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Pancreatic Islet and Stellate Cells Are the Main Sources of Endocrine Gland-Derived Vascular Endothelial Growth Factor/Prokineticin-1 in Pancreatic Cancer

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Key Words
Pancreatic adenocarcinoma · Stellate cells · Endocrine gland-derived vascular endothelial growth factor (EG-VEGF)

Abstract
Aims: Endocrine gland-derived vascular endothelial growth factor (EG-VEGF)/prokineticins have been identified as tissue-specific angiogenic factors. This study investigates the expression and localization of EG-VEGF and its receptors in pancreatic tissues and pancreatic stellate cells (PSCs). ***Methods:*** mRNA levels of EG-VEGF/prokineticin 1 (PK1), prokineticin 2 (PK2) and their receptors 1 (PKR1) and 2 (PKR2) were measured in pancreatic tissues, pancreatic cancer cell lines and PSCs by quantitative reverse-transcriptase polymerase chain reaction (QRT-PCR). Protein expression of PK1, PKR1 and PKR2 was assessed in pancreatic tissues by immunohistochemistry. Growth factor-induced secretion of EG-VEGF was measured by ELISA. ***Results:*** QRT-PCR analysis in bulk tissues of normal pancreas, chronic pancreatitis and pancreatic ductal adenocarcinoma showed no significant difference of PK1 mRNA levels, whereas PK2 mRNA was barely detectable. High PK1 mRNA levels were observed only in cultured PSCs and microdissected islet cells, but not in cancer cells, and PK1 protein was localized mainly in islets and cancer-associated stromal cells. PKR1 and PKR2 proteins were present in endothelial cells of small blood vessels. TGF-β_1 and PDGF-BB specifically stimulated PK1 secretion in PSCs. ***Conclusions:*** Islet and/or PSC-derived PK1 might act through its receptors on endothelial cells to increase angiogenesis in pancreatic diseases.

Introduction

Angiogenesis is a complex multistep process leading to the formation of new blood vessels, which is essential during fetal development, in the female reproductive cycle, and for tissue repair [1]. Angiogenesis also plays a pivotal role in the growth, invasion, and metastasis of malignant tumors, including pancreatic cancer [2, 3]. A better understanding of angiogenic networks may facilitate better antiangiogenic cancer therapy [4].

One of the most specific and critical regulators of angiogenesis is vascular endothelial growth factor (VEGF),

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which regulates proliferation, permeability, and survival of endothelial cells [5, 6]. Since broad-spectrum angiogenic factors such as VEGF are known to play a critical role in tumorigenesis, the discovery of tissue-specific angiogenic factors may allow the development of tumor type-specific angiogenesis inhibitors [7].

Endocrine gland-derived VEGF (EG-VEGF) is a newly identified tissue-specific angiogenic molecule [8]. Although EG-VEGF functionally resembles and complements another angiogenic factor, VEGF, to regulate angiogenesis and permeability as well as to induce the formation of endothelial fenestration, the two molecules are structurally dissimilar and work through different receptors [8, 9]. It is currently known that EG-VEGF, also known as prokineticin 1 (PK1), is highly expressed in steroidogenic tissues, including the ovary, testis, adrenal gland, and placenta [8]. It has also been detected in other non-steroidogenic tissues such as human brain, colon, skeletal muscle, small intestine, spleen, thymus, liver, and uterus [10, 11]. PK1 is a member of a class of proteins that also includes prokineticin 2 (PK2) [12, 13]. PK1 and PK2 are known to bind two closely related G-protein-coupled receptors, PKR1 and PKR2. This binding leads to mobilization of calcium, stimulation of phosphoinositide turnover, and activation of the p44/42 MAPK signaling pathway, which together is consistent with the effects of PKs on smooth muscle contraction and angiogenesis [14, 15].

To date, it is unknown whether PK1 is expressed in pancreatic tissues and whether it plays a role in supporting angiogenesis and tumor growth in pancreatic cancer. In the present study we analyzed the expression of PK1 and its two receptors in different pancreatic tissues as well as in pancreatic stellate cells (PSCs).

