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Original Articles

Hypoxic stellate cells of pancreatic cancer stroma regulate extracellular matrix fiber organization and cancer cell motility

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ABSTRACT

Desmoplasia and hypoxia in pancreatic cancer mutually affect each other and create a tumor-supportive microenvironment. Here, we show that microenvironment remodeling by hypoxic pancreatic stellate cells (PSCs) promotes cancer cell motility through alteration of extracellular matrix (ECM) fiber architecture. Three-dimensional (3-D) matrices derived from PSCs under hypoxia exhibited highly organized parallel-patterned matrix fibers compared with 3-D matrices derived from PSCs under normoxia, and promoted cancer cell motility by inducing directional migration of cancer cells due to the parallel fiber architecture. Microarray analysis revealed that procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2 (PLOD2) in PSCs was the gene that potentially regulates ECM fiber architecture under hypoxia. Stromal PLOD2 expression in surgical specimens of pancreatic cancer was confirmed by immunohistochemistry. RNA interference-mediated knockdown of PLOD2 in PSCs blocked parallel fiber architecture of 3-D matrices, leading to decreased directional migration of cancer cells within the matrices. In conclusion, these findings indicate that hypoxia-induced PLOD2 expression in PSCs creates a permissive microenvironment for migration of cancer cells through architectural regulation of stromal ECM in pancreatic cancer.
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Introduction

The prognosis of pancreatic cancer remains dismal with a 5-year survival rate of 6% despite considerable progress in understanding its underlying genetic and molecular events [1]. A characteristic feature of pancreatic cancer is a dense desmoplastic stroma, which plays a crucial role in tumor aggressiveness and therapeutic resistance [2,3]. The desmoplastic microenvironment rich in extracellular matrix (ECM) is mainly produced by the activated phenotype of pancreatic stellate cells (PSCs). Activated PSCs produce paracrine growth factors, proteolytic enzymes, and ECM components, which promote proliferation, migration, and invasion of cancer cells. Moreover, ECM in the tumor-associated stroma creates a 'fortress-like' hypovascular barrier that impairs the delivery of chemotherapeutics [2,4–6].

Preclinical models have demonstrated the benefit of stromal depletion through blockade of paracrine Hedgehog signaling to improve drug delivery [4]; nonetheless, subsequent clinical trials targeting stromal desmoplasia in pancreatic cancer have failed due to paradoxical accelerated disease progression [7]. Recent experimental evidence provides insight into the failure of antistromal therapy in clinical trials, showing that stromal depletion may increase tumor aggressiveness and spread [8,9], implying that tumor stroma may be restrictive rather than supportive of tumor growth. Consequently, these studies strongly suggest that instead of aiming to ablate desmoplasia, a more effective approach is needed for targeting pancreatic cancer stroma.

The ECM in tumor stroma plays roles in biochemical and biomechanical interactions with cells that are required for key cellular events. Several groups have observed that tumor cells preferentially invade along aligned collagen fibers [10–12]. Recent evidence indicates that ECM architecture as well as composition is altered in cancer stroma and that these changes may accelerate tumor progression [13,14]. Rho-mediated alignment of dense collagen fibers perpendicular to the tumor boundary promotes invasion, whereas reticular collagen surrounding mammary glands restrains it [15]. However, the contribution of these stromal modifications to tumor

Abbreviations: PSC, pancreatic stellate cell; ECM, extracellular matrix; 3-D, three-dimensional; PLOD2, procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2; α-SMA, α-smooth muscle actin; HIF-1α, hypoxia-inducible factor-1α; GFAP, glial fibrillary acidic protein.

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progression and the genetic and molecular mechanisms underlying these alterations remain elusive in pancreatic cancer.

Pancreatic cancer contains an area of hypoxia, which has also been proposed as an important microenvironmental factor for tumor progression [16,17]. Hypoxia in pancreatic cancer is formed partly through fibrogenic effects of PSCs [18,19] as well as a result of inefficient tumor vascular supply and a high metabolic need for oxygen. Recent studies suggest that hypoxic conditions concomitantly exist in pancreatic cancer cells and surrounding stroma [20,21]. PSCs in pancreatic cancer stroma respond to hypoxia by increasing hypoxia-inducible factor-1α (HIF-1α) protein level and producing soluble factors and ECM components [19,22], whereas the effect of hypoxia in PSCs within tumor desmoplasia on ECM mechanical properties such as fiber alignment has not been reported.

In this study, we explored the possibility that hypoxia may be causally involved in the biomechanical properties of PSC-derived tumor stroma. In PSC-derived cancer stroma, hypoxia regulated ECM fiber architecture. We further showed that ECM produced by PSCs under hypoxia facilitated the directional migration of pancreatic cancer cells, and we link this activity to the parallel fiber architecture.

