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Activation of IGF/IGF-IR signaling pathway fails to induce epithelial-mesenchymal transition in pancreatic cancer cells

E.P. Kopantzev [a, b, *], M.R. Kopantseva [a, b], E.V. Grankina [a, b], A. Mikaelyan [c, d], V.I. Egorov [a, e], E.D. Sverdlov [a]

[a] M. M. Shemyakin and Yu. A. Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Ul. Miklukho-Maklaya 16/10, 117997, Moscow, Russia
[b] A. V. Vishnevsky Institute of Surgery, Ul. B. Serpuchovskaya 27, 115998, Moscow, Russia
[c] Institute of Developmental Biology, Russian Academy of Sciences, Ul. Vavilova 26, 119991, Moscow, Russia
[d] Skolkovo Institute of Science and Technology, Skolkovo Innovation Center, Skolkovo, 143025, Russia
[e] Bakhrushin Brothers Moscow City Hospital, Department of Surgical Oncology, Ul. Stromynka 7, 107014, Moscow, Russia

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ABSTRACT

Background: Pancreatic cancer stromal cells produce various protein factors, which presumably provide cancer cells with drug resistance and may influence their ability to form metastasis via induction of epithelial-mesenchymal transition (EMT). The goal of our project was to study the effects of IGF-I on expression of protein markers of epithelial and mesenchymal differentiation, and on expression of transcriptional regulators of EMT in pancreatic cancer cell lines.
Methods: We used Western blot analysis to study the expression patterns of epithelial and mesenchymal protein markers in pancreatic cancer cell lines, which have been stimulated with IGF-I for various periods of time. The ELISA technique was employed to determine the concentration of IGF-I in conditioned media. Additionally, the effect of IGF-I on proliferation of pancreatic cancer cells was measured via MTS technique.
Results: We investigated the effect of IGF/IGF-IR signaling pathway activation on expression levels of cell differentiation markers in five pancreatic cancer cell lines (AsPC-1, BxPC-3, Capan-2, MiaPaCa-2 and Panc1). The IGF-I stimulation led to phosphorylation of IGF-IR and activation of PI-3K/Akt signaling cascade. At the same time our results reveal that the activation of IGF/IGF-IR signaling pathway in pancreatic cancer cells does not induce a significant shift in cell phenotype towards mesenchymal differentiation and does not induce a decrease in expression levels of epithelial protein markers.
Conclusions: Our results demonstrate that IGF-I does not function as an effective inductor of EMT in pancreatic cancer cell lines and that stimulation of IGF-I/IGF-IR signaling pathway does not lead to EMT associated changes in cell differentiation.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal forms of cancer. The survival rate among patients with this diagnosis is extremely low [1]. The tumor can be surgically removed in only 20% of the cases, while the rest of the patients with locally advanced or metastatic tumors are subjected to low-efficient gemcitabine-based chemotherapy [2,3]. Established risk factors for PDAC include cigarette smoking, chronic pancreatitis, obesity and long-standing diabetes mellitus [4,5].

Pancreatic ductal adenocarcinoma is primarily characterized by the significant presence of tumor stroma that can comprise more than 70% of full volume of the tumor [6,7]. Stromal microenvironment cells produce various protein factors, which presumably provide cancer cells with drug resistance and may also influence their ability to form metastasis [7]. Recent studies have demonstrated that protein factor IGF-I, which is frequently overexpressed in pancreatic tumor microenvironment in many ways may determine drug resistance in PDAC cells and their ability to migrate and

* Corresponding author. M. M. Shemyakin and Yu. A. Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Ul. Miklukho-Maklaya 16/10, 117997, Moscow, Russia.
E-mail address: kopantzev@ibch.ru (E.P. Kopantzev).

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form metastases [8–10].

Pancreatic cancer is characterized by resistance to standard chemotherapy drugs and relatively early formation of metastases. The initiation of metastases formation is traditionally linked to epithelial-mesenchymal transition (EMT), a process that can be induced in tumor cells via secreted protein factors from tumor microenvironment [11]. Additionally, it is considered that the phenotype transformation of tumor cells as a result of EMT may be followed by the appearance of slow-dividing cells, displaying some properties of cancer stem cells and an increased resistance to standard chemotherapy [11,12]. In the present study, we examined the effect of IGF-I factor on expression of protein markers of epithelial and mesenchymal differentiation, as well as on expression of transcriptional regulators of EMT in five pancreatic cancer cell lines. Our results indicate that despite the presence of functional IGF-I/IGF-IR signaling pathway in these cell lines, stimulation of cells with IGF-I does not lead to a pronounced decrease in epithelial protein marker expression, nor does it lead to an increased expression of mesenchymal markers.

