PANCE4 regulates apoptosis in human pancreatic cancer
Panc-1 cells via the mitochondrial signaling pathway

XIAO-FENG TIAN, GUO-MIN HUANG, HONG-LIANG ZANG and HONG CAO

Department of General Surgery, Xinmin Branch of The China-Japan Union Hospital, Jilin University, Changchun, Jilin 130021, P.R. China

Received April 11, 2015; Accepted April 7, 2016

DOI: 10.3892/mmr.2016.5885

Abstract. Previous studies have demonstrated the overexpression of paired basic amino acid cleaving enzyme 4 (PACE4) mRNA in prostate cancer tissues. This overexpression is correlated with higher circulating protein levels in certain patients, however, the role of PACE4 in apoptosis and the potential molecular mechanisms of pancreatic cancer remain to be elucidated. The aim of the present study was to investigate the effect and potential molecular mechanisms of PACE4 on apoptosis in the Panc-1 pancreatic cancer cell line. Cell proliferation was assessed using a Cell Counting Kit-8 assay. Apoptotic nuclear shrinkage was monitored using Hoechst 33258 staining. Caspase-3/7 activities were measured using a colorimetric caspase-glo 3/7 assay. Alterations in protein expression were monitored using Western blot analysis. The results indicated that PACE4 small interfering (si)RNA inhibited cell proliferation and activated caspase-3/7 activities. In addition, PACE4 siRNA significantly increased apoptosis via the activation of caspase-3 and the downregulation of anti-apoptotic proteins, X-linked inhibitor of apoptosis protein and phosphorylated-Akt. In addition, the results showed deregulation of the B cell lymphoma-2 (Bcl-2)-associated X protein/Bcl-2 ratio which led to the release of cytochrome c following PACE4 siRNA transfection. In conclusion, PACE4 siRNA may exert antitumor activity through the mitochondrial pathway and is expected to be a promising therapeutic strategy for the treatment of pancreatic cancer.

Introduction

Pancreatic cancer, one of the most aggressive types of human malignancy, has a poor prognosis with a five-year survival rate of <5% worldwide (1). It is characterized by rapid disease progression without specific symptoms, thus, early diagnosis and curative treatment are almost impossible (1,2). The standard treatment strategies for pancreatic cancer include surgery, which is restricted to early disease stages, radiation and/or gemcitabine-based chemotherapies. However, pancreatic cancer is among the most intrinsically resistant types of malignancy to radiation and chemotherapy, resulting in an overall survival rate of <7%. Therefore, scientists and oncologists are attempting to identify novel and more efficient anti-pancreatic cancer agents or adjuvants (3-5). A previous report showed that ~90% of patients succumb to the disease within 1 year following diagnosis, and the five-year survival rate is <5% worldwide (6). The incidence of pancreatic cancer has been increasing in previous years, demonstrating the importance of investigating its pathogenesis. Alterations in gene and protein expression levels, and the activation of signaling pathways are associated with the occurrence and progression of pancreatic cancer (7).

Proprotein convertases (PCs) are a family of enzymes, which are responsible for the activation of numerous protein precursors. At present, nine PCs have been identified, namely, furin, paired basic amino acid cleaving enzyme 4 (PACE4), PC1/3, PC2, PC4, PC5/6, PC7, PCSK9 and SKI-1/SIP (8). PACE4 is considered to be important in the development and progression of cancer. It activates several biologically relevant substrates; and a number have been shown to be significantly involved in tissue homeostasis and cancer growth (9,10). Among these are numerous metalloproteinases, growth factors, growth factor receptors and adhesion molecules, which are directly associated with tumor development (11-14). PACE4 is expressed at low levels in several mammalian tissues and has been demonstrated to be upregulated in certain tumor cell lines, including murine squamous cell carcinoma (15). In addition, mice overexpressing PACE4 have been found to exhibit tumors with increased growth rates (16).

Previously, two independent studies demonstrated the overexpression of PACE4 mRNA in prostate cancer tissues (17,18). This overexpression was correlated with higher circulating protein levels in certain patients (18). However, the possible role of PACE4 in apoptosis and the potential molecular mechanisms of pancreatic cancer remain to be elucidated. In the present study, molecular silencing with small interfering (si)RNA was used to knock down endogenously expressed
PACE4 in the Panc-1 cell line, following which cell proliferation and the apoptotic response was examined.

