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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),

X.J. and I.A. contributed equally to this study.

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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

(for PK1) or normal rabbit serum (for PKR1 and PKR2). Under these conditions, no specific immunostaining was detected (data not shown).

PSC Culture Conditions and Detection of PK1

PSCs were trypsinized and seeded into 6-well plates. Upon reaching 95% confluence, cells were washed with PBS, and 2 ml of serum-free medium was added into each well. After 24 h, cells were washed again and 2 ml of serum-free medium was added into each well.

For growth factor stimulation, bone morphogenetic protein 2 (BMP2) (300 ng/ml), transforming growth factor-β_1 (TGF-β_1) (5 ng/ml), fibroblast growth factor-1 (FGF-1) (50 ng/ml), fibroblast growth factor-2 (FGF-2) (50 ng/ml), platelet-derived growth factor-AA (PDGF-AA) (50 ng/ml), or platelet-derived growth factor-BB (PDGF-BB) (50 ng/ml) was added into the culture medium. A selective inhibitor of human vascular β-type PDGF receptor tyrosine kinase (DMPQD) and a selective inhibitor of TGF-β type-I receptor (LY 364947) (Tocris Bioscience, Bristol, UK) was used to analyze growth factor-induced secretion of PK1. For co-culture experiments, PSCs were cultured with different PDAC cell lines. After 48 h of incubation, the supernatant was collected, centrifuged (1,500 rpm/5 min/RT) and stored at –80°C. The PK1 concentration in the supernatant was measured using a human PK1 ELISA Development Kit (900-K244, PeproTech, London, UK) according to the manufacturer's instructions.

Statistical Analysis

Statistical analysis and graph presentation were performed using the GraphPad Prism 4 Software (GraphPad, San Diego, Calif., USA). For comparisons of groups, one-way analysis of variance (ANOVA) for random measures was applied, followed by Dunn's post-hoc test. The level of statistical significance was set at $p < 0.05$.

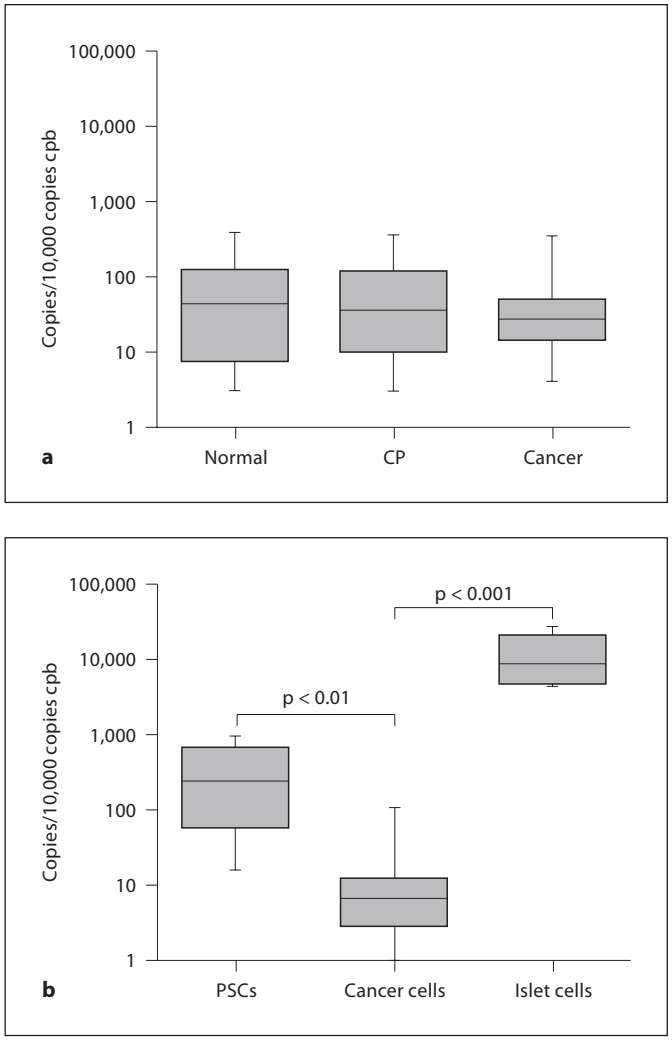

Fig. 1. PK1 mRNA expression levels in pancreatic tissues, pancreatic cancer cell lines and primary PSCs: real-time quantitative RT-PCR analysis of mRNA levels for PK1 in normal, CP and PDAC tissue samples (**a**) as well as in pancreatic cancer cell lines, PSCs and microdissected pancreatic islet cells (**b**) was performed as described in the Methods section. Number of cDNA copies is normalized to 10,000 copies of cyclophilin B (bars: range; box: IQR).