Methods

Materials and cells

Unless otherwise specified, chemicals were obtained from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA). Sera and cell culture media were obtained from Invitrogen (Invitrogen Corporation, Carlsbad, CA, USA). Primary antibodies were as follows: rabbit polyclonal H-63 anti-N-Cadherin, rabbit polyclonal H-108 anti-E-Cadherin, rabbit polyclonal H-102 anti-β-Catenin, mouse monoclonal C51 anti-Cytokeratin-8, mouse monoclonal DCS-6 anti-Cyclin D1, mouse monoclonal B-8 anti-SMAD4, mouse monoclonal 0411 anti-GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse monoclonal V9 anti-vimentin (Sigma-Aldrich), rabbit monoclonal D23H3 anti-IGF-IRβ, rabbit monoclonal C14A11 anti-P-IGF-IRβ (Y980), rabbit monoclonal DA7A8 anti-P-IGF-IRβ (Y1135), rabbit monoclonal C67E7 anti-panAkt, rabbit monoclonal 244F9 anti-P-Akt (T308), rabbit monoclonal D9E anti-P-Akt (S473), rabbit monoclonal D5Z5Z anti-GSK-3β, rabbit monoclonal D85E12 anti-P-GSK-3β (S9), rabbit monoclonal 137F5 anti-p44/42 MAPK (Erk1/2), rabbit monoclonal D13.14.4E anti-P-p44/42 MAPK (T202/Y204), mouse monoclonal BA17 anti-Keratin 19, mouse monoclonal L40C6 anti-Slug, mouse monoclonal L70G2 anti-Snail, rabbit monoclonal D80D3 anti-ZEB1, rabbit monoclonal D43B4 anti-SMAD2, rabbit monoclonal 138D4 anti-P-SMAD2(S465/467), rabbit monoclonal 9F3 anti-β-Tubulin and rabbit monoclonal D1H2 anti-Histone H3 (Cell Signaling, Danvers, MA, USA). Secondary goat anti-mouse and goat anti-rabbit IgG antibodies conjugated to HPR were obtained from Santa Cruz Biotechnology.

AsPC-1, BxPC-3, Capan-2, MiaPaCa-2 and Panc1 human pancreatic cancer cell lines were obtained from American Type Cell Collection (ATCC, Manassas, VA, USA) and maintained in Dulbecco's modified Eagle medium/Ham's F12 (DMEM/F12, 1:1) containing 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Prior to stimulation experiments medium was changed to DMEM/F12 (1:1) with 0.5% serum for 24 h. Serum-starved cells were treated with recombinant human IGF-I (Sigma–Aldrich, 100 ng/ml) for the designated time. Primary stromal cell cultures of the pancreatic tumors were obtained using digestion of the ground tumor tissue with an enzyme mixture (collagenase/dispase/DNAse) as described by us earlier. Stromal cells were cultured in DMEM/F12 (1:1) medium supplemented with 10% fetal calf serum, 100 μg/ml streptomycin, and 100 U/ml penicillin. Stromal cells at passages 4–6 of culturing were used in the experiments. All cells were cultured at 37 °C in humidified atmosphere of 95% air and 5% CO_2.

Western blot analysis and IRS-1 immunoprecipitation

Cell lysates were prepared from subconfluent cultures of IGF-I stimulated and control cells. To this end, cells were washed twice with cold PBS and lysed for 30 min in NP-40 lysis buffer (1% NP-40, 0.2% sodium deoxycholate, 200 mM NaCl, 100 mM Tris-HCl, pH 7.5, 2 mM EDTA, 1 mM EGTA, 10 mM NaF, 1 mM Na_3VO_4, 1 mM AEBSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 10 μg/ml antipain) on ice. The protein concentration in the lysates was determined using a Micro BCA protein assay kit (Pierce, Rockford, IL, USA). Equal amounts of cell lysates (20 μg of protein) were boiled in SDS sample buffer consisting of 1% SDS, 2% 2-mercaptoethanol, and 62 mM Tris-HCl, pH 6.8, subjected to SDS electrophoresis in 10–15% polyacrylamide gels and then electrotransferred to a PVDF Immobilon-P membrane (Millipore, Bedford, MA, USA) using a Bio-Rad Trans-Blot SD cell (Bio-Rad Laboratories, Hercules, CA, USA). After this, the membranes were blocked with 5% skimmed milk in PBS-T (PBS containing 0.1% Tween 20) for 1 h at room temperature, incubated in PBS-T containing 5% skimmed milk and the relevant primary antibody overnight at 4 °C and finally washed three times with PBS-T. Mouse monoclonal anti-GAPDH antibody was used as a loading control. After washing, the membranes were incubated in PBS-T containing 5% skimmed milk and a goat anti-mouse or anti-rabbit antibody HRP conjugates (Santa Cruz, 1:5.000) for 1 h at room temperature. The membranes were finally washed with PBS-T, and specific signals were visualized using a Clarity Western ECL (Bio-Rad) and a Bio-Rad ChemiDoc Touch imager station. The digital images of Western blot bands were quantified by densitometric analysis using the Bio-Rad Image Lab (Version 5.2.1) software program, and the expression levels were normalized to GAPDH, Beta-Tubulin and Histone H3 expression levels.

For immunoprecipitation with IRS-1 antibodies (H-165, Santa Cruz Biotechnology), cells were grown in DMEM/F12 (1:1) medium containing 10% FCS and then incubated for 24 h in medium containing 0.5% FCS before stimulation with IGF-I (100 ng/ml) for 10 min. Cells were lysed in ice-cold NP-40 lysis buffer with 0.1% SDS. Cell lysates (500 μg) were incubated for 1 h at 4 °C with IRS-1 antibodies (10 μg/ml), followed by incubation with Protein A/G-Agarose PLUS (Santa Cruz Biotechnology) on a rotating device at 4 °C overnight. Precipitates were washed three times with ice-cold NP-40 lysis buffer, resuspended in loading buffer and heated for 5 min at 95 °C. After centrifugation the supernatants were subjected to Western blotting. Membranes were probed with anti IRS-1 (59G8, Cell Signaling) or anti-*p*-Tyr (PY99, Santa Cruz Biotechnology) antibody.

