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Nicotine enhances the malignant potential of human pancreatic cancer cells via activation of atypical protein kinase C

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ABSTRACT

Background: Pancreatic cancer (PC) is the most lethal malignancy among solid tumors, and the most common risk factor for its development is cigarette smoking. Atypical protein kinase C (aPKC) isozymes function in cell polarity, proliferation, and survival, and have also been implicated in carcinogenesis. However, the involvement of aPKC in PC progression and the effect of nicotine, a major component of cigarette smoke, on the biological activities of aPKC remain to be fully elucidated.
Methods: We investigated the effects of nicotine on the proliferation, migration and invasion of the human PC cell lines Panc1 and BxPC3. We analyzed aPKC localization and activity by immunohistochemistry and in vitro kinase assays, respectively, to assess their involvement in the regulation of PC progression. Moreover, we examined the effect of nicotine on implanted peritoneal tumors of PC cells in mice.
Results: Nicotine enhanced cell proliferation, migration and invasion in Panc1 and BxPC3 cells. In nicotine-treated PC cells, the aPKC was significantly activated. We also found that nicotine induced phosphatidylinositol 3-kinase (PI3K) signal activation, and a specific inhibitor of the nicotine acetylcholine receptor (nAChR) as well as knockdown of nAChR prevented nicotine-mediated Akt phosphorylation and aPKC activation. In a peritoneal dissemination model of PC, nicotine-treated mice had larger tumors and increased numbers of nodules. Immunohistochemistry showed enhanced expression levels of aPKC and phosphorylated Akt in nodules from nicotine-treated mice.
Conclusions and general significance: Nicotine induces aberrant activation of aPKC via nAChR/PI3K signaling in PC cells, resulting in enhancement of cellular proliferation, migration and invasion.

1. Introduction

Among all types of solid cancers, pancreatic cancer (PC) is the most lethal [1]. PC is the fourth leading cause of cancer death in the United States and Japan, as indicated by the estimated 40,600 and 32,800 deaths in 2015, respectively [2,3]. The low five-year survival rate of PC (7.8%) [4] is attributed to its characteristics, including aggressive progression and resistance to therapy [5,6].

Cigarette smoking (CS) increases the risk of PC by 70–260% compared with non-smokers. The proportion of PC caused by CS is estimated to be 25% [7]. Nicotine is a major component of cigarette smoke. It can be observed at a steady-state concentration of 0.2 μM in the serum of chronic smokers, and acutely increases to 10–100 μM in serum immediately after smoking [8–10]. Nicotine maintains tobacco dependence [11] by interacting with nicotine acetylcholine receptor (nAChR). It is classically known that nicotine exerts its action either in the motor endplate of muscle or the central nervous system that is responsible for tobacco addiction [12].

Atypical protein kinase C (aPKC) isozymes are reported to function in epithelial cell polarity, proliferation, and survival [13,14], and have been implicated in carcinogenesis [15,16]. Its expression levels correlate to poor prognoses of some human malignancies [17,18]. There is a series

Abbreviations: aPKC, Atypical protein kinase C; Par6, Partitioning defective-6; EMT, Epithelial-mesenchymal transition; PC, Pancreatic cancer; CS, Cigarette smoking; nAChR, Nicotine acetylcholine receptor; PI3K, Phosphatidylinositol 3-kinase; MCA, Mecamylamine hydrochloride; Ly, Ly294002; ζPS, ζ pseudo-substrate for aPKC; IP, Immunoprecipitation; Llgl2, Lethal (2) giant larvae.

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of reports demonstrating that enhanced aPKC activity is frequently observed in cancer cell invasion and metastasis [19,20]. Although nicotine is generally thought to have limited carcinogenicity, some studies indicate that nicotine exposure promotes tumor progression [21,22].

Because PC is one of the environmental cancers commonly associated with CS, we hypothesized that nicotine exposure might induce aPKC activation in PC cells, leading to PC progression. In the present study, we demonstrated that nicotine enhanced the proliferation, invasion and migration/metastasis of PC cells in vitro and in a mouse peritoneal dissemination model of PC. Furthermore, we identified nicotine-induced phosphatidylinositol 3-kinase (PI3K) signaling as an important activator of aPKC in the progression of human PC.

2. Materials and methods

2.1. Materials

The commercially available antibodies were as follows: anti-aPKCι (Clone 23) and anti-annexin II (Clone 5) mouse monoclonal antibodies (BD Biosciences, San Jose, CA); anti-aPKCζ (C20) and anti-partitioning defective-6 (Par6) β (H64) rabbit polyclonal antibodies and an anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 6C5) mouse monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA); anti-bromodeoxyuridine (BrdU) mouse monoclonal antibody (Medical & Biological Laboratories Co., Ltd., Nagoya, Japan); anti-phospho-aPKCζ T560 rabbit monoclonal antibody (Abcam Ltd., Cambridge, UK); anti-phospho-Akt S473 rabbit polyclonal antibody [immunohistochemistry (IHC) specific, #9277], anti-phospho-Akt S473 (#4060S) and anti-Akt (#9272) rabbit monoclonal antibodies, and anti-β-actin rabbit polyclonal antibody (#4967) (Cell Signaling Technology, Beverly, MA); Alexa Fluor 488-conjugated secondary antibody (Life Technologies Japan, Tokyo, Japan); horseradish peroxidase-conjugated secondary antibody (GE Healthcare Bio-Sciences, Piscataway, NJ; True Blot, Rockland Immunochemicals Inc., Limerick, PA). The phospho-specific mouse monoclonal antibody against S653 of human lethal (2) giant larvae (Llgl2) was produced against a phospho-peptide [KSLRES(-P)FRKLR] as described previously [23,24].