Results

PK1 and PK2 mRNA Levels in Human Pancreatic Tissues, Pancreatic Cancer Cell Lines and PSCs

Quantitative PCR was performed to evaluate the mRNA levels of PK1 and PK2 expression in normal pancreatic tissue samples (n = 20), CP tissue samples (n = 25) and PDAC samples (n = 43). The median (range) copy number of PK1 mRNA/10,000 cpb was 44 (3–389) in normal pancreas tissue samples, 36 (3–357) in CP tissue samples, and 27 (4–346) in PDAC samples (fig. 1a). Tissue samples from the normal pancreas had a median (range) PK2 mRNA copy number/10,000 cpb of 4.5 (0–65), while the median copy number was 2 (0–56) in CP tissues and 2 (0–56) in PDAC tissues (data not shown). No significant difference was found among the three different tissues for both PK1 and PK2 mRNA levels. However, pancreatic tissues revealed remarkably higher expression of PK1 compared to PK2.

Expression of PK1 and PK2 mRNA using QRT-PCR was also analyzed in 8 pancreatic cancer cell lines, primary PSCs and microdissected islet cells. PK1 mRNA expression was low in all the 8 pancreatic cancer cell lines, with a range of 1–105 copies/10,000 cpb (fig. 1b). In contrast, significantly elevated PK1 mRNA levels were found in different PSC clones (n = 8), with a median expression of 240 copies (fig. 1b). PK1 mRNA was also highly expressed in microdissected pancreatic islet cells (n = 5),

