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Original Articles

Reprogramming tumor stroma using an endogenous lipid lipoxin A4 to treat pancreatic cancer

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ABSTRACT

Pancreatic stellate cells (PSCs) are the precursors of cancer-associated fibroblasts (CAFs), which potentiate pancreatic tumor growth and progression. In this study, we investigated whether Lipoxin A4 (LXA4), an endogenous bioactive lipid, can inhibit the differentiation of human PSCs (hPSCs) into CAF-like myofibroblasts and thereby hPSC-induced pro-tumorigenic effects. LXA4 significantly inhibited TGF-β-mediated differentiation of hPSCs by inhibiting pSmad2/3 signalling. Furthermore, treatment with LXA4 abolished the paracrine effects (proliferation and migration of Panc-1 tumor cells) of hPSCs *in vitro*. These data demonstrated that LXA4 can interrupt pro-tumoral paracrine signalling of hPSCs. Furthermore, LXA4 treatment significant decreased the size and growth rate of 3D-heterospheroids comprised of hPSC and Panc-1 and these effects were exhibited due to inhibition of hPSC-induced collagen1 expression. *In vivo*, we examined the therapeutic efficacy of LXA4 in a co-injection (Panc-1 and hPSCs) subcutaneous tumor model. Intriguingly, LXA4 significantly abolished the tumor growth (either injected intratumor or intraperitoneally), attributed to a significant reduction in fibrosis, shown with collagen1 expression. Altogether, this study proposes LXA4 as a potent inhibitor for hPSCs which can be applied to reprogram tumor stroma in order to treat pancreatic cancer.

1. Introduction

Annually, around 90,000 people in Europe and 45,000 people in the U.S. get diagnosed with pancreatic ductal adenocarcinoma (PDAC), the most common form of pancreatic cancer [1]. Patients with PDAC have a 1 year survival rate of 20%, whereas the 5-year survival rate decreases to 7% [1]. In most PDAC, a surgical resection is not feasible, making radiotherapy or cytotoxic chemotherapy the golden standard treatment. However, these treatments are not effective and result in a survival benefit of only a few months [2,3]. One reason of the treatment failure is the abundant desmoplastic reaction in PDAC, also called the tumor stroma, which supports cancer progression, invasion and metastasis [4]. Novel therapeutic approaches for the treatment of PDAC aim to modulate the cellular components of the tumor stroma. Several different studies have shown an anti-tumoral effect by modulating the tumor stroma [5,6], whereas unexpectedly a complete depletion of the stroma genetically was shown to promote tumor growth [7]. Therefore, it has been suggested to dampen the activity of tumor stroma instead of its total depletion to gain therapeutic benefits [8].

Human pancreatic stellate cells (hPSCs) are the main precursors of pancreatic cancer-associated fibroblasts (CAFs), the key drivers of the pancreatic tumor stroma [5,9,10]. Quiescent hPSCs can be found within the connective tissue of the pancreas in low numbers and secrete only small amounts of extracellular matrix (ECM) proteins (e.g. collagen) [11]. In PDAC, quiescent hPSCs become activated, loose their cytoplasmic lipid storing capacity and start secreting

Abbreviations: Human pancreatic stellate cell, hPSC; Cancer-associated fibroblast, CAF; Pancreatic ductal adenocarcinoma, PDAC; Lipoxin A4, LXA4; Lipoxin B4, LXB4; Transforming growth factor β, TGF-β; Platelet-derived growth factor, PDGF; Extra cellular matrix, ECM; α-smooth muscle actin, α-SMA; Collagen1, Col1; Collagen1α1, Col1α1; Conditioned Medium, CM.

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high amounts of ECM components, leading to fibrosis which ultimately presents as a barrier for tumor drug penetration. In addition, differentiated hPSCs secrete various cytokines and growth factors which stimulate tumor cells and other stromal cells in favour of tumor progression [12]. Therefore, inhibition of hPSC activation might be beneficial for the treatment of pancreatic cancer.