RNA isolation, reverse transcription and quantitative PCR analysis

Total RNA from pancreatic cancer cells was isolated using Trizol reagent (Thermo Fisher Scientific, Inc. Waltham, MA, USA), purified with the RNeasy Mini Kit (Qiagen, Hilden, Germany) and used as a template for RT with the Mint-2 (Evrogen, Moscow, Russia) cDNA synthesis kit. The mRNA expression levels were evaluated using qPCRmix-HS SYBR mix (Evrogen, Moscow, Russia) and Applied Biosystems 7300 Real-Time PCR System (Thermo Fisher Scientific, Inc.). The primers for each target were examined to estimate the effectiveness of the PCR reactions using diluted cDNA prepared from untreated Panc1 cells. The reaction efficiencies for all used probes were between 92 and 95%. The results of RT-qPCR are presented as the mean of three independent experiments and normalized against *GAPDH* expression. Statistical analysis was performed using GraphPad Prism (Version 6.01) (GraphPad Software, San Diego, USA). The data are presented as the

means ± standard error of the mean (SEM). *P*-value equal or less than 0.05 was considered statistically significant. Primer sequences are presented in Supplementary Table ST1.

Cell proliferation assay and ELISA

AsPC-1, BxPC-3, Capan-2, MiaPaCa-2 and Panc1 cells were seeded onto 96-well flat-bottomed plates at a density of 1000 cells/well, then incubated at 37 °C for 24 h in DMEM/F12 (1:1) medium supplemented with 0.5% fetal calf serum. Subsequent to changing the medium with different concentrations of IGF-I (10 ng/ml, 50 ng/ml and 200 ng/ml), the cells were cultured for a further 48 h, and subjected to an MTS dye reduction assay (Promega, Madison, WI, USA). Absorbance was measured at 570 nm with a GENios Pro microplate reader (TECAN, Mannedorf, Switzerland). Statistical analysis was performed using GraphPad Prism (Version 6.01) (GraphPad Software, San Diego, USA). The data are presented as the means ± standard error of the mean (SEM). *P*-value equal or less than 0.05 was considered statistically significant.

To generate cell conditioned media, pancreatic cancer and stromal cells were cultured in low serum (0.5%) DMEM/F12 (1:1) medium for 48 h, supernatant was harvested, centrifuged at 4 °C and stored at −20 °C. The levels of secreted IGF-I and IGF-II in cell conditioned media were measured by ELISA kits (R&D Systems, Minneapolis, MN, USA) following the manufacturer's protocols.

Results

Epithelial and mesenchymal differentiation protein marker expression in pancreatic cancer cell lines

In this study we investigated the effect of IGF-I stimulation on five pancreatic cancer cell lines. These cell lines had different mutational statuses [13] and different levels of epithelial marker gene expressions. Fig. 1 shows Western blot analysis of epithelial and mesenchymal differentiation marker proteins expression levels in cell lines AsPC-1, BxPC-3, Capan-2, MiaPaCa-2 and Panc1, which have been cultivated in cell medium with 10% serum. Cell lines BxPC-3 and Capan-2 are characterized by a high level of E-cadherin expression. Cell lines AsPC-1 and Panc1 have much lower levels of E-cadherin protein expression, while in MiaPaCa-2 cells E-cadherin expression has not been detected at all. The overall expression level of epithelial adherent junction associated protein β-catenin correlated with expression levels of E-cadherin. All five cell lines also had various expression levels of cytokeratin-8 (CK-8) and cytokeratin-19 (CK-19). At the same time, three cell lines (AsPC-1, MiaPaCa-2 and Panc1) expressed vimentin (VIM), a known marker of mesenchymal lineage. Cell line Panc1 additionally expressed mesenchymal intercellular junction protein N-cadherin (Fig. 1a).

A moderate expression level of the IGF-I receptor (IGF-IRβ) was detected in untreated cells of all five cell lines. Furthermore, we detected low levels of the activated form of the receptor, phosphorylated at Tyr-1135, in lysates of AsPC-1, BxPC-3, MiaPaCa-2 and Panc1 cell lines (Fig. 1b). We also detected the phosphorylated form of the receptor (Tyr-980) in lysates of untreated cells AsPC-1 and BxPC-3 (Fig. 1b). A visible expression level of ZEB1 protein, which functions as a transcriptional regulator of EMT, was detected only in vimentin-positive cell lines: AsPC-1, MiaPaCa-2 and Panc1. Another transcriptional regulator of EMT, Snail (SNAI1), was detected in cell lines MiaPaCa-2 and Panc1, while Slug (SNAI2) was present only in lysates of cell line BxPC-3. Two other well known transcriptional regulators of EMT, TWIST1 and ZEB2, have not been detected in any of the cell lines we used in our study (data not provided in the paper).

