Human IGF-II ELISA (Boster Biological Technology, Ltd., Abingdon, UK), respectively.

WESTERN BLOT ANALYSIS

Protein was isolated from cells after 72 h of culture. A 10-μg sample of protein was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis, and transferred to a nylon filter. After a 30-min incubation with 5% skim milk, filters were incubated with primary antibodies for 1 h at room temperature (1:2,000). After 1 h of incubation with secondary antibodies at room temperature (1:2,000), the specific antigen-antibody complexes were visualized by enhanced chemiluminescence (GE Healthcare Japan). Rabbit polyclonal anti-IGF-IRβ antibody, rabbit polyclonal anti-PI3 kinase antibody, rabbit polyclonal anti-phosphorylated PI3 kinase antibody, rabbit polyclonal anti-ERK, and rabbit polyclonal anit-phosphorylated ERK antibodies were purchased from Cell Signaling Technologies Japan K.K., and mouse monoclonal anti-tubulin-α antibody was form Lab Vision (Fremont, CA). Rabbit polyclonal anit-Cyclin D1 antibody was purchased from Epitomics, Inc. (Burlingame, CA). Rabbit polyclonal anti-phosphorylated IGF-IRβ antibody was purchased from GenScript (Piscataway, NJ). Mouse monoclonal anti-IGF-I antibody was purchased from Abcam (Cambridge, MA). Mouse monoclonal anti-IGF-II antibody was purchased from R&D systems. Secondary antibodies (1:2,000), HRP-linked anti-rabbit antibody and HRP-linked anti-mouse antibody, were purchased from GE Healthcare Japan. The filter was reprobed with anti-tubulin-α antibody. The expression levels of phosphorylated IGF-IRβ were normalized with IGF-IRβ and analyzed using ImageJ64 imaging software (National Institutes of Health, Bethesda, MD).

CELL PROLIFERATION ASSAY

Cells were seeded in 96 well plates (Asahi Techno Glass) at a density of 1,000 cells/well. Twenty-four hours later, the medium was replaced by DMEM without FBS and with or without PPP (Calbiochem, Darmstadt, Germany), a specific IGF-IR inhibitor; LY294002 (Wako Pure Chemicals, Osaka, Japan), a PI3 kinase inhibitor; or PD98059 (Wako Pure Chemicals), a MAP kinase inhibitor [Tomizawa and Saisho, 2006]. After 72 h, the 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 bio-reduced by cells into a colored formazan product, the absorbance of which was analyzed at a wavelength of 490 nm with an iMark microplate reader (Bio-Rad, Hercules, CA).

SCRATCH ASSAY

Scratch assays were performed according to a previously reported procedure [Fujimoto et al., 2009]. Briefly, cells were spread onto 4-well chambers (Becton Dickinson, Franklin Lakes, NJ), cut with a sterile razor, and stained with hematoxylin and eosin 48 h later. Five images were taken with a light microscope (IMT-2) (Olympus, Tokyo, Japan). For each experiment, the number of cells migrating more than 100 μm per 100 μm cut surface was counted.

BRDU UPTAKE AND APOPTOSIS

5′-Bromo-2′-deoxyuridine (BrdU) uptake by cultured cells was analyzed with 5′-bromo-2′-deoxyuridine labeling & detection kit 2 (Roche Diagnostics, K.K., Tokyo, Japan). Cultured cells were labeled with BrdU at the concentration of 10 μM for 45 min. The labeled cells were immunostained with anti-BrdU antibody. HRP labeled anti-mouse antibody was incubated and the conjugates were visulalized with 3,3′-diaminobenzidine (DAB). Apoptotic cells were detected with Apoptosis in situ Detection Kit Wako (Wako Pure Chemicals). Analysis of apoptotic cells was based on the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) procedure, which consists of the addition of fluorescein-dUTP to the 3′ terminals of apoptotically fragmented DNA with TdT followed by immunochemical detection using an anti-fluorescein antibody conjugated with HRP and DAB as a substrate. Numbers of cells positive for BrdU or apoptosis were counted from five different fields on photographic images (AX80, Olympus).