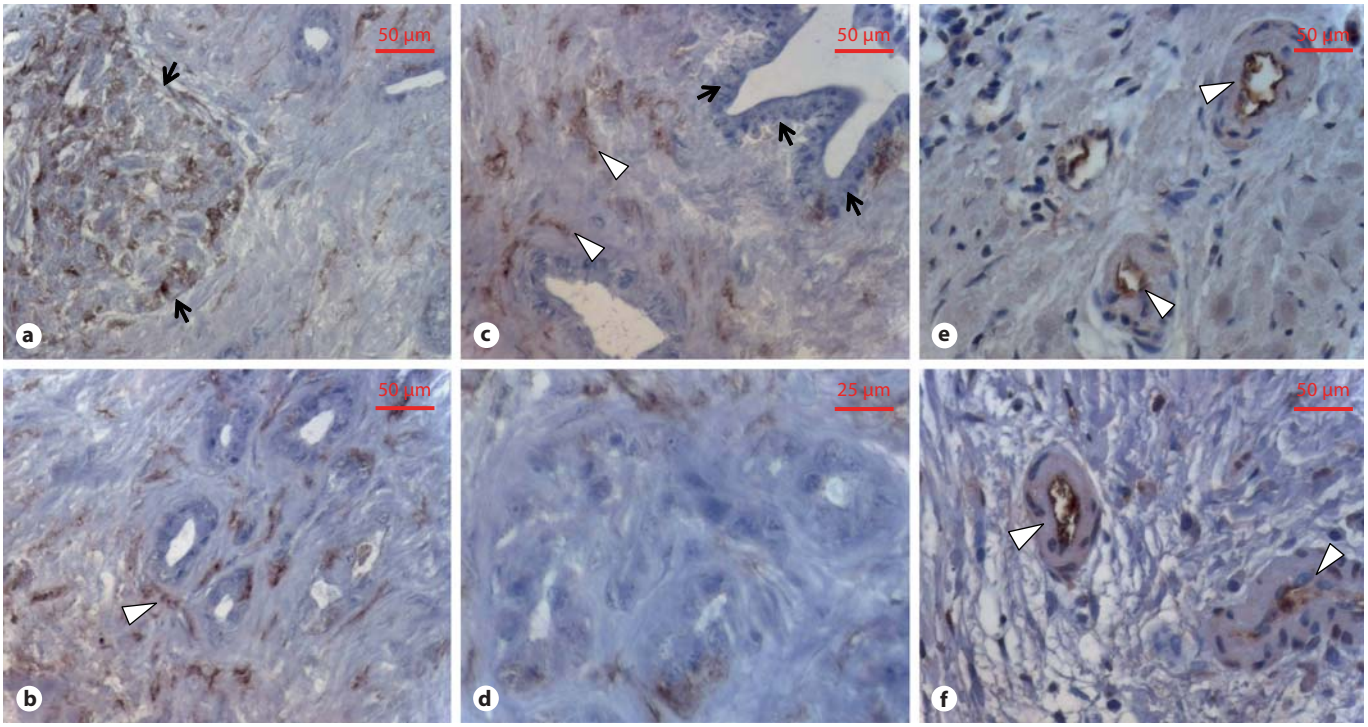

Fig. 2. PK1, PKR1 and PKR2 expression and localization in human PDAC tissues: immunohistochemistry was performed using PK1-, PKR1- and PKR2-specific antibodies as described in the Methods section. Pancreatic tissues displayed PK1 staining in islets (**a**) (arrows). PDAC tissues displayed PK1 staining in tumor-associated PSCs (**b**, **c**) (arrowheads, frozen tissues) and PKR1 (**e**) and PKR2 (**f**, paraffin-embedded tissues) staining in the small vessels (arrowheads). Note the absence of PK1 staining in cancer cells (**c** arrows, **d**).

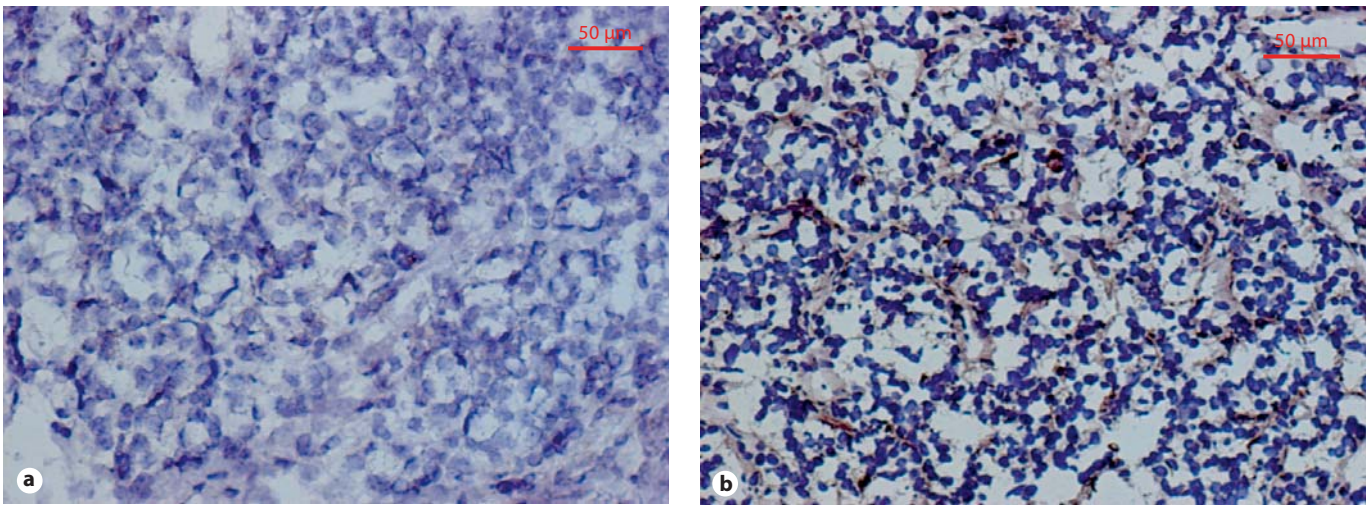

Fig. 3. Expression of PK1 in human CP and neuroendocrine tumor tissues: immunohistochemistry of PK1 in CP tissues (**a**) and pancreatic neuroendocrine tumors (**b**) was performed using PK1-specific antibodies as described in the Methods section.