Expression of IGF-I in conditioned mediums and the effect of IGF-1 on proliferation of pancreatic cancer cell lines

It is worth noting that none of the five cell lines secreted IGF-1 on a significant level. A low concentration of IGF-I has only been identified via ELISA method in conditioned media from BxPC-3 and Capan-2. At the same time a much higher level of IGF-I has been detected in conditioned mediums from two out of three stromal pancreatic cancer cell cultures (Supplementary Table ST2). Conditioned medium from Panc1 cells contained a detectable level of IGF-II protein, which has not been observed in conditioned mediums from other cell cultures in our experiments (Supplementary Table ST2). We have also determined the effect of IGF-I on cell proliferation of five pancreatic cancer cell lines (Fig. 2). In culture medium with low concentration of serum IGF-I significantly stimulated proliferation in AsPC-1 cells, had a less powerful stimulating effect on BxPC-3, MiaPaCa-1 and Panc-1 cells, and did not have a detectable stimulating effect on proliferation of Capan-2 cell culture (Fig. 2a).

The effect of short-term stimulation of cells with IGF-I on activation of IGF1/IGF-IR signaling pathway in pancreatic cancer cell lines

Before determining the long-term effects of IGF-I stimulation on

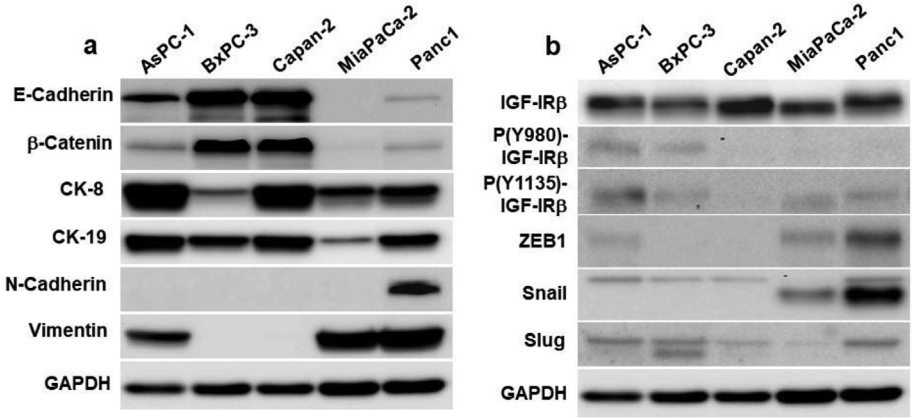

Fig. 1. (**a**) Western blot analysis of expression of epithelial (E-cadherin, β-catenin, CK-8 and CK-19) and mesenchymal (N-cadherin and vimentin) proteins in pancreatic cancer cell lines. (**b**) Western blot analysis of expression of IGF-IR protein and transcriptional regulators of EMT (ZEB1, Snail and Slug) in pancreatic cancer cell lines. Whole cell lysates were prepared from indicated pancreatic cancer cell lines grown in medium supplemented with 10% serum. GAPDH used as a loading control.

