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Ukrain Affects Pancreas Cancer Cell Phenotype in vitro by Targeting MMP-9 and Intra-/Extracellular SPARC Expression

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Key Words
Pancreatic ductal adenocarcinoma · Tumor invasion · Secreted protein acidic and rich in cysteine · Matrix metalloproteinases

Abstract
Background/Aims: We investigated whether the anticancer drug Ukrain (UK) is able to modulate the expression of some of the key markers of tumor progression in pancreatic cell carcinoma, in order to assess its potential therapeutic effect. ***Methods:*** Three cell lines (HPAF-II, PL45, HPAC) were treated with UK (5, 10 and 20 μM) for 48 h, or left untreated. Secreted protein acidic and rich in cysteine (SPARC) mRNA levels were assessed by real-time PCR. Matrix metalloproteinases (MMP)-2 and -9 activity was analyzed by SDS zymography; SPARC protein levels in cell lysates and supernatants were determined by Western blot. Cell cycle was determined by flow cytometric analysis, and invasion by matrigel invasion assay. ***Results:*** UK down-regulated MMP-2 and MMP-9, suggesting that UK may decrease pancreatic cancer cell invasion, as confirmed by the matrigel invasion assay. SPARC protein down-regulation in supernatants points to an inhibition by UK of extracellular matrix remodeling in the tumor microenvironment. At the same time, SPARC mRNA and cellular protein level up-regulation suggests that UK can affect cell proliferation by cell cycle inhibition, showing a cell cycle G2/M arrest in UK-treated cells. ***Conclusion:*** Our results suggest that UK modulates two major aspects involved in tumorigenesis of pancreatic cancer cells, such as extracellular matrix remodeling and cell proliferation.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive tumors, representing the fourth most common cause of cancer death in the Western world, with an estimated incidence of more than 40,000 cases

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per year in the United States [1]. The 5-year survival for all stages of the disease remains <5% [1, 2]. The high incidence of recurrence, lymph node metastases, hepatic metastases, and peritoneal dissemination determine the very poor prognosis of PDAC [3–5]. Current therapy for PDAC is surgery followed by adjuvant radiotherapy and chemotherapy for early-stage and palliative chemotherapy for advanced disease [2]. Gemcitabine is the standard drug in both the adjuvant and the palliative treatment [6, 7]. The variability of clinical response in PDAC patients may be attributed to interindividual differences in the pharmacokinetics and pharmacodynamics of gemcitabine as well as to resistance mechanisms to radiotherapy [8]. Therefore, predictive genetic markers to assess these differences are urgently needed. PDAC usually exhibits an intense desmoplastic reaction and stromal cells release cytokines, growth and angiogenic factors which favor tumor growth and influence chemoresistance [9]. During tumor progression, the phenotypic switch of pancreatic epithelial cells to mesenchymal cells, the so-called epithelial-to-mesenchymal transition (EMT), plays a pivotal role. The loss of epithelial characteristics and the acquisition of a mesenchymal phenotype render tumor cells invasive and able to disseminate, forming distant metastasis [10, 11].

The anticancer drug Ukrain (UK; NSC 631570) contains alkaloids of greater celandine (*Chelidonium majus*, a member of the Papaveraceae family) [12] made water soluble by derivatization with thiotepa. Some clinical investigations suggest that UK, given as a single drug or in combination with conventional chemotherapy, exerts beneficial effects in the treatment of a range of solid tumors including the colon, rectum, breast, pancreas, bladder, and ovary [13–17]. It was shown that UK is a safe and effective drug in the treatment of unresectable pancreatic cancer [16, 18]. When used as adjuvant chemotherapy in combination with gemcitabine, UK seems to lead to a prolonged survival [18].

Considering the biological features of pancreatic cancer, we aimed to investigate whether UK is able to modulate the expression of some of the key markers involved in the mechanisms leading to tumor progression in vitro on three pancreatic cancer cell lines (HPAF-II, HPAC, PL-45), in order to assess its potential therapeutic effect on pancreas cancer. Our results suggest that UK modulates the invasive potential of pancreas cancer cells and their ability to affect extracellular matrix remodeling of the tumor environment.

Materials and Methods

Cell Cultures

For this study, three human pancreatic cancer cell lines were used: HPAF-II, HPAC, PL-45 (American Type Culture Collection, ATCC). All the considered cell lines were obtained from pancreatic adenocarcinoma. Cells were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine, antibiotics (100 U/ml penicillin, 0.1 mg/ml streptomycin), and 0.25 μg/ml amphotericin B. Each sample was cultured in duplicate.

Drug Preparation

UK (kindly provided by Nowicky Pharma, A-1040 Vienna, Austria) was obtained as a 5-mg water-soluble *C. majus* alkaloid thiophosphoric acid derivative in 5 ml water for injection. According to the manufacturer's instructions, the stock solution was stored at room temperature.

Ukrain Treatment

Pancreas cancer cells were treated at 80% of confluence with three final concentrations of UK (5, 10 and 20 μM). Untreated cultures served as controls (CT). CT and treated cells were incubated for 48 h in serum free medium and then harvested by trypsinization (trypsin 0.25%; EDTA 0.1% in PBS). Each cell line was cultured in duplicate. Cell viability was determined by Trypan blue staining.

Real-Time PCR

Total RNA was isolated by a modification of the acid guanidinium thiocyanate-phenol-chloroform method (Tri-Reagent, Sigma, Italy). 1 μg of total RNA was reverse-transcribed in 20 μl final volume of reaction mix (Bio-Rad, Segrate, Italy). mRNA levels for secreted protein acidic and rich in cysteine (SPARC) were assessed. GAPDH was used as endogenous control to normalize the differences in the amount of total RNA in each sample.

The primer sequences, designed with Beacon Designer 6.0 Software (Bio-Rad, Italy), were the following: GAPDH: sense CCCTTCATTGACCTCAACTACATG, antisense TGGGATTTCCATTGATGACAAGC; SPARC: sense GCGAGCTGGATGAGAACAACAC, antisense GTGGCAAAGAAGTGGCAGGAAG.

Amplification reactions were conducted in a 96-well plate in a final volume of 20 μl per well containing 10 μl of 1× SYBR Green Supermix (Bio-Rad, Italy), 2 μl of template, 300 pmol of each primer, and each sample was analyzed in triplicate. The cycle threshold (Ct) was determined and gene expression levels relative to that of GAPDH were calculated by the $2^{-}\Delta\Delta^{Ct}$ method.

SDS Zymography

Culture media were mixed 3:1 with sample buffer (containing 10% SDS). Samples (15 μg of total protein per sample) were run under non-reducing conditions without heat denaturation onto 10% polyacrylamide gel (SDS-PAGE) co-polymerized with 1 mg/ml of type I gelatin. The gels were run at 4°C. After SDS-PAGE, the gels were washed twice in 2.5% Triton X-100 for 30 min each and incubated overnight in a substrate buffer at 37°C (Tris-HCl 50 mM, $CaCl_2$ 5 mM, NaN_3 0.02%, pH 7.5). The matrix metalloproteinase (MMP) gelatinolytic activity was detected after staining the gels with Coomassie brilliant blue R250, as clear bands on a blue background.