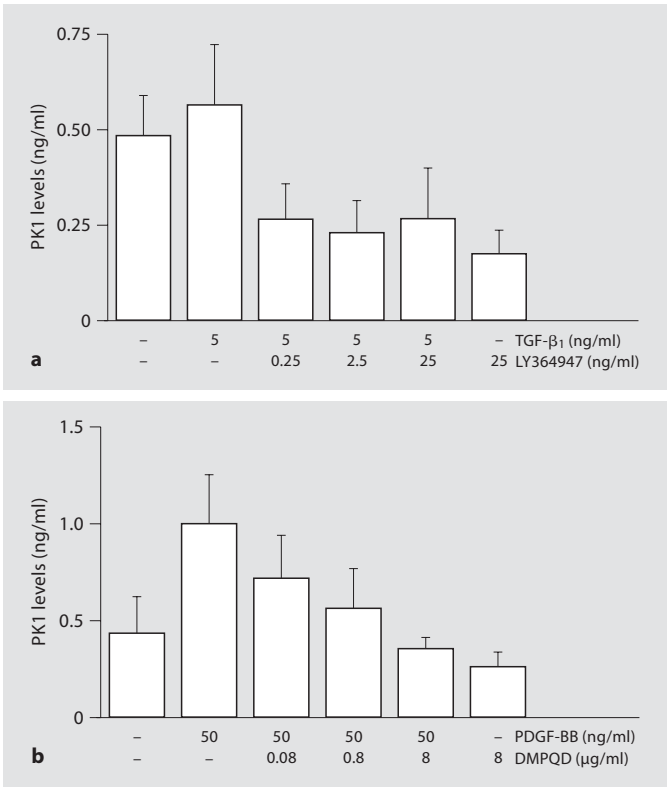

Fig. 4. Growth factor regulation of PK1 secretion in PSCs: primary cultured PSCs were treated with growth factors and with different dose of specific receptor inhibitors as indicated, and PK1 concentration in the supernatant was measured by ELISA. Data are presented as mean ± SEM of three independent experiments.

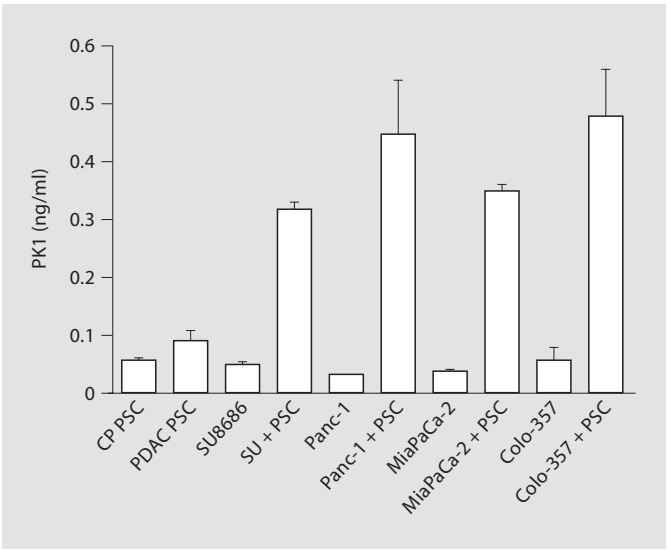

Fig. 5. PDAC cell regulation of PK1 secretion in PSCs: primary cultured PSCs were co-cultured with different PDAC cell lines and the concentration of PK1 in the supernatants was measured by ELISA. Data are presented as mean ± SEM of two independent experiments.

with a median copy number of 8,466 (fig. 1b). Pancreatic cancer cell lines and PSCs did not express PK2 mRNA (data not shown).

PK1, PKR1 and PKR2 Localization in Human Pancreatic Tissues

Immunohistochemistry was performed to determine the distribution of PK1 in 10 normal pancreatic tissues and 15 PDAC tissues. Since PK2 mRNA expression was very low/absent in all tissues and cells analyzed, immunohistochemistry was not carried out for this protein. In normal pancreatic tissues, PK1 immunostaining was only present in islets (fig. 2a). Expression was not detectable in normal pancreatic ductal or acinar cells (data not shown). In PDAC tissues, PK1 staining was present in tumor-associated stromal cells, i.e. PSCs (fig. 2b, c). In contrast, cancer cells were devoid of PK1 immunoreactivity (fig. 2c, d).