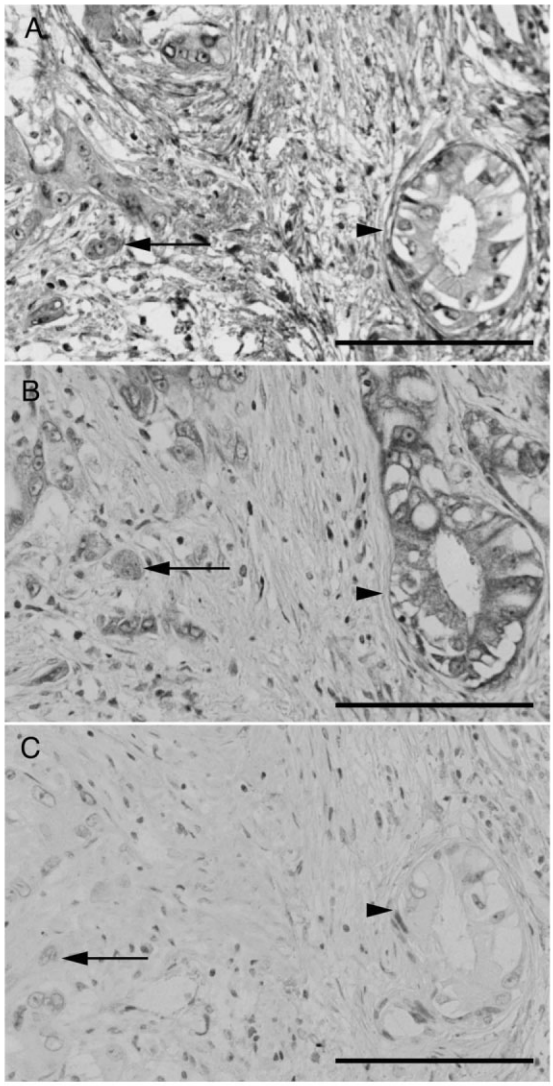

Fig. 1. Immunostaining. Pancreatic cancer tissue was stained with IGF-IRβ. Pancreatic cancer cells (arrows) invade fibrous tissues far apart from cancer cell nests (arrow heads). A: Hematoxylin and eosin (H&E) staining, (B) immunostaining with anti-IGF-IRβ, (C) negative control (without primary antibody). Original magnification: ×50, scale bar: 1 mm.

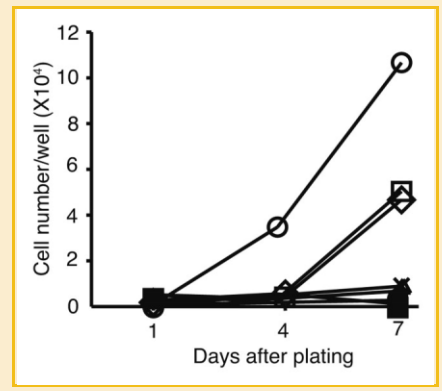

Fig. 2. Growth curve of pancreatic cancer cell lines. Cell numbers were counted on days 1, 4, and 7 after plating of 10^4 cells onto each well of 6-well plates in media without FBS. NOR-P1 cells grew most rapidly. MIA-Paca2 (●), Panc-1 (■), NOR-P1 (○), PK-45H (□), PK-1 (▲), PK-59 (□),

STATISTICAL ANALYSIS

Statistical analysis was performed by one-factor analysis of variance with JMP 8.0 (SAS Institute Japan, Tokyo, Japan). $P < 0.05$ was considered statistically significant.

