研究成果報告書

PPARγ ligandによる消化器癌細胞増殖抑制の分子メカニズム

18590666

平成18年度～平成19年度科学研究費補助金
（基盤研究(C)） 研究成果報告書

平成20年4月

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＜はしがき＞

平成１８年度から２年間に、科学研究費（基盤 C）の助成のもとに本研究（PPARgamma ligand による消化器癌細胞増殖抑制の分子メカニズム）は行われました。得られた知見は断片的ですが、ここにこれまでの研究成果をまとめ、各分野の専門家の先生に率直なご批判をいただければ幸いであります。

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交付決定額（配分額）

（金額単位：円）

	直接経費	間接経費	合 計
平成 18 年度	2,200,000	0	2,200,000
平成 19 年度	1,300,000	390,000	1,690,000
総 計	3,500,000	390,000	3,890,000

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研究成果

Increased expression of E-cadherin by troglitazone through down-regulation of MEK-ERK signaling pathway in human pancreatic cancer cells

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Abstract Troglitazone, a PPARγ ligand, inhibits cell invasion in human pancreatic cancer cells. However, little is known about the mechanisms of the reduced cell invasive activity by troglitazone. In the present study, we tested a hypothesis that E-cadherin expression might be influenced by troglitazone since E-cadherin is established as one of the most important molecules that are implicated in cancer cell invasion. Effect of troglitazone on the expression of E-cadherin and possible transcriptional regulators for E-cadherin was examined in human pancreatic cancer cells. We next examined whether MEK-ERK signaling pathway is involved in the expression of E-cadherin and cell motility since troglitazone down-regulates MEK-ERK signaling in human pancreatic cancer cells as shown in our recent report. Real-time PCR, western blotting and immunohistochemistry showed troglitazone increased expression of E-cadherin mRNA and protein in cultured cells. MEK inhibitors, PD98059 and U0126 increased E-cadherin mRNA expression and potently inhibited cell migration. These results suggest that troglitazone increases expression of E-cadherin at least in part by down-regulation of MEK- ERK signaling in human pancreatic cancer cells, which might be involved in the troglitazone-induced inhibition of cell invasive activity.

Introduction

Pancreatic cancer is one of the most lethal malignancies and a large majority of pancreatic cancer patients present with metastatic disease or advanced local disease, precluding a curative surgical resection[1]. It is also shown that chemotherapy has not resulted in a significant survival benefit, with a median survival of 4 months [2].

These evidence indicates a novel therapeutic approach must be developed for pancreatic cancer.

Peroxisome proliferator-activated receptor γ (PPARγ) is a member of the nuclear receptor superfamily that includes receptors for steroids, thyroid hormone, vitamin D and retinoic acid [3]. PPARγ is expressed at high levels in adipose tissue and functions as a key molecule of adipocyte differentiation. In addition to adipose tissue, PPARγ expression is detected in a wide variety of tumor cells including pancreatic cancer cells [4-10]. With regard to pancreatic cancer cells, we have done a series of experiments and have demonstrated that human pancreatic cancer cells such as PK-1 cells express functional PPARγ as a transcriptional factor and that activation of PPARγ by its ligand including troglitazone induced G1 arrest and inhibited cell invasive activity [9-11]. Thus PPARγ is involved in cellular behavior in pancreatic cancer cells. Based on these evidence, PPARγ ligands are considered to be effective for pancreatic cancer treatment. Although we and other investigators demonstrated that PPARγ ligands inhibit pancreatic cancer cell invasion [11-13], precise mechanisms of the inhibition of invasive activity by PPARγ ligands is poorly understood.

Several classes of proteins are participating when cells exhibit an invasive phenotype [14]. This includes cell-cell adhesion molecules (CAMs) like members of the immunoglobulin and calcium-dependent cadherin families and integrins [15]. Notably, all of these adherence interactions convey regulatory signals to the cell [16]. One widely observed alteration in cell-to-environment interaction in pancreatic cancer involves E-cadherin, which couples adjacent cells by E-cadherin bridges [17]. E-cadherin function is lost in a majority of pancreatic tumors [18]. In addition, accumulating evidence indicate that the presence of E-cadherin is considered as an important suppressor of invasion and metastasis. Based upon these evidence, we made a hypothesis that E-cadherin expression would be involved in the inhibition of cancer cell invasion by PPARγ ligands. The present study was therefore performed to clarify whether troglitazone influences E-cadherin expression in human pancreatic cancer cells.