Lipoxins are part of a family of inflammation resolving endogenous lipid mediators which are locally secreted by immune cells such as neutrophils and macrophages in response to infection, injury or inflammatory stimuli [13–16]. Lipoxins are synthesized from membrane arachidonic acid via biochemical synthesis by two major routes. The first route of synthesis starts in platelets where leukotriene A4 is converted into lipoxin by 12-LO. The second major route involves the action of either 5-LO in neutrophils and 15-LO in erythrocytes and reticulocytes. Arachidonic acid is converted into 15-hydroperoxyeicosatetraenoic acid (15-HPETE), which is then converted into lipoxin A and lipoxin B [17]. Based on an aspirin-triggered reaction in which aspirin acetylates cyclooxygenase-2 (COX-2) to form 15R-HPETE, and is eventually metabolized by 5-LO epi-lipoxin, lipoxin A4 (LXA4) and lipoxin B4 (LXB4) are formed [18,19]. Lipoxins and epi-lipoxins function by binding to the high-affinity G protein-coupled lipoxin A4 (LXA4) receptor formyl peptide receptor 2 (FPR2)/ALX [14]. Via this route, LXA4 can resolve inflammation already at low nanomolar concentrations and additionally activates anti-bacterial mechanisms [20,21].

There are little evidences that LXA4 may possess anti-fibrotic effects. In mesangial cells in kidney, it inhibited platelet-derived growth factor (PDGF)–dependent TGF-β production, the expression of fibrosis promoting genes in renal mesangial cells [22] and Akt/PKB activation and cell cycle progression in mesangial cells [23]. Additionally, LXA4 attenuated experimental renal fibrosis [24] and inhibited epithelial to mesenchymal transition of renal epithelial cells in proximal tubules [25]. Also, LXA4 has been shown to inhibit connective tissue growth factor-induced proliferation and TGF-β-dependent pro-fibrotic activity in human lung (myo)fibroblast [26,13]. Yet, there is no study showing the inhibitory effects of LXA4 on hPSC differentiation and its impact on pancreatic tumor growth.

Considering the anti-fibrotic activity of LXA4, in this study, we hypothesized that LXA4 might be an interesting biomolecule to inhibit hPSCs activation and their pro-tumorigenic effects. We show that the LXA4 specific receptor FPR2/ALX is overexpressed in activated hPSCs. Furthermore, we demonstrate the inhibitory effect of LXA4 on the hPSC activation, migration and then hPSC-induced paracrine effect on pancreatic cancer cells. Next, we showed the inhibitory effects of LXA4 on the growth and extracellular matrix (ECM) deposition in stromal-rich 3D-heterospheroids. Eventually, we examined the therapeutic efficacy of LXA4 *in vivo* in a co-injection tumor model which resulted in a reduced tumor growth and intra-tumoral ECM deposition after the treatment with LXA4.

2. Materials and methods

2.1. Cells

Primary human pancreatic stellate cells (ScienCell, Carlsbad, USA), were cultured in complete Stellate Cell Medium (supplemented with 2% FBS, 1% Penicillin/Streptomycin and 1% Stellate Cell Growth Supplements (SteCGS)) (ScienCell). Panc-1 cancer cells were cultured in Dulbecco's Modified Eagles medium (DMEM) High Glucose (4.5 g/l) with L-Glutamine (GE Healthcare, Vienna, Austria) supplemented with 10% FBS (Lonza, Verviers, Belgium), 100 μg/ml penicillin/streptomycin (Sigma Aldrich). AsPc-1 cells were cultured in complete RPMI Medium (GE Healthcare, Vienna, Austria) supplemented with 10% FBS (Lonza), 100 μg/ml penicillin/streptomycin (Sigma). The cells were maintained at 37 °C in a humidified 5% CO2 atmosphere.

