Inhibition of cell invasion and morphological change by troglitazone in human pancreatic cancer cells

WATARU MOTOMURA1, MIHO NAGAMINE2, SATOSHI TANNO2, MITSUKO SAWAMUKAI1, NOBUHIKO TAKAHASHI2, YUTAKA KOHGO1, and TOSHIKATSU OKUMURA2

1 Third Department of Internal Medicine, Asahikawa Medical College, Asahikawa, Japan
2 Department of General Medicine, Asahikawa Medical College, Asahikawa 078-8510, Japan

Introduction

Peroxisome proliferator activated receptor γ (PPARγ) is a nuclear hormone receptor that provides a strong link between lipid metabolism and the regulation of gene transcription.1-3 Increasing evidence shows that PPARγ is implicated in a wide variety of biological functions in addition to lipid metabolism. For instance, PPARγ activation by its selective ligands inhibits inflammatory responses in macrophages,4 angiogenesis,5 atherosclerosis,6 and cancer-cell growth. With regard to the inhibition of cancer-cell growth, it has been shown that thiazolidinediones, which are selective PPARγ ligands,7 induced growth arrest and apoptosis in colon cancer, liposarcoma, prostate cancer, breast cancer, lung cancer, gastric cancer, pancreatic cancer, lymphoma, and leukemia.8-22 These results suggest that PPARγ activation may be implicated in the growth of malignant tumors, and that PPARγ could be considered as a possible target molecule for the treatment of human malignant tumors.

The metastatic cascade consists of a series of steps, i.e., cells leaving the primary tumor, invading the local tissue, entering the circulation, resting at a distant vascular bed, extravasating into the target organ interstitium and parenchyma, and proliferating as a secondary colony.23-25 Among these steps, tumor cell invasion is an important step. Little is known, however, about the role of PPARγ in cancer cell invasion. The possibility that PPARγ is implicated in cancer-cell invasion should be clarified so that we can understand the pathophysiological relevance of PPARγ in tumor biology and to determine whether we could use PPARγ ligands as anti-cancer therapy in the near future.

Pancreatic cancer is one of the most lethal malignancies,26 and a large majority of pancreatic cancer patients present with metastatic disease or advanced local disease, precluding a curative surgical resection. It has also been shown that chemotherapy has not resulted in a

Background. We have recently demonstrated that peroxisome proliferator activated receptor (PPAR) γ activation by its selective ligand, troglitazone, potently inhibited cell proliferation in human pancreatic cancer cells. The present study was performed to clarify the role of PPARγ in cell invasion/motility in human pancreatic cancer cells. Methods. Cell invasive activity was assessed by an in vitro invasion assay, using a Transwell chamber, and by a wound-healing assay, in the human pancreatic cancer cell lines, PK-1 and PK-9. Cell morphology and actin structure were evaluated by phase-contrast and fluorescence microscopy. Results. PPARγ activation by troglitazone inhibited cell invasion and cell migration in PK-1 and PK-9 cells. We also examined the effect of troglitazone on cell morphology and actin structure because of its effect on cell motility. The size of PK-1 and PK-9 cells that had been incubated with troglitazone became smaller, and the in shape changed from flat to spindle, followed by round. The troglitazone-induced cell rounding was reversible by replacement with troglitazone-free medium. Rhodamine-phalloidin staining revealed a decreased number of actin filaments in PK-1 cells treated with troglitazone. In cells treated with mycalolide B, an actin depolymerizing agent, troglitazone failed to induce cell rounding. Conclusions. These results suggest that PPARγ activation by troglitazone inhibited cell motility and changed cell morphology through modulating actin organization.

Key words: cell invasion, troglitazone, PPARγ, pancreatic cancer, cell morphology

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Reprint requests to: T. Okumura
significant survival benefit, with a median survival of 4 months.\textsuperscript{27} This evidence indicates that a novel therapeutic approach must be developed for pancreatic cancer. To establish a therapy using PPAR\textsubscript{γ} ligands for pancreatic cancer, we have very recently demonstrated that human pancreatic cancer cells, such as PK-1 cells, expressed PPAR\textsubscript{γ} and that PPAR\textsubscript{γ} expressed in the PK-1 cells was functional as a transcriptional factor; further, PPAR\textsubscript{γ} activation by troglitazone induced G1 cell-cycle arrest through an increase of p27\textsuperscript{kip1}, a cyclin-dependent kinase inhibitor.\textsuperscript{18} These results suggest that PPAR\textsubscript{γ} activation by troglitazone could be considered as a therapeutic approach for pancreatic cancer. Thus, characterization of PK-1 cells has already been demonstrated, with special reference to the role of PPAR\textsubscript{γ} in cell proliferation. Based upon this evidence, we tested the hypothesis, using PK-1 cells, that PPAR\textsubscript{γ} activation may play a role in cancer-cell invasion.