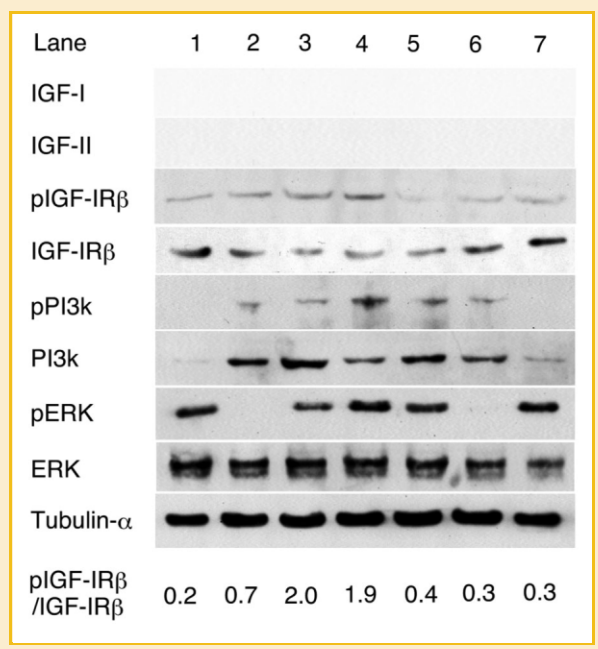

Fig. 3. Western blot analysis. Proteins extracted from pancreatic cancer cells were analyzed with anti-IGF-IRβ or anti-phosphorylated IGF-IRβ (pIGF-IRβ) antibodies. Under serum-free conditions, all the pancreatic cancer cell lines expressed IGF-IRβ, and the IGF-IRβ was phosphorylated. Tubulin-α was used as a loading control. Lane 1: MIA-Paca2, 2: Panc-1, 3: NOR-P1, 4: PK-45H, 5: PK-1, 6: PK-59, 7: KP-4. pIGF-IRβ/IGF-IRβ: The expression levels of phosphorylated IGF-IRβ normalized against those of IGF-IRβ.

RESULTS

Pancreatic cancer cells were detected as small clusters invading surrounding fibrous tissues (Fig. 1 A). These cells were positive for IGF-IRβ (Fig. 1 B), while there were no IGF-IRβ-positive cells in the negative control (Fig. 1 C). To further investigate the intriguing mechanism of fibrous tissue invasion of cancer cells, pancreatic cancer cells cultured in serum-free media were used as a model system.

Seven pancreatic cancer cell lines were cultured in serum-free media, and cell proliferation was assessed. Culture of PK45H, PK-1, and NOR-P1 in serum-free media showed that NOR-P1 had the fastest growth, and NOR-P1 was therefore used for further experiments (Fig. 2).

The expression of IGF-IRβ was analyzed in the pancreatic cancer cell lines based on its expression in fibrous tissues in pancreatic cancer (Fig. 3), and the results showed positive expression in all cell lines analyzed. Unexpectedly, IGF-IRβ was phosphorylated in pancreatic cancer cells, suggesting that IGF-IR was activated even in serum-free media. The expression levels of phosphorylated IGF-IRβ were normalized with those of IGF-IRβ. NOR-P1 showed higher expression level of phosphorylated IGF-IRβ than MIA-Paca2 and PK-1. PI3 kinase was not phosphorylated in MIA-Paca2 and KP4. ERK was not phosphorylated in PANC1 and PK-59.

TABLE I. Concentration of IGF-I and IGF-II in the Medium.

	IGF-I (ng/ml)	IGF-II (ng/ml)
MIA-Paca2	0.30 ± 0.05	0.30 ± 0.05
Panc-1	0.23 ± 0.04	0.26 ± 0.07
NOR-P1	0.45 ± 0.04*	0.37 ± 0.04*
PK-45H	0.26 ± 0.07	0.20 ± 0.02*
PK-1	0.17 ± 0.06*	0.23 ± 0.05
PK-59	0.24 ± 0.04	0.25 ± 0.03
KP-4	0.38 ± 0.05	0.34 ± 0.09

Average ± standard deviation.
*$P < 0.05$ (one factor ANOVA), n = 3.

The concentration of IGF-I and IGF-II in the serum-free media from the cell culture was analyzed using ELISA (Table I). All the cell lines produced less than 1 ng/ml of IGF-I or IGF-II. NOR-P1 had higher concentration of IGF-I and IGF-II in than the other cell lines.