Materials and methods

Reagents and antibodies. The following polyclonal antibodies were purchased from Proteintech Group, Inc. (Wuhan, China): Rabbit anti-human cleaved caspase-3 (19677-1-AP; 1:3,000), rabbit B cell lymphoma (Bel)-2 (12789-1-AP; 1:3,000), rabbit Bel-2-associated X protein (Bax; 50599-2-1g; 1:3,000), rabbit cytochrome c oxidase subunit IV and X-linked inhibitor of apoptosis protein (XIAP; 10037-1-1g; 1:2,000) antibodies, and the mouse anti-GAPDH (MM-0163-P; 1:1,000) antibody. Polyclonal rabbit anti-human PACE4 (AB151562; 1:3,000) and cytochrome c (ab154476; 1:3,000) antibodies were purchased from Abcam (Cambridge, UK). Polyclonal rabbit anti-human phosphorylated-Akt (p-Akt; 13038; 1:2,000) and Akt (4685; 1:3,000) antibodies were purchased from Cell Signaling Technologies, Inc. (Danvers, MA, USA). Anti-rabbit (15134-1-AP) and anti-mouse (30000-0-AP) IgG-horseradish peroxidase antibodies were purchased from Proteintech Group, Inc. A Cell Counting Kit-8 (CCK-8) and Hoechst 33258 were purchased from Beyotime Institute of Biotechnology (Haimen, China). Other reagents were of analytical grade.

Cell culture and small interfering RNA transfection. Human pancreatic cancer Panc-1 cells were obtained from American Type Culture Collection (Manassas, VA, USA). The cells were routinely grown in Dulbecco's modified Eagle's medium (DMEM; GE Healthcare Life Sciences, Beijing, China) containing 10% fetal bovine serum (FBS; GE Healthcare Life Sciences, Beijing, China) was used to isolate the mitochondria and cytosol, according to the manufacture's protocol. Following transfection, as described above, the cells (5x10⁴ cells) were collected by centrifugation at 600 x g for 5 min at 4°C, washed twice with ice-cold phosphate-buffered saline and then resuspended in 500 µl isolation buffer (Tiangen Biotech Co., Ltd., Beijing, China) containing protease inhibitors for 10 min on ice. The cells were then mechanically homogenized using a Dunce grinder (ATX810; Tienan Biotech Co., Ltd.). The unbroken cells, debris and nuclei were discarded by centrifugation at 800 x g for 10 min at 4°C. The supernatants were centrifuged at 12,000 x g for 15 min at 4°C. The supernatant of the cytosol was collected and the pellet fraction mitochondria was dissolved in 50 µl lysis buffer (Sigma-Aldrich).

Western blot analysis. The Panc-1 cells were transfected, as described above, and were then lysed using radioimmunoprecipitation assay buffer (Sigma-Aldrich). A Bicinchoninic Acid Protein Assay kit (NEPS045-2; Beijing Dingguo Changsheng Biotechnology Co., Ltd., Beijing, China) was used to measure the protein concentrations. The total protein (0.2 µg) was loaded onto 12% SDS-PAGE gels (Tiangen Biotech Co., Ltd.) and transferred onto polyvinylidene fluoride membranes (Sigma-Aldrich). The membranes were probed overnight at 4°C with the indicated primary antibodies in Tris-buffered saline with Tween-20 (Tiangen Biotech Co., Ltd.), containing 1% bovine serum albumin (w/v; Gibco; Thermo Fisher Scientific, Inc.). The blots were then incubated for 1 h with anti-rabbit or mouse secondary antibodies at 37°C. The immune complexes were detected using an ECL Detection kit (32132; Thermo Fisher Scientific, Inc.) and quantified using a scanning densitometer (SD4; Tobias Associates, Inc., Ivyland, PA, USA) with molecular analysis software (Quantity One 1-D Analysis software; version 4.6.9; Bio-Rad Laboratories, Inc., Hercules, CA, USA). Quantification of band density was additionally performed using Quantity One 1-D Analysis software with normalization to the GAPDH signal. The expression levels of the proteins of interest in the
various treatment groups were expressed relative to those under non-treated conditions.