Since PK1 was present in PSCs of PDAC tissues, we analyzed the expression of the two PK1 receptors – PKR1 and PKR2 – in PDAC tissues. Immunohistochemical analysis revealed localization of PKR1 (fig. 2e) and PKR2 (fig. 2f) in endothelial cells of small vessels in PDAC but not in non-malignant pancreatic tissues. These proteins were also absent in ducts, islets, acinar cells, PSCs and cancer cells of pancreatic tissues (data not shown). Interestingly, expression of PK1 was absent in stromal cells of CP tissues (fig. 3a). In contrast, analysis of pancreatic neuroendocrine tumors revealed strong expression of PK1 in stromal cells of these neoplastic tissues (fig. 3b).

Growth Factor Regulation of PK1 Secretion in PSCs

Several growth factors can stimulate the secretion of VEGF and thus enhance angiogenesis in pancreatic cancer. In this study, we analyzed whether PK1 secretion in PSCs was regulated by growth factors. The concentration of PK1 without treatment was 0.42 ± 0.19 ng/ml in the supernatant of PSCs (fig. 4a, b). TGF-β_1 as well as PDGF-BB treatment increased secretion of PK1 by PSCs: 0.55 ± 0.16 ng/ml for TGF-β_1 (fig. 4a) and 0.98 ± 0.27 ng/ml for PDGF-BB (fig. 4b). Moreover, specific blockage of TGF-β and PDGF receptors in PSCs significantly suppressed secretion of PK1 induced by these growth factors (fig. 4a, b). Secretion of PK1 in cell supernatants did not signifi-

cantly change after treatment with BMP2, FGF-1, FGF-2 and PDGF-AA (data not shown). Next we sought to compare expression of PK1 between PSCs isolated from CP and PDAC tissues. PDAC stellate cells displayed approximately 50% higher secretion of PK1 than CP-derived PSCs (fig. 5). Since the secretion of PK1 from PSCs was found to be growth factor regulated and since pancreatic cancer cells are known to produce different growth factors [18], next we examined the release of PK1 under co-culture conditions. To this end, cancer tissue-derived PSCs were utilized (fig. 5, second column). These experiments revealed that co-culturing of these PSCs with different pancreatic cancer cells resulted in a 5- to 13-fold upregulation of PK1 release (fig. 5).

Discussion

PDAC is the fourth leading cause of cancer deaths in the USA, with a median survival of <6 months and a dismal 5-year survival rate of <5% when all stages are combined. Its lethal nature stems from its tendency to rapidly disseminate to the lymphatic system and distant organs, and from its resistance to conventional and targeted therapies [19, 20].

PSCs are myofibroblast-like cells found in the areas of the pancreas that have exocrine function. PSCs are known to be crucially involved in the development of pancreatic fibrosis/desmoplasia a characteristic feature of CP and pancreatic cancer [21–23]. The key event in the pathogenesis of fibrosis represents a transition process of quiescent PSCs into a myofibroblast-like phenotype, associated with cell activation in terms of proliferation and synthesis of profibrogenic substances [22, 23]. Studies have shown that the interaction of stellate and cancer cells promotes progression of pancreatic cancer [21–27]. Previously we have shown that PSCs secrete VEGF [28], therefore PSCs might also play an important role in angiogenesis of PDAC.

Recently, PK1/EG-VEGF, a new angiogenic factor expressed mainly in normal endocrine cells, has been identified [13]. In normal human tissues, PK1 is expressed only by hormone-producing cells such as the ovaries, testes and placenta [13]. Like VEGF, PK1 is involved in angiogenesis in these tissues. Studies in endocrine gland-related cancers, such as ovarian carcinoma [11], Leydig cell tumor [29] and prostate cancer [30], demonstrate that PK1 could be involved in carcinogenesis via regulation of angiogenesis. In addition, PK1 expression was found to be upregulated in colon cancer cell lines that express estrogen receptor β, promoting cell proliferation and liver metastasis in colorectal cancers [31]. In the present study, we demonstrate absence of PK1 in normal ductal cells and cancer cells of pancreatic tissues. Furthermore, eight cultured pancreatic cancer cell lines displayed PK1 levels at the threshold of detection. Therefore, cancer cell-derived PK1 does not seem to play a relevant role in PDAC. In addition, we analyzed expression of PK1 in pancreatic neuroendocrine tumor tissues. Similarly to ductal adenocarcinoma, neuroendocrine tumors of the pancreas displayed PK1 expression only in the stromal cells. It is currently not known to what extent PSC-derived VEGF [28] versus PSC-derived PK1 (present study) contributes to angiogenesis in vivo, especially with respect to the complex interaction of PSCs with pancreatic cancer cells and their contribution to angiogenesis [4].