To examine the effects of IGF-I and IGF-II, cell proliferation was assessed and western blot analysis was performed in cells cultured

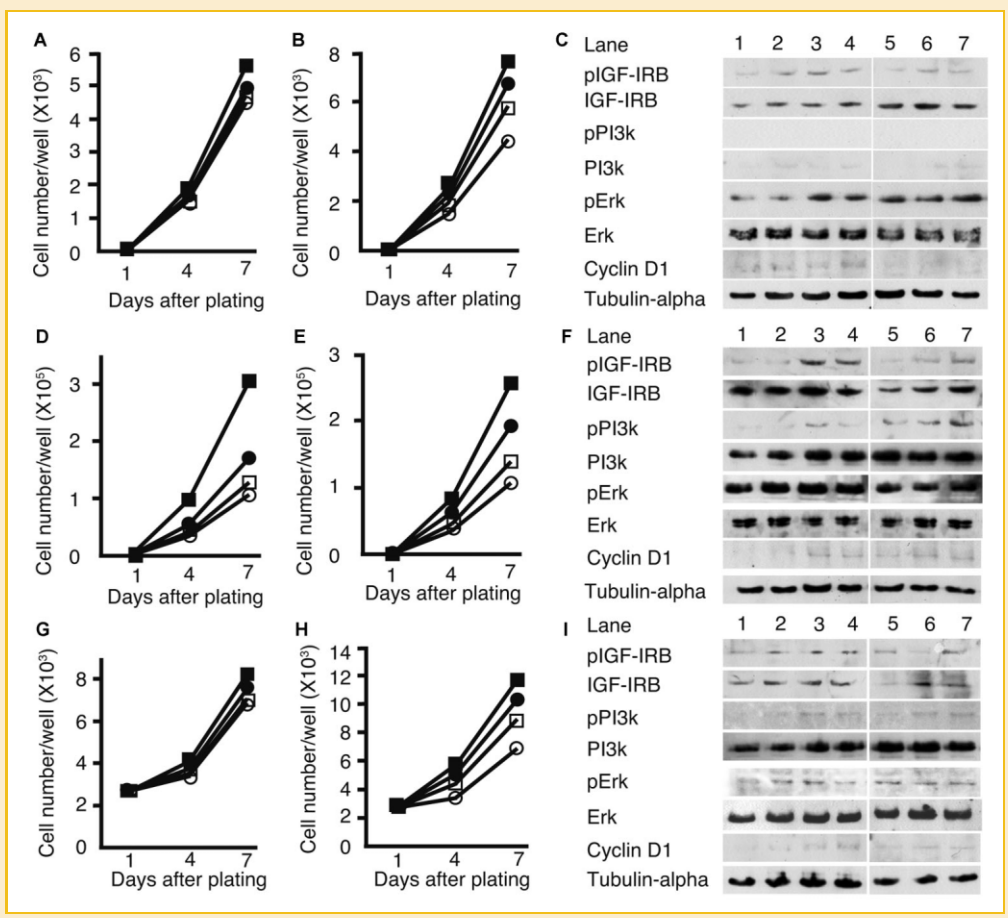

Fig. 4. Stimulation with IGF-I or IGF-II. MIA-Paca2 (A–C), NOR-P1 (D–F), and PK-1 (G–I) cells were cultured with IGF-I (A, D, G) or IGF-II (B, E, H), and cell numbers were counted. Protein was isolated from the cells after 2-days stimulation with IGF-I or IGF-II, and subjected to western blot analysis (C, F, I). (○): 0 ng/ml, (□): 2 ng/ml, (●): 20 ng/ml and (■): 200 ng/ml of IGF-I or IGF-II. n = 3. Lane 1: 0 ng/ml, 2: IGF-I at 2 ng/ml, 3: IGF-I at 20 ng/ml, 4: IGF-I at 200 ng/ml, 5: IGF-II at 2 ng/ml, 6: IGF-II at 20 ng/ml, 7: IGF-II at 200 ng/ml.