Materials and Methods

Cell culture

Human pancreatic cancer cell lines, PK-1 [19] weas cultured in RPMI-1640 medium (GIBCO, Grand Island, NY) supplemented with 100 U/ml penicillin, 100

μg/ml streptomycin, 2.5 μg/ml amphotericine, and 10 % fetal bovine serum. Cells were incubated at 37° C in a humidified atmosphere of 5 % CO_2 in air.

Chemicals

Troglitazone was kindly provided from Sankyo Pharmaceutical Co. (Tokyo, Japan), and was dissolved in dimethyl sulfoxide (DMSO). MEK inhibitors, U0126 and PD98059 [20, 21],were purchased from Promega Biosciences, Madison, WI, USA. GW9662, a PPARγ antagonist [22], was obtained from SIGMA, St Louis. MO USA.

RNA isolation and first strand cDNA synthesis

Total RNA was isolated from cultured cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA). RNA was reverse-transcribed using the RETROscript (Ambion, Austin, Texas, USA). One μg of total RNA was mixed with 2 μl of Random decamers and nuclease-free water in a total volume of 12 μl and heated at 80° C for 3 min. The mixture was then chilled on ice and incubated with 2 μl of 10× RT buffer, 4μl dNTP mix, 1 μl RNase inhibitor, and 1 μl reverse transcriptase, at 44° C for 60min. The reaction mixtures were further incubated for 10 min at 92° C. The cDNA was stored at –30° C until used for real-time PCR.

Real-time PCR

Real-time PCR were performed in 7500 Real Time PCR system (Applied Biosystems, Foster City, CA, USA) following the manufacturer's suggested procedure. Human 18S ribosomal RNA was used as endogenous amplification control. The following Taqman probe of E-cadherin (Hs00170423_m1) and 18S ribosomal RNA were purchased from Applied Biosystems (Foster City, CA, USA).

Western Blotting Analysis

Fifty μg of protein extracted from PK-1 cells were separated by 4-20% SDS-PAGE. After electrophoresis, the proteins were transferred to PVDF membrane (Millipore Corporation, Bedford, MA, USA), blocked overnight in Block Ace at 4° C, reacted with primarily polyclonal antibody against human actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and monoclonal antibody against human E-cadherin (BD bioscience, San Diego, CA, USA) for 1 h, washed with TBS-T, reacted

with secondary polyclonal antibody against rabbit or mouse IgG (Chemicon International, Temecula, CA, USA) for 1 h and washed with TBS-T. After reaction with horseradish peroxidase-conjugated anti-rabbit or mouse IgG immune complexes were visualized by using the ECL plus detection reagents (Amersham Internationa, NJ, USA)

Immunofluorescence Analysis

Immunofluorescence for E-cadherin was performed in PK-1 cells. After treatment of troglitazone at several doses, PK-1 cells were fixed at room temperature for 20 min with 4% paraformaldehyde in PBS, permeabilized with 0.1% TritonX-100 and blocked in BlockAce (Dainippon Seiyaku, Osaka, Japan) for 1 h at room temperature and then incubated in primary antibody overnight at 4^{O} C. Anti E-cadherin monoclonal antibody was used 2 μg/ml. After washing PBS-T, each cell were incubated with Alexa Fluor 488 goat anti-mouse IgG and TO-PRO-3 (Molecular Probes, Eugene, Oregon, USA) for 1 h at room temperature. Immunodetection was carried out using confocal microscopy.

Migration assay

Migration assay of cancer cells towards endothelial cell-conditioned medium was measured using transwell chambers (Costar, Cambridge, MA) with 8 mm diameter, tissue culture-treated filters with 8 μm pores according to the method of Repesh [23] with some modifications. Tumor cells (5×10^5 ml-1) were suspended in RPMI supplemented with 0.1 % bovine serum albumin and the cell suspensions (100 μl) were then placed into the upper compartment of a transwell chamber. RPMI and 20 μg/ml dose of fibronectin were then placed into the lower compartment. After incubation for 15 h, cells that penetrated through the filters were counted. Each filter was fixed with 4 % parafolmaldehyde in Dulbecos phosphated 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 number of cells in the lower transwell compartment and on the lower side of filter were determined, and chemotaxis was expressed as the number of cells penetrating through the filter per 5×10^4 cells added to the upper compartment.

Statistical analysis

The results are expressed as mean ± SEM. Statistical analysis was performed by repeated measures ANOVA and subsequent Fisher's LSD test. $P < 0.05$ was considered statistically significant. Correlation analysis was performed by Supearman correlation.

