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Sterol regulatory element-binding protein 1 inhibitors decrease pancreatic cancer cell viability and proliferation

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Sterol regulatory element-binding protein1 (SREBP1) is a key regulatory factor that controls lipid homeostasis. Overactivation of SREBP1 and elevated lipid biogenesis are considered the major characteristics in malignancies of prostate cancer, endometrial cancer, and glioblastoma. However, the impact of SREBP1 activation in the progression of pancreatic cancer has not been explored. The present study examines the effect of suppression of SREBP1 activation by its inhibitors like fatostatin and PF429242 besides analyzing the impact of inhibitory effects on SREBP1 downstream signaling cascade such as fatty acid synthase (FAS), hydroxymethylglutaryl-CoA reductase (HMGCoAR), stearoyl-CoA desaturase-1 (SCD-1), and tumor suppressor protein p53 in MIA PaCa-2 pancreatic cancer cells. Both fatostatin and PF429242 inhibited the growth of MIA PaCa-2 cells in a time and concentration-dependent manner with maximal inhibition attained at 72 h time period with IC_{50} values of 14.5 μM and 24.5 μM respectively. Detailed Western blot analysis performed using fatostatin and PF429242 at 72 h time point led to significant decrease in the levels of the active form of SREBP1 and its downstream signaling proteins such as FAS, SCD-1 and HMGCoAR and the mutant form of tumor suppressor protein, p53, levels in comparison to the levels observed in vehicle treated control group of MIA PaCa-2 pancreatic cells over the same time period. Our *in vitro* data suggest that SREBP1 may contribute to pancreatic tumor growth and its inhibitors could be considered as a potential target in the management of pancreatic cancer cell proliferation.

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1. Introduction

Pancreatic cancer is the 4th leading cause of cancer-related death in the USA with 6% survival rate in 5 years [1]. The low survival rate is due to early recurrence and metastasis of pancreatic carcinoma, and also the late stage diagnosis due to its asymptomatic nature [2]. Over the past few years, several targets such as the epidermal growth factor receptor (EGFR), Phosphatidylinositol 3-kinase (PI3K), protein kinase B (Akt) the mechanistic target of rapamycin (mTOR) and B-Raf have been developed and tried for the inhibition of different cancers. Currently, the EGFR inhibitor, erlotinib, in combination with gemcitabine has been approved for the treatment of pancreatic cancer [3]. While inhibiting these upstream targets has been successful for treating several types of cancers, these therapies are either ineffective or lead to a development of resistance over time. Knowing the complex tumor biology of pancreatic cancer, identification of new targets and therapies is an urgent unmet medical need.

It is now a well-established fact that tumor cells readjust their metabolic pathways to meet the need of the cancer cells [4]. In malignant cells, mainly lipid metabolism gets reprogrammed to meet high metabolic demands of cancer cells and the lipogenic phenotype is a significant characteristic of tumors. Tumor cells also divert glucose and glutamate metabolism pathways to serve in lipid biogenesis process [5]. Sterol regulatory element-binding proteins (SREBPs) are key transcription factors that control lipid homeostasis [6]. There are two types of SREBPs, SREBP1, which mainly regulates fatty acid synthesis, and SREBP2, which activates cholesterol synthesis [7]. The elevated lipid biogenesis regulated by SREBP1 is considered a major characteristic of malignancies including prostate cancer, endometrial cancer, and glioblastoma [8,9]. SREBP1 acts as a central regulator in integrating glucose metabolism regulated by PI3K/Akt signaling pathway and fatty acid

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synthesis by Myc-regulated glutamine metabolism [10]. The active N-terminal region of SREBP1 localized in the nuclei upregulates expression of several downstream target genes such as fatty acid synthase (FAS) and steaoryl-CoA-desaturase-1 (SCD-1) [11]. The oncogenic receptor tyrosine kinases (RTK)/PI3K/Akt pathway increases the expression of SREBP-1 which in turn upregulates ATP citrate lyase (ACL), Acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) to promote fatty acid synthesis and increased low density lipoprotein receptor expression to increase cholesterol uptake by these malignant cells. The fatty acids and phospholipids promote cell growth and survival. A recent study also showed that inhibition of upstream events such as EGFR, PI3K, and Akt activation lead to drastic reduction in the expression of SREBP1 in cancer cells [11]. While a clear positive correlation between SREBP-1 level and pancreatic cancer cell proliferation has been addressed [6], whether the inhibitors of SREBP1 would affect pancreatic cell has not been addressed so far.

Therefore, we analyzed the effect of two known inhibitors of SREBP1, fatostatin and PF429242, on human pancreatic cancer cell line, MIA PaCa-2 cells. Fatostatin is a non-sterol synthetic diarylthiazole derivative which inhibits SREBP1 maturation and its nuclear translocation [12]. PF429242 is a reversible, competitive aminopyrrolidineamide inhibitor of site-1 protease (S1P), which inhibits endogenous SREBP processing [13]. We investigated the effect of these two inhibitors on pancreatic MIA PaCa-2 cells' viability as well as their effects on SREBP1 activation and its key downstream targets.