2.2. Western blot

To evaluate the expression of the LXA4-specific surface receptor FPR2 and the hPSC activation marker α-SMA, hPSCs were seeded into a 12 well plate at a seeding density of 40.000 cells/well. The next day hPSCs were starved for 24 h and subsequently activated with 5 ng/ml TGF-β for 48 h. To evaluate the effect of LXA4 on TGF-β-mediated Smad 2/3 and phosphorylated Smad 2/3 (pSmad2/3) levels in hPSCs, cells were seeded into a 12 well plate at a seeding density of 40.000 cells/well. The cells were starved for 24 h, treated with 10 nM LXA4 (Biomol, Hamburg, Germany) and activated with 5 ng/μl TGF-β for 30 min. In general, cells were lysed using 1× SDS-lysis buffer, cell lysis was centrifuged at 10,000 g for 10 min, and the supernatant was collected for Western blot analysis. To analyse the expression of MMP-2 in conditioned medium, 1 ml conditioned medium was freeze dried using an Il-Shin TFD5503 Freeze Dryer (Scala Scientific, Ede, The Netherlands) and afterwards resuspended in 100 μl TBS (Thermo Scientific, Rockford, USA). Conditioned medium was mixed with Sample Reducing agent (Life Technologies, Carlsbad, USA) and LDS Sample Buffer (Life Technologies) and incubated at 95 °C for 10 min. Protein lysates and conditioned medium was loaded on a 10% Tris-Glycine gel (Thermo Scientific) and transferred onto PVDF membranes (Thermo Scientific). The blots were incubated with the required primary antibody (Supplementary Table 1) and incubated overnight at 4 °C, followed by incubation with species specific horseradish peroxidase (HRP) conjugated secondary and tertiary antibody for 1 h at RT. Proteins were detected with Pierce™ ECL Plus Western Blotting Substrate kit (Thermo Scientific) and exposed to FluorChem™ M System (ProteinSimple, San José, USA). The protein levels were quantified using Image J software (NIH, MD). To evaluate the amount of Smad 2/3 phosphorylation a pSmad 2/3/Smad 2/3 ratio was calculated.

2.3. Immunocytochemistry

To evaluate the effect of LXA4 on the expression of αSMA and collagen1 (Col1), hPSCs were seeded into a 24 well plate at a seeding density of 5.000 cells/well. The cells were starved, treated with 0.1, 1 and 10 nM LXA4 and TGF-β activated for 48 h. Next, cells were fixed and immunostained for αSMA, and collagen1 as described elsewhere [27]. Stained images were quantified for their positively stained area using ImageJ software.

To evaluate the effect of LXA4 on Smad2/3 and pSmad2/3 expression, hPSCs were seeded into a 24 well plate at a seeding density of 10.000 cells/well. The cells were starved and activated with TGF-β as described previously. After 30 min of incubation with TGF-β, cells were washed with phosphate buffered saline (PBS) (Lonza, Verviers, Belgium) and fixed with 4% paraformaldehyde (Sigma Aldrich) at room temperature for 10 min. Next, cells were washed 3 times with PBS and permeabilized with ice cold HPLC grade methanol (Fisher Scientific) at −20 °C for 10 min. After washing 3 times with PBS cells were blocked with blocking buffer consisting of 1 vol % bovine-serum albumin (BSA) (VWR, Radnor, PA, USA) + 0.3 vol % Triton-X (Sigma Aldrich) in PBS for 1 h. Subsequently, the cells were incubated with monoclonal anti-Smad2/3 or monoclonal anti-pSmad2/3 primary antibodies diluted in blocking buffer and incubated over night at 4 °C. Next, cells were washed 3 times with PBS and incubated with fluorescent secondary antibodies diluted in blocking buffer for 1 h at RT. Cells were washed once with PBS and stained for DAPI by incubation with NucBlue Fixed Cell Stain ReadyProbes Reagent (Life Technologies),