Results

We first examined the effect of troglitazone on E-cadherin mRNA expression in human pancreatic cancer cells. As shown in Figure 1A, real-time PCR studies demonstrated that E-cadherin mRNA expression was dose-dependently up-regulated 24 h after troglitazone treatment in PK-1 cells. Next, we evaluated protein expression of E-cadherin by western blotting and immunohistochemistry. As demonstrated in Figure 1B and 1C, western blotting and immunostaining show that troglitazone increased E-cadherin protein expression in a dose-dependent fashion in PK-1 cells. Based on the findings of immunostaining, E-cadherin protein is localized in the surface of PK-1 cells where it is required for cadherin-mediated cell-cell adhesion. These results suggest that PPARγ activation by its ligand increases E-cadherin expression in surface of human pancreatic cancer cells.

Next, we have examined the effect of GW9662, a PPARγ antagonist, on E-cadherin mRNA expression stimulated by troglitazone in PK-1 cells to clarify whether the elevated mRNA expression of E-cadherin by troglitazone is mediated through a PPARγ-dependent mechanism. As shown in Table 1, although GW9662 by itself did not change E-cadherin nRNA expression, GW9662 significantly but not completely blocked the up-regilation of E-cadherin mRNA expression by troglitazone.

To further clarify the mechanism by which troglitazone up-regulates E-cadherin expression in PK-1 cells, a role of MEK-ERK signaling pathway was examined. MEK-ERK inhibitors, PD98059 and U0126, dose-dependently increased expression of E-cadherin mRNA in PK-1 cells (upper panels in Figure 2). To evaluate a possibility that inhbition of MEK-ERK signaling pathway by PD98059 or U0126 influences pancreatic cancer cell invasion, a migration assay was performed. A dose-related experiment demonstrated that PD98059 or U0126 at tested doses potently inhibited cell migration in PK-1 cells (lower panels in Figure 2).

Discussion

We have already demonstrated that human pancreatic cancer cells express functional PPARγ and PPARγ activation by its specific ligands such as troglitazone induced growth arrest and inhibited cell invasion in human pancreatic cancer cells [9-11]. These results suggest that PPARγ is a possible molecular target for pancreatic cancer treatment and PPARγ ligands such as thiazolidinediones may be relevant for cancer therapy. However molecular mechanisms by which troglitazone exerts its inhibitory action of cell invasion are largely unknown. The present study was performed to test a hypothesis that E-cadherin may be involved in the troglitazone-induced inhibition of cell invasion in human pancreatic cancer cells.

First, the present study clearly demonstrated that PPARγ activation by troglitazone increased E-cadherin mRNA and protein expression in PK-1 cells. Epithelial-mesenchymal transition (EMT) is implicated in the progression of cancer cells [14]. E-cadherin plays a vital role in EMT in cancer cell progression [14]. For instance, reduced expression of E-cadherin increases in cancer cell invasive activity. We have shown by an *in vitro* migration assay in the previous study [11] that troglitazone inhibited cell migration in PK-1 cells. These results suggest that troglitazone up-regulates E-cadherin expression, thereby reducing cell invasive activity in human pancreatic cancer cells.

Although troglitazone functions as a ligand for PPARγ [3], troglitazone exerts its action through a PPARγ–independent mechanism in some cases [24, 25, 26]. We therefore examined whether elevated E-cadherin mRNA by troglitazone indeed depends upon PPARγ activation. As demonstrated in this study, a PPARγ antagonist, GW9662 partially but not completely blocked the troglitazone-induced stimulation of E-cadherin mRNA expression in PK-1 cells. We would therefore speculate that both PPARγ-dependent and –independent mechanisms might be involved in the up-regulation of E-cadherin mRNA expression by troglitazone in PK-1 cells. Further investigation should be needed to clarify the PPARγ–independent mechanism .

Mitogen-activated protein kinases (MAPKs) are essential components of the intracellular signal transduction pathways that regulate cell proliferation and cell motility [27]. There are three subgroups of MAPKs, extracellular signal related kinases (ERKs), c-Jun N-terminal protein linases (JNKs) and p38 MAPKs. We have

recently demonstrated that among these three kinases, troglitazone specifically inhibited ERK signaling pathway in PK-1 cells [28]. It has been reported that ERK signaling pathway regulates E-cadherin expression. Homma et al. [29] have demonstrated that upon treatment with ERK kinase (MEK) inhibitor PD98059, E-cadherin accumulated in the area of intercellular contact zone. In contrast, constitutively active MEK-transfected human hepatocellular cancer cells showed reduced E-cadherin expression, suggesting that inhibition of MEK-ERK signaling increases E-cadherin expression in cancer cells. The present study clearly demonstrated that either troglitazone or MEK-ERK inhibition by its specific inhibitors increased expression of E-cadherin in PK-1 cells. Considering our recent evidence that troglitazone potently inhibited MEK-ERK signaling pathway in PK-1 cells [28], we would speculate that troglitazone suppresses MEK-ERK signaling pathway, followed by increased expression of E-cadherin in PK-1 cells.