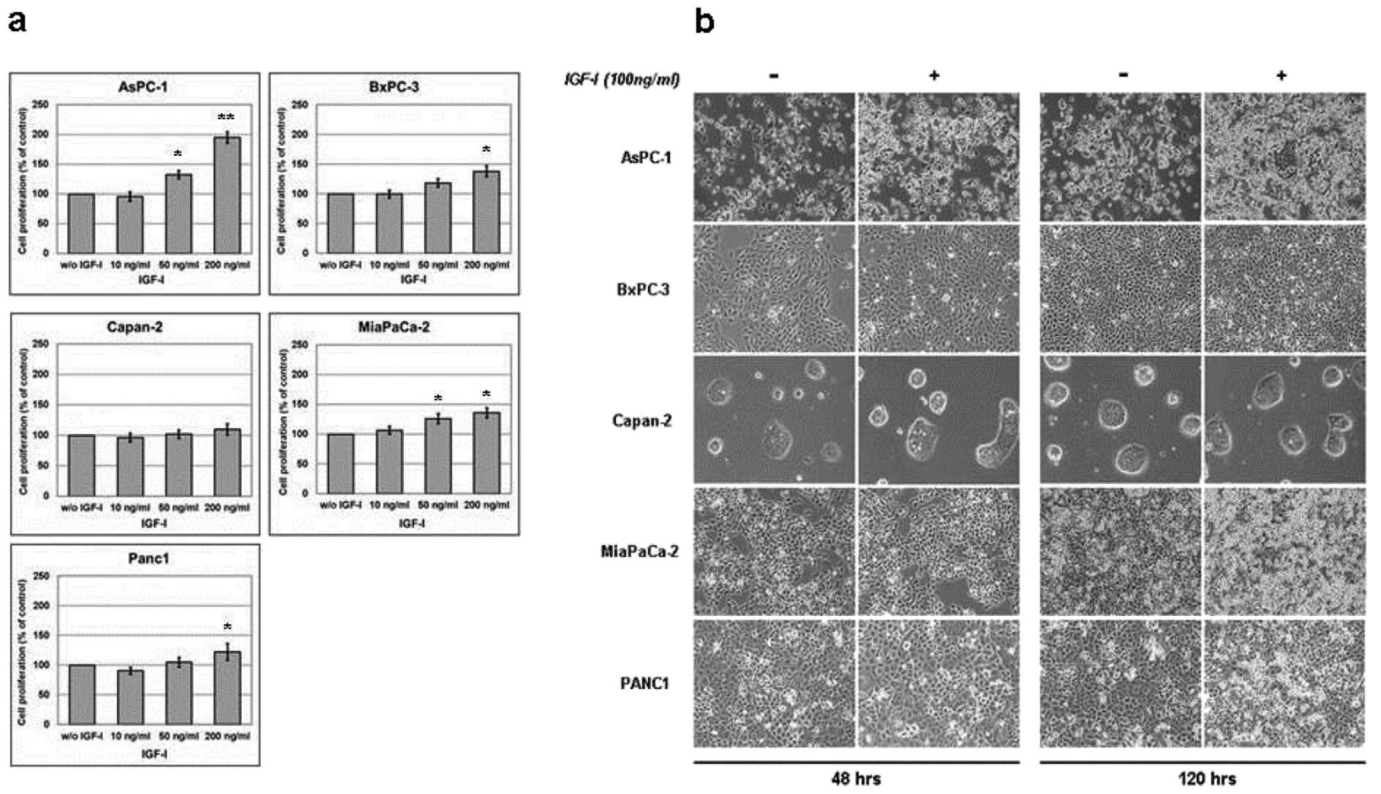

Fig. 2. (**a**) Effect of different concentrations of IGF-I on cell proliferation of pancreatic cancer cell lines (AsPC-1, BxPC-3, Capan-2, MiaPaCa-2 and Panc1). After incubation for 24 h in DMEM/F12 (1:1) medium containing 0.5% FCS cells were stimulated with 10 ng/ml, 50 ng/ml and 200 ng/ml of IGF-I for 48 h. Cell growth was determined using the MTS dye reduction assay. After subtracting background values, data were expressed as the percentage increase of control cell growth. Data are means ± SEM from 3 technical replicates and representative of at least 3 experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with untreated control (unpaired Student's t-test). (**b**) Cellular morphology of AsPC-1, BxPC-3, Capan-2, MiaPaCa-2 and Panc1 control untreated cells and cells treated with IGF-I. After incubation for 24 h in DMEM/F12 (1:1) medium containing 0.5% FCS cells were stimulated with 100 ng/ml of IGF-I for 4, 48 and 120 h. Original magnification X100 (Phase contrast).

expression levels of epithelial and mesenchymal differentiation markers, we conducted an experiment to test the effect of short-term IGF-I stimulation on cell lines AsPC-1, MiaPaCa-2 and Panc1 in culture medium with low serum concentration (Fig. 3). Cells in culture medium with 0.5% serum were treated with IGF-I (100 ng/ml) and lysed after 5, 10 and 20 min of treatment. This stimulation with IGF-I led to rapid tyrosine phosphorylation of IGF-IR receptor, adaptor protein IRS-1 (Supplementary Figure SF1) and phosphorylation Akt-kinase, which indicates that the PI-3K/Akt signaling cascade has been activated in treated cells. IGF-I stimulation in the investigated cell lines also led to serine phosphorylation of GSK-3β, a downstream target of activated Akt-kinase. Additionally, IGF-I treated Panc1 cells had an increased level of MAPK (ERK1/2) phosphorylation. At the same time, IGF-I stimulation did not lead to a significant increase in MAPK phosphorylation in IGF-I treated AsPC-1 and MiaPaCa-2 cells, relative to untreated control cells (Fig. 3).

The effect of long-term stimulation of cells with IGF-I on expression levels of epithelial and mesenchymal differentiation markers in pancreatic cancer cell lines

Before starting our experiments on IGF-I-treated cells we conducted a preliminary experiment aimed at inducing EMT in Panc1 and AsPC-1 cells via TGF-β2 (Supplementary Fig. 2, SF2). Treatment of Smad4-positive Panc1 cells with TGF-β2 leads to morphological changes in these cells that are characteristic for EMT, induces a decrease in the level of CDH1 expression and an increase in Snail expression level. Treatment of Smad4-negative AsPC-1 cells did not induce a decrease in the level of CDH1 expression, nor did it induce any changes in cell morphology. In order to investigate the long-term effects of IGF-I stimulation on expression of protein markers of epithelial and mesenchymal differentiation cell lines AsPC-1, BxPC-3, Capan-2, MiaPaCa-2 and Panc1 were incubated for 4, 48 and 120 h in the presence of IGF-I in cell culture medium with 0.5% serum concentration. Lysates of treated and control cells were then analyzed to determine levels of expression of markers of epithelial (E-cadherin, CK-8 and CK-19) and mesenchymal differentiation (vimentin and N-cadherin), levels of expression of transcriptional regulators of EMT (Snail, Slug and ZEB1) and of activated IGF-IR and Akt kinase, and finally levels of expression of Cyclin D1 and β-catenin proteins (Fig. 4 and Supplementary Figure SF3).

Treatment of cells with IGF-I did not change levels of vimentin expression in Vimentin-positive cell lines (AsPC-1, MiaPaCa-2 and Panc1) and did not induce vimentin expression in vimentin-negative cell lines (BxPC-3 and Capan-2). The expression level of N-cadherin protein in IGF-I treated Panc1 cells did not change relative to its expression level in control cells. The quantity of N-cadherin mRNA in IGF-I treated Panc1 cells also did not change relative to control cells (Supplementary Figure SF4).