Previous studies have highlighted the role of the interaction of tumor and stroma in cancer progression [26, 32, 33]. Since PSCs are the main component of stroma in pancreatic cancer, we also examined the expression of PK1 in these cells. PSCs expressed PK1 mRNA and secreted PK1 protein. Moreover, this expression was predominant not in quiescent myofibroblasts, but in PSCs activated by pancreatic cancer cells, i.e., PSCs adjacent to the cancer cells, suggesting that although pancreatic cancer cells did not secrete PK1 themselves, the interaction of cancer cells with PSCs might stimulate PSCs to release PK1. Many growth factors are secreted by pancreatic cancer cells, which participate in tumor development and activation of PSCs [22–24]. Here, we demonstrate that TGF-β_1 and PDGF-BB can specifically stimulate PSCs to secrete PK1. Interestingly, inhibition of the TGF-β receptor downregulated even basal PK1 production, which could be result of inhibition of TGF-β_1 produced by PSC itself [26]. In addition, co-culture of PSCs with pancreatic cancer cells strongly induces PK1 secretion, suggesting that growth factors released by cancer cells might stimulate PSCs, which results in secretion of PK1 by PSCs.

One of the characteristic features of solid tumors is hypoxia. Hypoxia selects cancer cells that are more resistant to radiotherapy and chemotherapy. It has previously been reported that adrenal carcinoma cell lines exposed to low oxygen concentration display a strong upregulation of both VEGF and PK1 genes, and that this response is potentially mediated by HIF-1 [34]. In the present study, we investigated whether hypoxia could stimulate PK1 secretion in PSCs. Unlike adrenal carcinoma cells, PSCs exposed to hypoxia did not increase PK1 secretion (data not shown). Therefore, we hypothesize that hypoxia may stimulate PSCs to secrete VEGF, but not PK1.

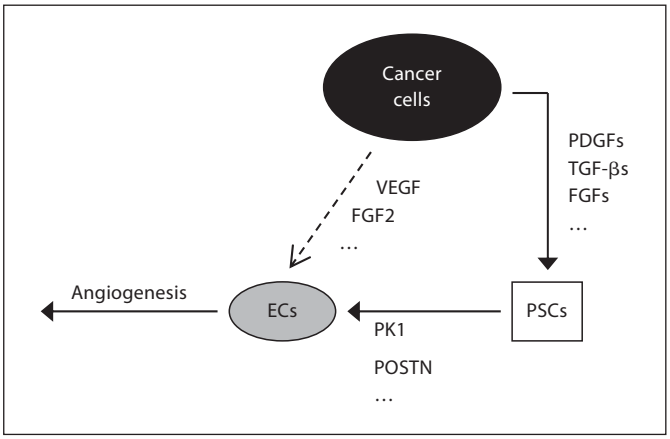

Fig. 6. Schematic model of direct and indirect effects of cancer cells on angiogenesis. PSCs = Pancreatic stellate cells; ECs = endothelial cells.

Two small, highly identical (80–90%) G-protein-coupled receptors of the neuropeptide Y (NPY) receptor class are known as the cognate receptors for PK1 and the related peptide PK2 [14, 15]. These receptors were designated as EG-VEGFR-1/ZAQ and EG-VEGFR-2/GPCR73, or prokineticin receptor-1 (PKR1) and PKR2 [14, 15]. Transcripts, and presumably both proteins, for PKR1 and PKR2 are expressed in adrenal cortex capillary endothelial cells [35], where PK1 promotes proliferation, survival, and chemotaxis through binding to these two receptors. However, appreciable expression was absent in other endothelial cell types, such as human umbilical vein endothelial cells [8]. In the present study, we demonstrate expression of PKR1 and PKR2 in endothelial cells of small vessels in pancreatic cancer tissues. Therefore, similarly to VEGF and its receptors, the PK/PKR system represents a paracrine organization, where ligands produced by non-endothelial cells and the receptors are selectively expressed in the vascular endothelium.

In conclusion, pancreatic cancer cells have the potential to increase PK1 production in PSCs via certain growth factors like TGF-β_1 and PDGF. PK1 in turn might act on endothelial cells, thereby contributing to angiogenesis (fig. 6). Thus, this represents another – indirect way – for pancreatic cancer cells to stimulate angiogenesis.

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