To further clarify whether inhibition of the MEK-ERK signaling is implicated in inhibition of cell invasive activity in PK-1 cells, we have demonstrated a couple of findings in this and our previous studies. Treatment of PK-1 cells with a MEK inhibitor, U0126 or PD98059 completely blocked ERK1/2 phosphorylation as demonstrated in our recent study [28] and significantly inhibited cell migration as shown in the present study, indicating that inhibition of the MEK-ERK signaling pathway by itself is sufficient to induce inhibition of cell motility in PK-1 cells. Recent reports have demonstrated that MEK-ERK inihibitors, PD98059 and U0126 inhibited cell invasion in cultured malignant pleural mesothelioma and human melanoma cells [30, 31], being in agreement with our present finding on human pancreatic cancer cells. Together with our previous data that treatment of PK-1 cells with troglitazone inhibited MEK-ERK signaling[24], the present results suggest that PPARγ activation by its ligand, troglitazone, increases E-cadherin expression possibly through inhibition of MEK-ERK signaling pathway, thereby suppressing cell invasive activity in human pancreatic cancer cells.

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Figure legends

Figure 1. E-cadherin mRNA and protein expression by troglitazone in PK-1 cells. (A) Dose response effect of troglitazone on E-cadherin mRNA expression in human pancreatic cancer cells. PK-1 cells were treated with several doses of troglitazone and 24 h after treatment, E-cadherin mRNA expression was detected by real-time PCR. Data are expressed as the mean ± SEM of 6 experiments. *p <0.01 when compared with DMSO alone. (B) Dose response effect of troglitazone on E-cadherin protein expression in human pancreatic cancer cells. PK-1 cells were treated with several doses of troglitazone and 48 h after treatment, E-cadherin protein expression was detected by western blot (B) or immunostaining (C).

Figure 2. Effect of MEL-ERK inhibitoirs, PD98059 and U0126, on E-cadherin mRNA expression and cell migration in PK-1 cells.
Dose response effect of MEK-ERK inhibitors, PD98059 (A) and U0126 (B) on E-cadherin mRNA expression (upper panel) and cell migration (lower panel) in human pancreatic cancer cells. PK-1 cells were treated with several doses of the inhibitors and 24 h after treatment, E-cadherin mRNA expression was detected by real-time PCR. Data are expressed as the mean ± SEM of 6 experiments. *p< 0.01 when compared with DMSO alone. Migration assay was performed in PK-1 cells that had been treated with several doses of the inhibitors and cell invasive activity was evaluated at 15 h. Data are expressed as the mean ± SEM of 6 experiments. * p <0.01 when compared with DMSO alone.

Table 1

Effect of GW9662, a PPARγ antagonist, on the troglitazone-induced stimulation of E-cadherin mRNA expression in human pancreatic cancer cells

	E-cadherin mRNA relative expression
DMSO	2.10 ± 0.18
Troglitazone	7.34 ± 0.56 *
GW9662 + DMSO	2.41 ± 0.21
GW9662 + Troglitazone	5.24 ± 0.28 **, ***

PK-1 cells were treated with troglitazone (100 μM) or DMSO, and simultaneously with or without GW9662 (20 μM), a PPARγ antagonist. Twenty four h after treatment, E-cadherin mRNA expression was detected by real-time PCR. Data are expressed as the mean ± SEM of 5or 6 experiments. * $p < 0.01$, when compared with DMSO alone. ** $p < 0.01$, when compared with GW9662 + DMSO, *** $p < 0.01$, when compared with Troglitazone.