Panc1 and BxPC3 cells were purchased from the American Type Culture Collection (Manassas, VA). Dulbecco's modified Eagle's medium (DMEM), Roswell Park Memorial Institute (RPMI) 1640 and phosphate-buffered saline (PBS) were purchased from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). Nicotine was purchased from Wako Pure Chemical industries, Ltd. (Osaka, Japan). BrdU was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Fetal bovine serum (FBS) and mecamylamine hydrochloride (MCA), an antagonist of nAChR, were purchased from Sigma-Aldrich (St. Louis, MO). L-Glutamine and a penicillin streptomycin-mixed solution were purchased from Nacalai Tesque (Kyoto, Japan). ζ Pseudo-substrate of aPKC (ζPS) was purchased from Invitrogen (San Diego, CA). Ly294002 (Ly), a PI3K inhibitor, was purchased from Carbiochem (San Diego, CA). The nicotine, MCA and ζPS were dissolved in distilled water. The Ly was dissolved in dimethylsulfoxide. MTT [3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] was purchased from Dojindo Molecular Technologies (Kumamoto, Japan). Can Get Signal, an immunoreaction enhancer solution, was purchased from Toyobo (Osaka, Japan). All other chemicals used were of analytical grade.

2.2. Cell culture

Panc1 cells were grown in DMEM supplemented with 10% heat-inactivated FBS, 4 mM L-glutamine, and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin) at 37°C in a humidified incubator with 5% CO_2. BxPC3 cells were grown in RPMI 1640 supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, and antibiotics. In experiments for membrane-rich fractionation, immunoprecipitation (IP), and IP-kinase assays, spiral scratches were made on cell monolayers, and samples were collected after the indicated incubation times. All cells were subjected to assays before passage 10. The present experiments were performed using the culture medium supplemented with antibiotics unless specified otherwise.

2.3. Cell viability assay (MTT assay)

Cell viability was determined by MTT assays according to the manufacturer's protocol with some modifications. After attachment of cells (5×10^3/well) to a 96-well plate for 24 h, the cells were treated with nicotine and inhibitors (MCA, Ly, or ζPS) in culture medium with 0.5% FBS for 72 h. Then, the cells were treated with MTT in culture medium containing 10% FBS for 3 h at 37°C. After discarding the medium, 100 μL of 0.05 M HCl in 2-propanol was added to each well, and the plate was shaken to dissolve the dark blue crystal. MTT reduction was measured with a microplate reader (Bio-Rad, Berkeley, CA) at 570 nm. The absorbance of untreated control cells was defined as 100% live cells.

2.4. BrdU incorporation assay

The cells were treated with nicotine and inhibitors as described in the above cell viability assay. The BrdU incorporation in the cells after a 2-h pulse was determined as described previously [25].

2.5. Wound healing assay

The cell migration rate was determined by a wound healing assay. Briefly, 2×10^4 cells/well were plated in 24-well plates and gently scratched with a 200-μL pipette tip to produce a wound when the cells had formed a confluent monolayer. The cells were then incubated for 24 h in 0.5% FBS-containing medium. The wounds were observed under a phase contrast microscope (TS100; Nikon, Tokyo, Japan), and the wound healing rate was determined by measuring the scratched area and calculated as (mean area at 0 h − mean area at 24 h) / (mean area at 0 h).

2.6. Cell invasion assay

Cell invasion was analyzed using a Transwell assay (8-μm pore size; CORNING, Corning, NY). Transwell inserts equipped with cell-permeable membranes were placed in 24-well plates. The upper side of the membranes was then coated with type I collagen (Koken, Tokyo, Japan) for 2 h at 37°C to block the pores in the membrane. Panc1 and BxPC3 cells (1×10^4/well) cultured in serum-free medium for 24 h were seeded in the Transwell inserts filled with DMEM containing 0.5% FBS as well as nicotine and/or inhibitors (MCA, Ly, or ζPS). Each well of the 24-well plates was filled with culture medium containing 10% FBS as well as nicotine and/or inhibitors (MCA, Ly or ζPS). Cells were incubated for 24 h at 37°C, and then the membranes were fixed in 3.7% formaldehyde for 10 min. The remaining cells on the upper side of the membranes were removed with a cotton swab. After treatment with Triton X-100, the nuclei of cells that had traversed the cell-permeable membranes were stained with 4′,6-diamidino-2-phenylindole (DAPI). Five visual fields were randomly selected from each membrane and photographed under a microscope (BZ53; Olympus, Tokyo, Japan) at 200× magnification. The number of migrating or invading cells was then counted. Each assay was performed in triplicate. The experiments were performed independently at least three times.

2.7. Immunofluorescence analysis

Cells grown on coverslips were washed twice in PBS, fixed with 3.7% formaldehyde for 10 min at room temperature, permeabilized with 0.2% Triton X-100 in PBS for 10 min, and then blocked with 10% FBS in PBS for