with IGF-I or IGF-II in the serum-free media (Fig. 4). On day 7, cell proliferation in the NOR-P1 line cultured in 200 ng/ml of IGF-I showed a 2.9-fold increase compared with untreated cells, while growth of the MIA-Paca2 and PK-1 cell lines was increased by 1.3 and 1.2 times, respectively. When MIA-Paca2, NOR-P1, and PK-1 cells were stimulated with 200 ng/ml of IGF-II, cell numbers were 1.7, 2.4, and 1.7 times higher than those of the untreated controls. PI3 kinase was not phosphorylated with IGF-I or IGF-II in MIA-Paca2. Phosphorylation of ERK increased in MIA-Paca2. Phosphorylation of PI3 kinase increased while that of ERK did not change in NOR-P1 and PK-1.

Next, we used inhibitors of IGF-IRβ, PI3 kinase, and MAP kinase to identify possible candidates for molecular therapy (Fig. 5). Cell proliferation was inhibited by PPP, LY294002, and PD98059 to $14.3 \pm 0.6\%$ ($P < 0.05$), $31.2 \pm 1.8\%$ ($P < 0.05$), and $80.1 \pm 2.5\%$ ($P < 0.05$) of the controls, respectively.

Suppression of cell motility would inhibit pancreatic cancer cell invasion to fibrous tissues. Cell migration was analyzed using scratch assays (Fig. 6 A). PPP, LY294002, and PD98059 suppressed cell motility to $7.2 \pm 1.8\%$ ($P < 0.05$), $0 \pm 0\%$ ($P < 0.05$), and $70.1 \pm 8.0\%$ ($P < 0.05$), respectively (Fig. 6 B).

We next addressed the mechanism of suppression of cell proliferation. BrdU incorporation in serum-free media was $65.0 \pm 9.7\%$ while that in PPP, LY294002, and PD98059 treated cells was $33.7 \pm 6.5\%$ ($P < 0.05$), $30.6 \pm 2.7\%$ ($P < 0.05$), and $46.7 \pm 7.7\%$ ($P < 0.05$), respectively (Fig. 7 A). The percentage of apoptotic cells in serum-free media was $4.0 \pm 1.5\%$, while in PPP-, LY294002-, and PD98059-treated cells, the values were $25.9 \pm 13.5\%$ ($P < 0.05$), $8.9 \pm 3.3\%$ ($P > 0.05$), and $3.4 \pm 0.9\%$ ($P > 0.05$), respectively (Fig. 7 B). BrdU uptake was suppressed with each inhibitor, while apoptotic cells increased significantly with PPP and marginally with LY294002.

Finally, we analyzed the downstream pathways of IGF-IRβ (Fig. 8). PPP and LY294002 suppressed phosphorylation of PI3 kinase and expression of Cyclin D1. PD98059 suppressed phosphorylation of ERK, but did not suppress Cyclin D1.

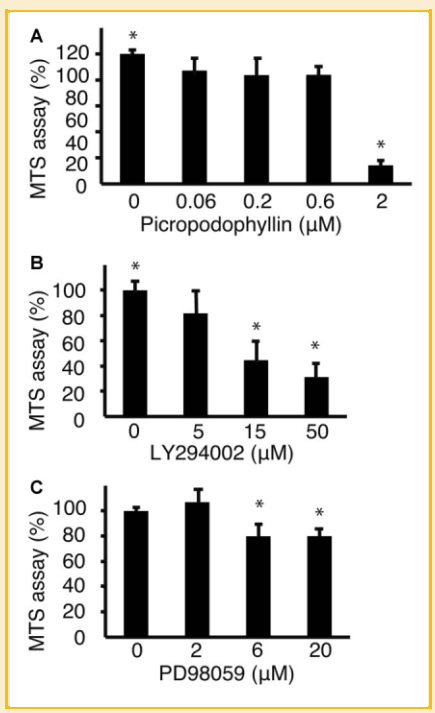

Fig. 5. MTS assay. Suppression of cell proliferation was analyzed with NOR-P1 cells grown in serum-free media with inhibitors of IGF-IRβ (picropodophyllin), PI3 kinase (LY294002), or MAP kinase (PD98059). *: $P < 0.05$ (one-factor ANOVA), n = 3.