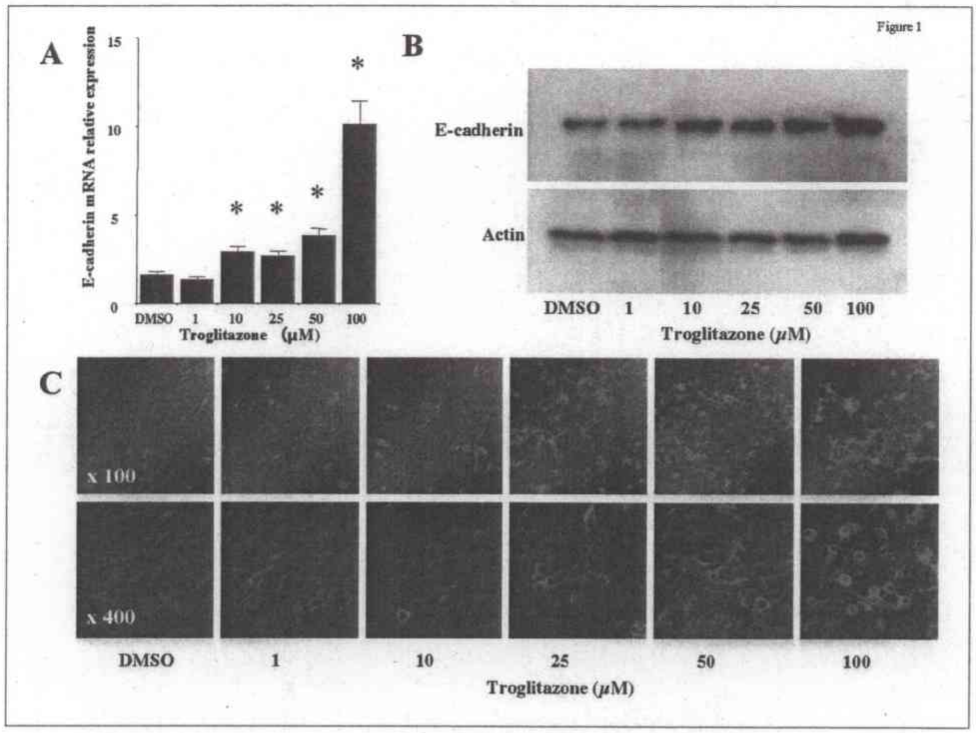
Figure 1
A
E-cadherin mRNA relative expression
15
10
5
0
DMSO 1 10 25 50 100
Troglitazone (μM)
B
E-cadherin
Actin
DMSO 1 10 25 50 100
Troglitazone (μM)
C
x 100
x 400
DMSO 1 10 25 50 100
Troglitazone (μM)

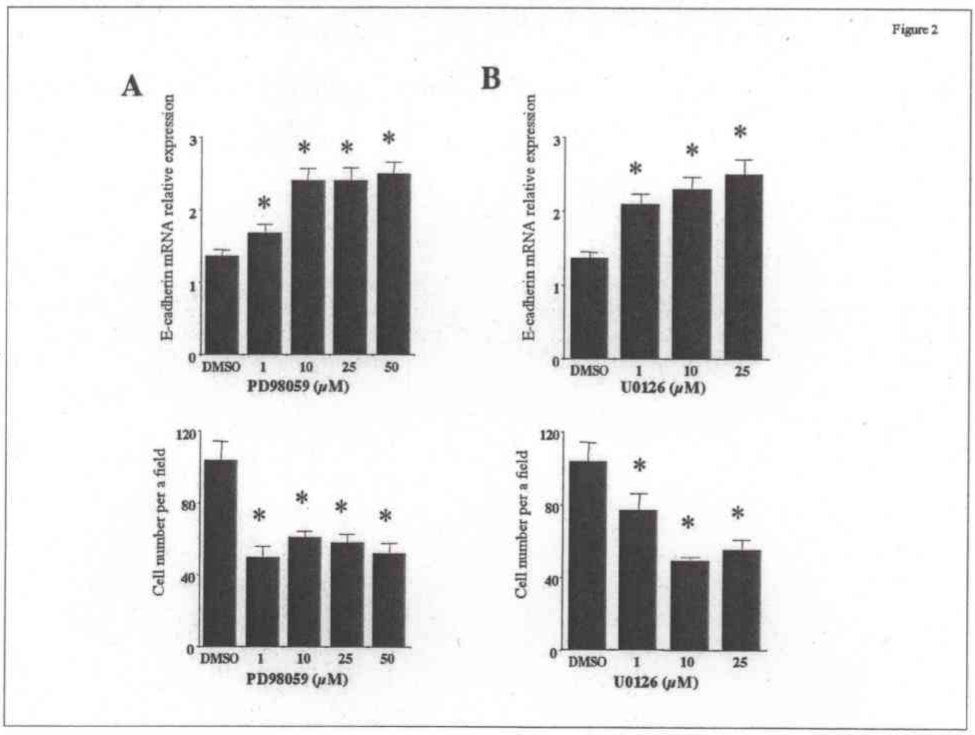
Figure 2
A
E-cadherin mRNA relative expression
3
2
1
0
DMSO 1 10 25 50
PD98059 (μM)
Cell number per a field
120
80
40
0
DMSO 1 10 25 50
PD98059 (μM)
B
E-cadherin mRNA relative expression
3
2
1
0
DMSO 1 10 25
U0126 (μM)
Cell number per a field
120
80
40
0
DMSO 1 10 25
U0126 (μM)