Stimulation of cells with IGF-I did not lead to a noticeable decrease in expression of epithelial differentiation marker proteins (E-cadherin, CK-8 and CK-19). On the other hand, the relative amount of E-Cadherin mRNA in Panc1 cells, stimulated with IGF-I for 48 h, was lower than the levels of the same mRNA in untreated control cells (Supplementary Figure SF4). However, this was not accompanied by a decrease in E-Cadherin protein levels in IGF-I-treated cells. A decrease in relative levels of CK-19 was observed

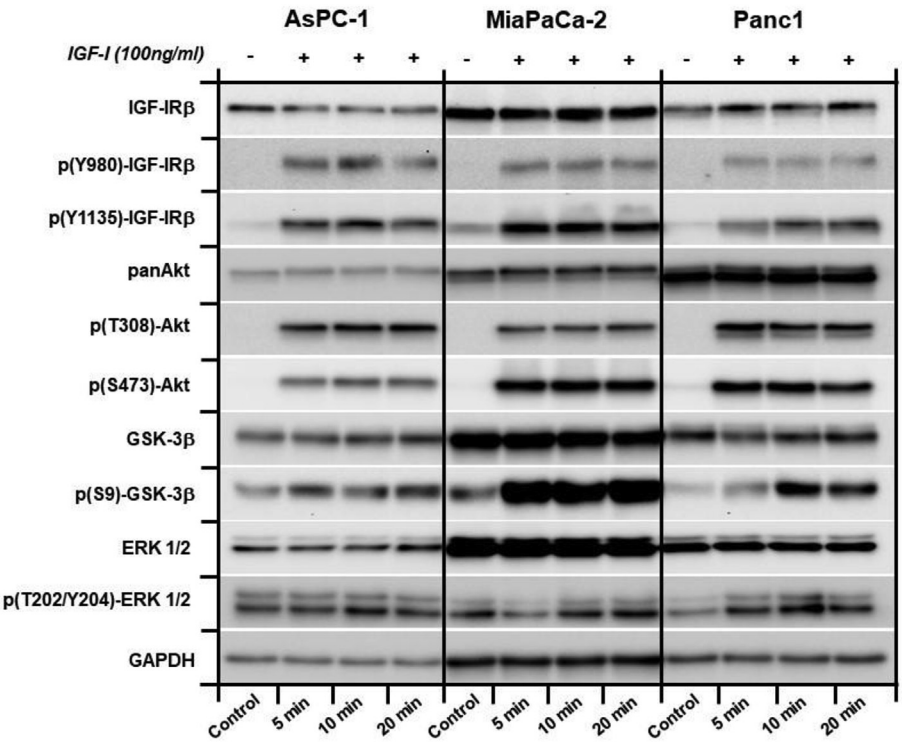

Fig. 3. Phosphorylation of IGF-IR, Akt, GSK-3β and ERK1/2 in AsPC-1, MiaPaCa-2 and Panc1 cells. After incubation for 24 h in DMEM/F12 (1:1) medium containing 0.5% FCS cells were stimulated with 100 ng/ml of IGF-I for 5, 10 and 20 min. Control untreated and treated cells were lysed and analyzed by Western blotting with specified antibodies directed against phosphorylated proteins. GAPDH used as a loading control.

exclusively in cell line MiaPaCa-2 after 120 h of IGF-I treatment. Therefore, we concluded that the treatment of experimental cell lines with IGF-I does not lead to changes in epithelial and mesenchymal differentiation protein marker expression, which are characteristic traits of the EMT process. It is also worth noting that IGF-I did not induce any noticeable changes in cell morphology that are characteristic for EMT in pancreatic cancer cell cultures we have used for analysis (Fig. 2b).

Relative expression levels of transcriptional regulator of EMT protein ZEB1 did not change in any of the cell lines that received stimulation with IGF-I. At the same time, the expression level of Snail protein was higher in IGF-I stimulated MiaPaCa-2 cells than in untreated cells from the same cell line. In Panc1 cells the expression level of Snail was increased transiently at 4 h of IGF-I stimulation, and was at the same level as in control cells at 48 and 120 h of stimulation. At the same time, it should be noted that the quantity of SNAI1 mRNA in IGF-I treated Panc1 and MiaPaCa-2 cells did not change relative to control cells (Supplementary Figure SF4). Also relative expression level of transcriptional regulator protein Slug was increased transiently at 4 h of IGF-I stimulation in AsPC-1 cells.