Material and Methods

Tissue Sampling

Pancreatic ductal adenocarcinoma (PDAC) tissue specimens (n = 43) and chronic pancreatitis (CP) tissue samples (n = 27) were obtained from diseased patients who underwent pancreatic resections. Normal human pancreatic tissue samples (n = 20) were obtained through an organ donor program from previously healthy individuals. Freshly removed tissues were fixed in paraformaldehyde solution for 24 h and embedded in paraffin for histological analysis. In addition, a portion of the tissue samples was preserved in RNAlater (Ambion Europe Ltd, Huntingdon, Cambs., UK), or snap-frozen in liquid nitrogen immediately upon surgical removal and maintained at –80°C until use. The Human Subjects Committee of the University of Heidelberg, Germany, approved all studies. Written informed consent was obtained from all patients.

Cell Culture

Pancreatic cancer cell lines were grown routinely in RPMI medium (Aspc-1, BxPc-3, Capan-1, Colo-357, SU8686 and T3M4) or DMEM medium (MiaPaCa-2 and Panc-1), supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin (Invitrogen, Karlsruhe, Germany). Primary PSCs isolated from pancreatic tissues using the outgrowth method [16] were cultured in special culture medium (40% DMEM with 1 g/l glucose, 40% F12, 20% fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin) [16]. Activated PSCs of the third passage were used. Cells were maintained at 37°C in a humidified incubator with 5% CO_2.

Real-Time Quantitative Polymerase Chain Reaction

For real-time quantitative PCR (QRT-PCR) analysis, all reagents and equipment for mRNA/cDNA preparation were supplied by Roche Applied Science (Mannheim, Germany). mRNA was prepared by automated isolation using the MagNA Pure LC Instrument and Isolation Kit I (for cells at approximately 80% confluence) and kit II (for tissues). cDNA was prepared using the First-strand cDNA Synthesis Kit for RT-PCR (AMV) according to the manufacturer's instructions. QRT-PCR was carried out using the LightCycler FastStart DNA SYBR Green kit. The number of specific transcripts was normalized to the housekeeping gene cyclophilin B (cpb) and presented as copies/10,000 copies cpb. All primers were obtained from Search-LC (Heidelberg, Germany).

Immunohistochemistry

For immunostaining of EG-VEGF/PK1, frozen tissue sections (5 μm thick) were fixed in acetone at –20°C for 10 min. After washing with 10 mM Tris-HCl, 0.85% NaCl, and 0.1% BSA, pH 7.4, tissue sections were incubated with 0.2% Triton-X 100 for 15 min. Paraffin-embedded tissue sections (3 μm thick) were subjected to immunostaining of prokineticin receptor 1 (PKR1) and prokineticin receptor 2 (PKR2). Tissue sections were deparaffinized in Roticlear® and rehydrated in progressively decreasing concentrations of ethanol. Antigen retrieval was performed by boiling with citrate buffer (pH 6.0) for 15 min. Thereafter, slides were cooled to room temperature and then placed in deionized water for 5 min. Slides were placed in washing buffer and subjected to immunostaining [17]. Endogenous peroxidase activity was quenched by incubating the slides in methanol containing 3% hydrogen peroxide, followed by washing in deionized water for 10 min, after which the sections were incubated at 4°C overnight with the primary antibodies (anti-PK1, MAb 1209, R&D Systems, Minneapolis, Minn. USA; anti-PKR1: 1:200, anti-PKR2: 1:1,000, Abcam, Cambs., UK) diluted in Dako antibody diluent (S3022, Dako Corp., Carpinteria, Calif., USA). The slides were rinsed with washing buffer and incubated with EnVision+ System-labeled polymer HRP anti-mouse antibody or anti-rabbit antibody (Dako) for 30 min at room temperature. Tissue sections were then washed in washing buffer and subjected to 100 μl DAB-chromogen substrate mixture (Dako), followed by counterstaining with hematoxylin. Sections were washed, dehydrated in progressively increasing concentrations of ethanol, and mounted with xylene-based mounting medium. Slides were visualized using the Axioplan 2 imaging microscope (Carl Zeiss Lichtmikroskopie, Göttingen, Germany). Additionally, to confirm the specificity of the primary antibodies, tissue sections were incubated in the absence of the primary antibody and with negative control mouse IgG2a