**Materials and methods**

**Cell culture**

The human pancreatic cancer cell lines PK-1\textsuperscript{28} and PK-9 were obtained from Tohoku University (Sendai, Japan) and the Riken Cell Bank (Tokyo, Japan), respectively. As shown in our previous study, both cell lines expressed PPAR\textsubscript{γ}.\textsuperscript{18} The pancreatic cancer cells were cultured in RPMI-1640 medium (GIBCO, Grand Island, NY, USA) supplemented with 100 U/ml penicillin, 100\(\mu\)g/ml streptomycin, 2.5\(\mu\)g/ml amphotericin, and 10% fetal bovine serum. Cells were incubated at 37°C in a humidified atmosphere of 5% CO\textsubscript{2} in air.

**Chemicals**

Troglitazone, a selective ligand for PPAR\textsubscript{γ}, was kindly provided by Sankyo Pharmaceutical (Tokyo, Japan) and was dissolved in dimethyl sulfoxide (DMSO) with a final concentration of 0.05% in the culture medium.

**Cell invasion assay**

Invasion assay of cancer cells towards endothelial cell-conditioned medium was performed, using Transwell chambers (Costar, Cambridge, MA, USA) with an 8-mm diameter and tissue culture-treated filters with 8-\(\mu\)m pores, according to the method of Repesh,\textsuperscript{29} with some modifications. Tumor cells (5 \(\times\) 10\(^3\) ml\(^{-1}\)) were suspended in RPMI supplemented with 0.1% bovine serum albumin; the cell suspensions (100\(\mu\)l) were then placed into the upper compartment of a Transwell chamber. RPMI and fibronectin (15\(\mu\)g/ml dose) were then placed in the lower compartment. After incubation for 12 h, cells that had penetrated through the filters were counted. Each filter was fixed with 3% paraformaldehyde in Dulbecco’s phosphate buffered saline (DPBS) and stained in Giemsa solution. After the cells attached to the upper side of the filter were removed by wiping with a cotton swab, the cells attached to the lower side of the filter were counted, using a microscope. The total numbers of cells in the lower Transwell compartment and on the lower side of the filter were determined, and chemotaxis was expressed as the number of cells penetrating through the filter per 5 \(\times\) 10\(^4\) cells added to the upper compartment.

**Cell migration assay**

Cell migration was measured by an in vitro wound assay, according to a previous report.\textsuperscript{30} Cells were plated in complete medium on 24-well plates. Initial plating was adjusted to yield subconfluent monolayers at the same cell density as that used for the cell invasion assay after 24 h. The monolayers were then wounded by scratching 1-mm lines with a plastic scriber and, after the monolayers were washed, they were incubated for various times in complete medium. The experiment was terminated by fixing the cells, followed by staining with hematoxylin. Migration was evaluated by measuring the distance of the cell-free area in four randomly chosen areas.

**Observation of cell morphology**

First, the time-course of changes in cell morphology induced by troglitazone was assessed. PK-1 and PK-9 cells were cultured with complete medium containing 100\(\mu\)M troglitazone, and cell morphology was observed by phase-contrast microscopy 0, 24, 48, or 72 h after the beginning of culture. Next, to evaluate whether the morphological change of PK-1 cells induced by troglitazone was reversible, PK-1 cells were cultured with 100\(\mu\)M of troglitazone for 72 h and then with troglitazone-free medium for 24 h, after which cell morphology was observed.

In a separate set of experiments, we analyzed the effect of troglitazone on actin structure. PK-1 cells were treated with or without 100\(\mu\)M troglitazone and then the cells on coverslips were rinsed twice with a cytoskeletal stabilizing buffer (4 M glycerol in 25 M piperazine-1,4-bis(2-ethanesulfonic acid) [PIPES], pH 6.9), 1 M ethylene glycol-tetraacetic acid [EGTA], and 1 M magnesium chloride), incubated in the same buffer containing 0.2% Triton X-100 for 5 min at 20°C to extract soluble proteins, and fixed for 45 min at 20°C in 3.7% formaldehyde in PBS. After two 10-min washes in PBS, the cells were incubated with rhodamine-conjugated phalloidin (Amersham, Tokyo, Japan),