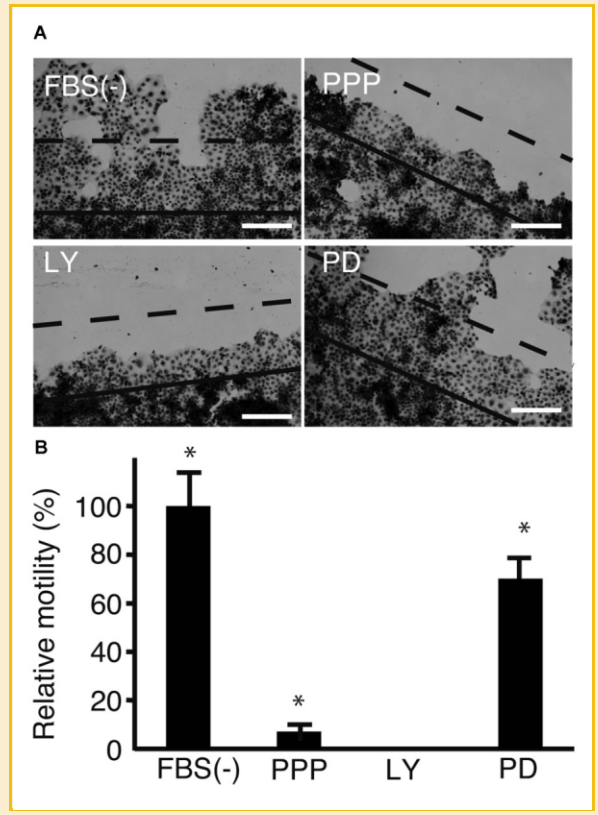

Fig. 6. Scratch assay. Cell motility was analyzed using the scratch assay. Cells were treated with different inhibitors, and those that migrated more than 150 μm were counted and normalized against untreated controls (relative motility). Relative motility decreased with inhibitor treatment, especially with PPP and LY294002. Solid line: Edge of scratch, dotted line: 150 μm from a solid line. FBS(−): Serum-free media, PPP: Picropodophyllin (2 μM), LY: LY294002 (50 μM), PD: PD98059 (20 μM). Original magnification: ×10, scale bar: 100 μm. *: $P < 0.05$ (one-factor ANOVA), n = 3.

DISCUSSION

IGF-IRβ is phosphorylated in surgical breast cancer specimens and is correlated with poor prognosis [Law et al., 2008]. IGF-IR is expressed in the cell membrane in pancreatic cancer tissues but not in surrounding non-tumor tissues [Bergmann et al., 1995; Ouban et al., 2003]. Our data clearly indicated that invading pancreatic cancer cells expressed IGF-IRβ (Fig. 1). NOR-P1 showed the

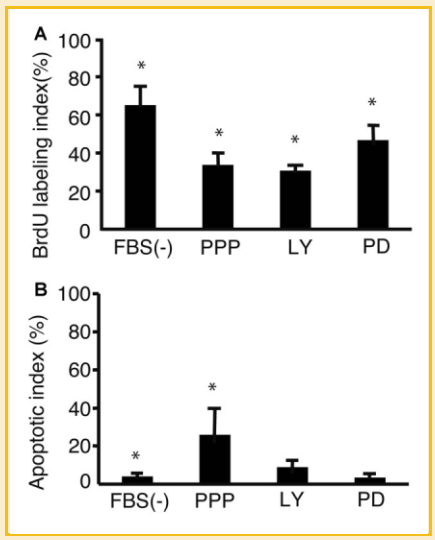

Fig. 7. BrdU uptake and apoptosis. To reveal the mechanism of suppression of cell proliferation, BrdU uptake and apoptosis were analyzed. BrdU-positive cells (BrdU labeling index) or apoptotic cells (apoptotic index) were counted out of 100 cells. The BrdU labeling index decreased with PPP, LY294002, and PD98059. The apoptotic index increased with PPP and LY294002. FBS(−): Serum-free media, PPP: Picropodophyllin (2 μM), LY: LY294002 (50 μM), PD: PD98059 (20 μM). *: $P < 0.05$ (one-factor ANOVA), n = 3.