Treatment of cell lines with IGF-I led to long-term activation of IGF-IR, which was demonstrated by the presence of tyrosine phosphorylated receptor in cell lysates after 4 h of incubation. The activated form of the receptor, phosphorylated at Tyr-980 and Tyr-1135, was detected in IGF-treated AsPC-1, BxPC-3 and Panc1 cells, in samples that received up to 120 h of stimulation. At the same time, the activated receptor, phosphorylated at Tyr-980, was not detected in IGF-I stimulated Capan-2 cells, in samples that received 48 or more hours of IGF1 treatment. It is peculiar that long-term stimulation of all cell lines with IGF-I led to a decrease in relative levels of IGF-IR in cell lysates at 48 and 120 h of incubation. The activated form of Akt kinase was present in cell lysates of AsPC-1, BxPC-3, Capan-2 and MiaPaCa-2 cell lines by 4 h of stimulation with IGF1. We only detected a low level of phosphorylated Akt kinase (Thr-308) in IGF-I treated Panc1 cells by 4 h of IGF-stimulation. At the same time, this cell line displayed IGF-I induced phosphorylation of Akt-kinase at Ser-473 for up to 120 h of IGF-I stimulation. A similar phenomenon was observed in IGF-I stimulated MiaPaCa-2 cells. In AsPC-1 and Capan-2 cell lines IGF1-actived Akt-kinase was present up to 48 h of IGF-I stimulation. Interestingly, in IGF-I stimulated BxPC-3 cells, at 48 and 120 h of treatment, the levels of activated Akt-kinase, phosphorylated at Thr-308 и Ser-473, were significantly lower than in untreated control cells.

Stimulation of all cell lines with IGF-I led to a transient increase in expression level of Cyclin D1 protein at 4 h of incubation, which can, most likely, be linked to short-term stimulation of cell proliferation. Interestingly, in MiaPaCa-2 cells, at 120 h of IGF-I stimulation, the levels of Cyclin D1 protein were significantly lower than in untreated control cells. Additionally, treatment of cells with IGF-I did not lead to changes in β-catenin levels in cell lines AsPC-1, BxPC-3, Capan-2 and Panc1. The expression level of β-catenin was slightly decreased only in MiaPaCa-2 cells at 48 and 120 h, compared to untreated control cells.

Discussion

Epithelial-mesenchymal transition (EMT) is a reversible biological process during which epithelial cells transform phenotypically. As a result of EMT, epithelial cells lose their properties as organized, layered polarized cells, the expression of specific epithelial intercellular junction proteins in these cells decreases. Eventually they become highly mobile and gain mesenchymal cell specific morphology [14–16]. EMT is essentially a type of cell reprogramming process, during the course of which highly differentiated epithelial cells lose their epithelial identity markers and start displaying properties of less differentiated mesenchymal cells,

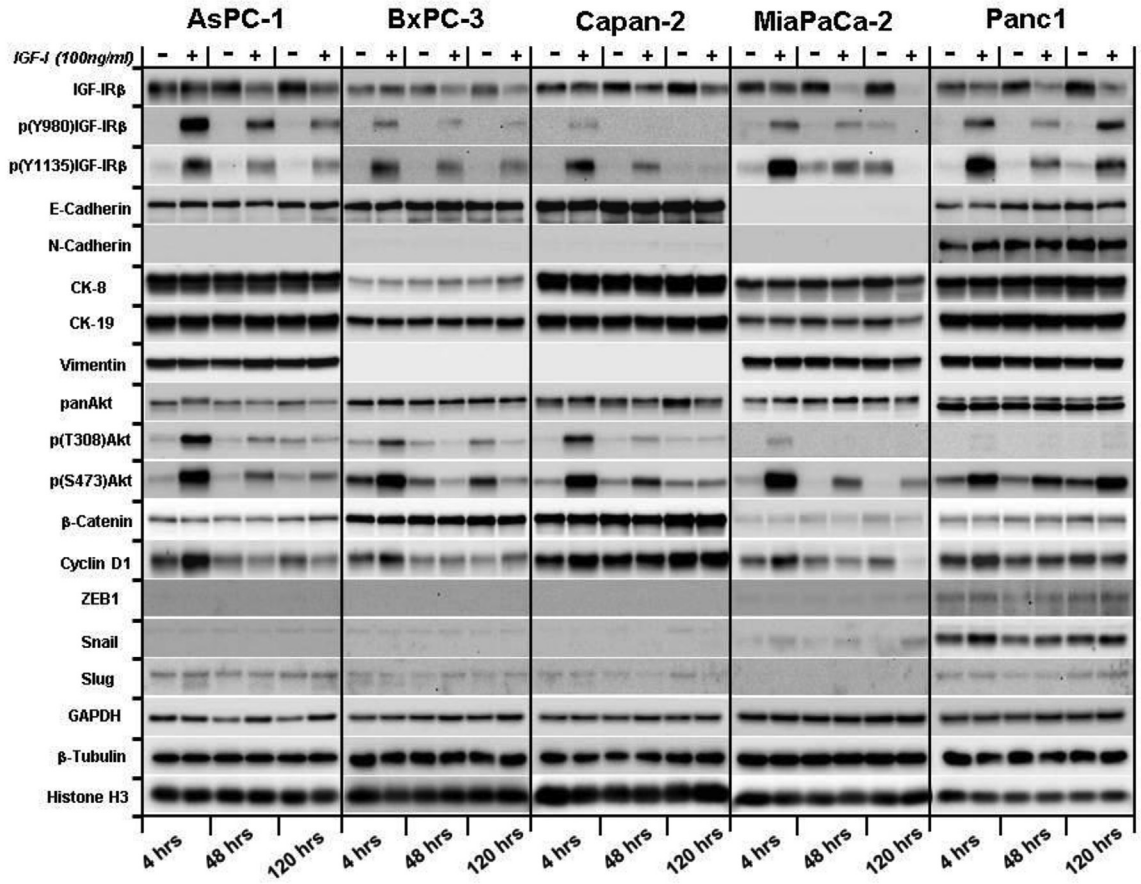

Fig. 4. Effect of long-term stimulation of pancreatic cancer cell lines with IGF-I. After incubation for 24 h in DMEM/F12 (1:1) medium containing 0,5% FCS cells were stimulated with 100 ng/ml of IGF-I for 4, 48 and 120 h. Control untreated and treated cells were lysed and analyzed by Western blotting with specified antibodies. Blots are representative of at least 3 experiments. GAPDH, beta-Tubulin and Histone H3 used as a loading controls.

which include the ability to locally invade tissues and migrate along a specific path, the heightened resistance to apoptosis, and the synthesis of extracellular matrix proteins specific for cells of mesenchymal lineage [16].