Statistical analysis. All data are presented as the mean ± standard deviation, and were analyzed using Student’s t-test and one-way analysis of variance to determine the levels of significance. P<0.05 was considered to indicate a statistically significant difference. Statistical analysis was performed using SPSS/Windows 11.0 software (SPSS Inc., Chicago, IL, USA).

Results

PACE4 reduces Panc-1 cell proliferation. Panc-1 cell proliferation was examined using a CCK-8 assay following transfection of the cells with PACE4 siRNA or control siRNA. As shown in Fig. 1, PACE4 siRNA inhibited cell proliferation, with a significant reduction observed 48 h post-transfection, compared with that in the control siRNA-treated group (P<0.05). Thus, these data indicated that PACE4 may affect cellular proliferation in Panc-1 cells.

PACE4 siRNA induces the apoptosis of Panc-1 cells. In the present study, the Panc-1 cells were transfected with PACE4 siRNA. The results of the Western blot analysis indicated that the protein expression of PACE4 was inhibited in the PACE4 siRNA-transfected group, compared with the control siRNA-transfected group (Fig. 2A and B). In order to evaluate whether the proliferation inhibition induced by PACE4 siRNA in the Panc-1 cells was associated with apoptosis, the present study examined the morphological changes in the cells using Hoechst 33258 staining. The Panc-1 cells were transfected with PACE4 siRNA for 48 h, and the apoptotic morphological changes were observed and compared with the appearances in the control group. In the control siRNA group, the nuclei of the Panc-1 cells were round and homogeneously stained (Fig. 2C). However, the PACE4 siRNA-transfected cells exhibited evident apoptotic characteristics, including cell shrinkage and membrane integrity loss or deformation, nuclear fragmentation and chromatin compaction of a late apoptotic appearance. Together, these data indicated that PACE4 siRNA induced apoptosis in the Panc-1 cells.

PACE4 siRNA induces apoptosis via a caspase-dependent pathway. Caspase-3 is a critical executioner of apoptosis, and its activation is essential for DNA fragmentation and a number of the typical biochemical and morphological changes observed in cells undergoing apoptosis. Therefore, to evaluate whether PACE4 siRNA-induced apoptosis is involved in the activation of caspase-3/7, the present study investigated the activities of caspase-3/7 through measuring the bioluminescence intensities. The activities of caspase-3/7 were significantly activated following PACE4 siRNA transfection (Fig. 3A).

To further assess the role of PACE4 in Panc-1 cell apoptosis, the present study evaluated the expression levels of apoptosis-associated proteins. These included pro-apoptotic cleaved caspase-3 (c-caspase-3), anti-apoptotic XIAP and p-Akt. The results of the Western blot analysis and subsequent statistical analysis indicated that PACE4 siRNA increased the levels of c-caspase-3 by ~2.2-fold (P<0.05). By contrast, the levels of XIAP and p-Akt were decreased by ~53% (P<0.05) and ~40% (P<0.05), respectively, following PACE4 siRNA transfection (Fig. 3B and C).

PACE4 siRNA induces apoptosis via the mitochondrial signaling pathway. In order to further understand the molecular mechanisms by which PACE4 siRNA exerts pro-apoptotic effects, the present study examined the protein expression of mediators in the mitochondrial signaling pathway. Initially, the present study determined whether PACE4 siRNA stimulated the release of cytochrome c into the cytosolic fraction in Panc-1 cells. As expected, cytochrome c was redistributed following PACE4 siRNA transfection. The level of cytochrome c in the mitochondria was significantly decreased by 64% (Fig. 4A and B; P<0.05). Correspondingly, the levels of cytochrome c in the cytosol were increased by 1.83-fold (Fig. 4C and D; P<0.05).

As the Bcl-2 family of proteins are critical in regulating the release of cytochrome c, the present study subsequently investigated the possible involvement of Bax and Bcl-2 in the process of PACE4 siRNA-mediated Panc-1 cell apoptosis. As shown in Fig. 4E and F, the level of Bax was significantly increased and the level of Bcl-2 was markedly decreased in the PACE4 siRNA-transfected cells, compared with the control cells. Statistical analysis showed that PACE4 siRNA increased the ratio of Bax/Bcl-2 by ~5.5-fold (P<0.05).