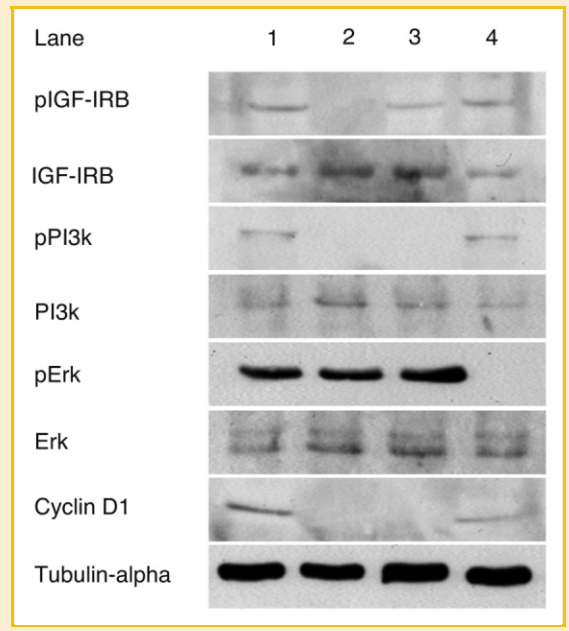

Fig. 8. Western blot analysis with inhibitors. NOR-P1 cells were incubated with picropodophyllin, LY294002, or PD98059 for 48 h and subjected to western blot analysis. Lane 1: No inhbitors, 2: Picropodophyllin at 2 μM, 3: LY294002 at 50 μM, 4: PD98059 at 20 μM.

strongest proliferative potential in serum-free media. NOR-P1 had high concentration of IGF-I and IGF-II and strong phosphorylation of IGF-IRβ. These data indicated that IGF-IRβ played a role in proliferation of NOR-P1 in serum-free media.

NOR-P1 was originally established from a cutaneous metastasis of a 79-year-old Japanese man with advanced pancreatic cancer [Sato et al., 2000]. NOR-P1 cells express vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF). Proliferation of NOR-P1 in serum-free media might be due to stronger phosphorylation of IGF-IRβ than the other cell lines and higher concentration of IGF-I and IGF-II secreted into the media in autocrine manner. PI3 kinase was not phosphorylated even with IGF-I or IGF-II in MIA-Paca2. On the other hand, phosphorylation of PI3 kinase increased with IGF-I and IGF-II in NOR-P1. Activated downstream pathway might be different among cell lines. It was suggested that PI3 kinase transferred proliferative stimulation from IGF-IRβ in NOR-P1. It was expected that inhibition of IGF-IRβ and PI3 kinase would suppress cell proliferation and motility.

PPP and LY294002 suppressed the cell proliferation as well as the motility of NOR-P1 cells in serum-free media, similar to their effect in hepatocellular carcinoma (HCC) [Tomizawa and Yokosuka, 2008]. It was suggested that IGF-IRβ and PI3 kinase were candidates for molecular therapy of pancreatic cancer cells proliferating in fibrous tissues like NOR-P1. The reasons for the lack of proliferative potential in the other cell lines in serum-free media, which was observed in NOR-P1, were not clear. However, the mechanism of proliferation could differ among cell lines.

Focal adhesion kinase (FAK) is located at focal adhesion contacts and is involved in the generation of actin–myosin mechanical forces. IGF-I enhances cell motility and down-regulates FAK, and PI3 kinase is the main direct downstream target of IGF-I [Zhang et al., 2005]. The effect of PPP and LY294002 on the suppression of motility may have occurred downstream of IGF-IR and involved FAK.

Analysis of the pathways downstream of IGF-IRβ might provide further insight into the motility of pancreatic cancer cells.

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