Pancreatic ductal adenocarcinoma is characterized by the presence of large amounts of tumor stroma [6,17]. The tumor microenvironment, altered during the tumor development and subsequent inflammation, frequently causes various phenotypic cell transformations, one of which is EMT [11]. Activated tumor microenvironment stromal cells secrete numerous protein factors and cytokines, which have various biological effects on tumor cells. IGF-I is one of the proteins secreted by tumor microenvironment cells. The biological effect of IGF-I on ductal adenocarcinoma cells is linked to inhibition of apoptosis and the development of resistance to anti-tumor compounds [10]. Additionally, it has been demonstrated via *in vitro* experiments that IGF-I stimulates cell proliferation and can enhance cell mobility and the invasive properties of cancer cells [8,18–20].

The goal of our current project was to study the effect of IGF-I on expression of protein markers of epithelial and mesenchymal differentiation and on expression of transcriptional regulators of EMT in several pancreatic cancer cell lines. The cell lines we used in our experiments were characterized by various levels of epithelial and mesenchymal differentiation. Cell lines BxPC-3 and Capan-2 had a high expression level of epithelial protein E-cadherin and did not express the mesenchymal differentiation marker vimentin at all. Cell lines AsPC-1 and Panc1 expressed vimentin and E-Cadherin, which indicates the intermediate (quasimesenchymal) differentiation state of those cells.

The immunoreactive protein IGF-I was practically undetected in conditioned medium from tumor cells, but was present in two out of three conditioned mediums from pancreatic ductal adenocarcinoma stromal cell cultures. This ELISA data demonstrates the absence of pronounced autocrine IGF-I/IGF-IR stimulation in the pancreatic cancer cell lines we used in our study. These results also demonstrate that the main source of IGF-I in pancreatic tumors is the stromal microenvironment cells.

Short-term stimulation of cells with IGF-I leads to activation of IGF-IR signaling pathway, which manifested itself in phosphorylation of intracellular tyrosine residues of IGF-IR, adaptor protein IRS-1 and phosphorylation of Akt kinase and GSK-3β proteins [21]. Untreated control cells from AsPC-1, MiaPaCa-2 and Panc1 cell lines were characterized by a high level of Ras/MAPK activation. However, IGF-I stimulation leads to a noticeable increase in phosphorylation of ERK1/2 proteins only in Panc1 cells. Akt-dependent inhibitory phosphorylation of GSK-3β leads to stabilization of Snail protein [22], which is the main inductor of EMT in many normal and tumor cells. Therefore, the goal of our next experiment was to test the effect of long-term IGF-I stimulation on expression of EMT marker proteins. The following analysis of EMT marker expression in cells that were incubated in the presence of IGF-I (for up to 5 days) did not show any significant shifts in their expression patterns in response to IGF-I. This data allowed us to conclude that activation of IGF-I/IGF-IR signaling pathway in analyzed pancreatic

cancer cells does not induce a significant shift in cell phenotype towards mesenchymal differentiation and does not induce a decrease in expression levels of epithelial protein markers. Interestingly, patterns of signaling protein activation in AsPC-1, BxPC-3, Capan-2, MiaPaCa-2 and Panc1 cell lines look quite similar by the fourth hour of IGF-I stimulation. Individual differences between cell lines that become visible at 48 and 120 h of IGF-I stimulation are, most likely, related to different mutational statuses of these cancer cell lines, their differentiation levels and varying sensitivity of activated IGF-IR to proteins that attenuate IGF-I signaling in stimulated cells.

In conclusion, despite the series of studies that demonstrated the ability of IGF-I to increase the mobility and invasiveness of pancreatic tumor cells [8,18–20], our results suggest that this effect of IGF-I may not be linked to cells gaining the mesenchymal phenotype via EMT. Also our data indirectly confirms the recent reports, which state that EMT may not be an indispensable step in the formation of metastasis in lung and pancreatic cancer [23,24]. However, there is a possibility that experimental induction of EMT requires a longer period of stimulation of cells with IGF-I or the employment of 3D cell cultivating systems. We also theorize that additional biologically active factors from pancreatic tumor microenvironment, such as ECM proteins or other soluble factors, may also make the effect of IGF-I more potent. All of these suggestions may become the leading hypotheses in our future experimental works.

Author contributions

E.P.K., M.R.K., and E.D.S. designed the experiments and provided intellectual input. M.R.K., E.V.G., A.M. and V.I.E. performed the experiments and collected data. E.P.K. wrote and edited the manuscript with input from the other authors. All authors discussed the results and conclusions drawn from them.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pan.2019.01.010.

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