Discussion

PACE4 has already been highlighted for its potential role in several types of neoplasia, including oral tongue carcinoma (19), hepatocellular carcinoma (20), glioma (21), skin cancer (16,22) and prostate cancer (17). Whereas previous studies have predominantly examined PACE4 overexpression, the present study focused on gene silencing as a predictive approach to define potential therapeutic benefits. In the present study, the effect of PACE4 siRNA on apoptosis in a cellular
model of pancreatic cancer was investigated. The results indicated that PACE4 siRNA inhibited the proliferation of Panc-1 cells. Based on the results of Hoechst 33258 staining, measurement of caspase-3/7 activities and Western blot analysis, it was concluded that PACE4 siRNA induced apoptosis in the Panc-1 cells. Thus, the present study provided the first
evidence, to the best of our knowledge, that PACE4 has an anti-apoptotic effect in pancreatic cancer cells.

As a primary executioner caspase in the majority of pathways of the caspase protein family, the activation of caspase-3 often results in the irreversible commitment of a cell to apoptosis. Therefore, the activation of caspase-3 is considered a reliable marker for cells undergoing apoptosis (23). The present study found that the activity of caspase-3/7 was significantly activated following PACE4 siRNA transfection (Fig. 3A). An effective strategy for destroying cancer cells is to induce cell apoptosis. XIAP, a member of the inhibitor of apoptosis protein family, contributes to the apoptosis resistance of cancer cells (24,25). Akt is a promoter of cell proliferation and survival, and has been found to be overexpressed in tumor formation (26). Thus, the present study investigated whether these apoptosis-associated proteins were involved in PACE4 siRNA-induced apoptosis. The results of this investigation confirmed the role of PACE4 in the apoptosis of Panc-1 cells, based on the following lines of evidence: PACE4 siRNA increased the apoptosis of cells by regulating the expression levels of the apoptosis-associated factors c-caspase-3, XIAP and p-Akt (Fig. 3B and C). The inactivation of XIAP and p-Akt by PACE4 siRNA may prevent the development and progression of cancer.

The Bcl-2 family of proteins are important in the apoptosis of cancer cell apoptosis (27,28). The Bcl-2 family can primarily regulate mitochondrial membrane permeabilization (29). The Bax/Bcl-2 ratio is usually regarded as a criterion for apoptosis (30). The results from the present study demonstrated that the level of cytochrome c in the mitochondria was significantly decreased (Fig. 4A and B), whereas that in cytosol was increased (Fig. 4C and D). In addition, PACE4 siRNA increased the levels of Bax and decreased the level of Bcl-2, leading to changes in the ratio of Bax/Bcl-2 (Fig. 4E and F). These results indicated that PACE4 siRNA had an effect on mitochondrial membrane stability. This was evidenced by the increased Bax/Bcl-2 ratio and the release of cytochrome c into the cytoplasm. Taken together, these data demonstrated

![Figure 4. Regulation of mediators in the mitochondrial pathway in apoptotic Panc-1 cells by PACE4 siRNA. Panc-1 cells were transfected with PACE4 siRNA or control siRNA for 48 h, and the levels of (A and B) cyto c in the mitochondria (COXIV as loading control) and (C and D) cytosol (GAPDH as a loading control) were measured using Western blot analysis. (E and F) PACE4 siRNA inhibited the expression of Bcl-2 and increased the expression of Bax, as detected by Western blot analysis. Intensities of the proteins of interest were measured for quantitative analysis. GAPDH was included as a loading control. The data are presented as the mean ± standard deviation (n=3). *P<0.05, vs. control. PACE4, paired basic amino acid cleaving enzyme 4; siRNA, small interfering RNA; COXIV, cytochrome c oxidase subunit IV; cyto c, cytochrome c; Bcl-2, B cell lymphoma-2; Bax, Bcl-2-associated X protein.](image-url)
that PACE4 siRNA may exert its anti-tumor activity through the mitochondrial signaling pathway (intrinsic pathway) in pancreatic cancer cells.

In conclusion, the results of the present study suggested PACE4 siRNA possesses anti-proliferation and apoptosis-inducing properties in human pancreatic cancer Panc-1 cells. The PACE4 siRNA-induced apoptosis of Panc-1 cells may be mediated through the mitochondria pathway. These results support the potential of PACE4 to be developed as a promising agent for the treatment of pancreatic cancer.

References