2. Materials and methods

MIA PaCa-2 cell line was obtained from Sigma-Aldrich (Sigma-85062806). Dulbecco's Modified Eagle Medium (Hyclone DMEM), fetal bovine serum (FBS) were purchased from HyClone (Cat No: SH30024.01). Cell Counting-8 (CCK-8) kit was purchased from Dojindo (Cat No: LT807). Fatostatin was obtained from TOCRIS BIOSCIENCE (Catalog No:4444). PF429242 was obtained from SIGMA (Catalog No: SML0667).

2.1. Maintenance of tumor cell line

MIA PaCa-2 cell line was authenticated by Sigma-Aldrich, Canada (Invoice No. 536284272; dated 06/22/2015). These cells were grown in Hyclone DMEM with 10% FBS and cultured in a humidified atmosphere of 5% CO_2 at 37 °C.

2.2. Cytotoxicity assay

MIA PaCa-2 cells (4000 cells/well) were seeded in 96-well plates with the medium mentioned above. After overnight attachment, the cells were treated with the medium containing either fatostatin (0–320 μM) or PF429242 (0–320 μM) for 24, 48 and 72 h (6 wells/concentration). Fatostatin (1 mM) was initially dissolved in dimethyl sulfoxide while PF429242 was dissolved in sterile water and further dilutions of each inhibitors were made in Dulbecco's modified essential medium. The viable cell number was measured using the CCK-8 assay kit. Absorbance was measured at 450 nm using a microplate reader (Spectral Max M5). The optical density of each well was measured to represent the proliferation of the cells. All the experiments were performed in triplicate.

2.3. Western blot analysis

MIA PaCa-2 cells were plated in 10 cm Petri dishes. After overnight attachment, the cells were treated with either vehicle (control), PF429242 or fatostatin for 72 h. The concentrations of fatostatin and PF429242 employed were chosen based on the IC50 values derived from preliminary studies. For total protein extraction, cells were lysed in RIPA buffer (Cell Signalling, #9806, 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 μg/ml leupeptin) supplemented with protease inhibitor cocktail (Cell Signaling, #5871). Normal pancreatic tissues were obtained from 2 months old male and female C57BL/6 mice. The mice were euthanized by cervical dislocation under mild anesthesia (isoflurane), the pancreas was excised out and processed for whole cell lysates similar to the Mia PaCa-2 cell lysates as mentioned above. Total protein extracts were quantified using the Bradford assay with bovine serum albumin as standard. Western blot experiments were carried out using the Mini Trans-Blot cell BIO-RAD. The following primary antibodies were used: anti-FAS (Santa Cruz, sc-48357; dilution 1:500), anti-SCD1 (Abcam, ab19862; dilution 1:1000), anti- HMGCoAR (Abcam, ab174830; dilution 1:1000), anti-SREBP1 (Santa Cruz, sc-365513, 1; 500) and anti-p53 (Abcam, ab1101; dilution 1:1000). Anti-beta Actin (Abcam,ab8227; dilution 1:1000) was used for normalization. All the experiments were performed in triplicate.

2.4. Statistical analysis

All the statistical analyses were conducted using GraphPad Prism 6 software (GraphPad Software, San Diego, CA). Data are presented as mean ± standard deviation (SD). For western blots, the protein expressions for various conditions were compared by one-way ANOVA test. P values < 0.05 were considered as statistically significant.

3. Results

First, we determined the cytotoxic effect of fatostatin and PF429242 in the MIA PaCa-2 cell line as a function of time by incubating them for varying time intervals of 24, 48 and 72 h at concentration ranges (10–320 μM) employing the CCK-8 assay. While significant inhibition ($P < 0.001$) was observed even at 24 h period of incubation using either agent at their lowest concentration (10 μM) employed in the study (Fig. 1A and B upper panel) both agents display concentration and time-dependent further increases in their inhibitory effects (Fig. 1A–F). The IC_{50} values were found to be 14.5 μM and 24.5 μM for fatostatin and PF429242, respectively at 72 h time of incubation.

Western blot analysis showed a significant increase in active form of SREBP1 ($P < 0.001$) and its downstream signaling proteins FAS ($P < 0.001$), SCD-1 ($P < 0.001$) and HMGCoAR ($P < 0.001$) in MIA PaCa-2 cells when compared to the levels of these protein seen in normal mice pancreatic tissue homogenates (N). Treatment with both fatostatin (IC_{50} 14.5 μM for 72 h) and PF429242 (IC_{50} 24.5 μM for 72 h) significantly decreased the levels of active form of SREBP-1 ($P < 0.05$ and $P < 0.01$, respectively; Fig. 2B), FAS ($P < 0.05$ and $P < 0.05$, respectively; Fig. 2C), SCD-1 ($P < 0.01$ and $P < 0.001$, respectively; Fig. 2D), HMGCoAR ($P < 0.01$ for PF429242; Fig. 2E) and p53 ($P < 0.05$ and $P < 0.001$, respectively; Fig. 2F) in MIA PaCa-2 cells in comparison to vehicle treated control group of MIA PaCa-2 cells maintained over the same time period.

4. Discussion

There is increasing evidence that cancer cells engage in increased lipogenesis to become independent of the systemic regulation, leading to rapid cell proliferation and enhanced tumor growth [7,10]. Therefore, recent studies have explored the role of

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