Materials and methods

Patients and pancreatic tissues

Pancreatic cancer tissues were obtained from patients who underwent pancreatic resection at our institution. The study was approved by the Ethics Committee of Kyushu University and conducted according to the Ethical Guidelines for Human Genome/Gene Research enacted by the Japanese Government and the Helsinki Declaration.

Cells and culture conditions

PSCs were established in our laboratory from fresh pancreatic cancer surgical specimens using the outgrowth method [23,24]. The isolated cells were identified as PSCs by their spindle-shaped morphology and expression of α-smooth muscle actin (α-SMA) and glial fibrillary acidic protein (GFAP), as described previously [25,26]. All of the established PSCs used for the assays were between passages 3 and 8. We also used a normal fibroblast cell line, MRC-5 (RIKEN BRC, Tsukuba, Japan) and the following six pancreatic cancer cell lines: AsPC-1, CFPAC-1, SW1990, and Capan-2 (American Type Culture Collection, VA, USA); PANC-1 (RIKEN BRC); and SUIT-2 (Health Science Research Bank, Osaka, Japan). Cells were maintained until subconfluent in DMEM supplemented with 10% FBS, penicillin, and streptomycin as described previously [27]. Hypoxic PSCs were maintained in a hypoxic incubator with a gas mixture containing 1% O_2, 10% CO_2, and 89% N_2. One percent O_2 was used as hypoxic conditions based on the data from direct oxygen measurements in human pancreatic cancer during surgery [16]. Oxygen levels in the culture chambers were continuously monitored.

3-D matrices produced by PSCs

Three-dimensional (3-D) matrices produced by PSCs were prepared as described previously [28,29]. Cells (2.0×10^5) were plated on chemically crosslinked gelatin on 35-mm tissue culture or glass-bottom dishes, and maintained at confluence for 5–6 days. Cells were removed from the matrix by alkaline detergent extraction buffer containing 0.5% Triton X-100 and 20 mM NH_4OH in PBS for 5 minutes, yielding cell-free 3-D matrices.

Immunofluorescence and confocal microscopy of labeled cells and matrices

PSCs and 3-D matrices were fixed with 4% paraformaldehyde for 5 minutes at room temperature, blocked with 3% BSA in PBS for 30 minutes, and incubated with rabbit anti-α-SMA (ab32575; 1:100; Abcam, Cambridge, UK), mouse anti-fibronectin (ab6328; 1:200; Abcam), rabbit anti-collagen I (bs-0578R; 1:100; Bioss, MA, USA), and rabbit anti-PLOD2 (21214-1-AP; 1:50; Proteintech, IL, USA) overnight at 4 °C. The cells and matrices were incubated for 1 hour with Alexa Fluor 488- or 546-conjugated secondary antibody (1:200; Molecular Probes, Thermo Fisher Scientific, MA, USA). Nuclear DNA was counterstained with DAPI. A laser scanning confocal microscope (A1R; Nikon, Tokyo, Japan) and a fluorescent microscope (BZ-9000; Keyence, Osaka, Japan) were used for immunofluorescence microphotography. Confocal image processing and analyses were performed with NIS-Elements software (Nikon).

Analysis of fibronectin fiber orientation

Images of 3-D matrices stained with anti-fibronectin antibody were analyzed using MetaMorph software (Molecular Devices, CA, USA). Counts of the total fibers, as well as each orientation angle relative to the *x* axis, were measured, and the angles were determined by approximating the relative angle every 10°. To allow for comparison of acquired images, the mode angle, representing the angle at which the largest score of fibers was observed, was arbitrarily set to 0°. To determine the relative distribution of fibers organized in parallel patterns, the percentage of fibers that were arranged in parallel within ±10° of the mode angle was calculated for each region.

Motility assay within cell-free 3-D matrices

Cancer cells were seeded in cell-free 3-D matrices and incubated for 6 hours, followed by recordings of cellular movements every 5 minutes for 6 hours using BZ-9000 (Keyence) with an environmentally controlled chamber that was maintained at 37 °C and 5% CO_2 (Tokai Hit, Fujinomiya, Japan). Individual cell dynamics were analyzed using MetaMorph (Molecular Devices).

Microarray analysis

Total RNA was isolated from cultured cells using a High Pure RNA Isolation Kit with DNase digestion (Roche Diagnostics, Mannheim, Germany). RNA quality was evaluated using the Experion microfluidic capillary electrophoresis system (Bio-Rad Laboratories, CA, USA) for microarray analysis. RNA was labeled and hybridized to the Agilent SurePrint G3 Human Gene Expression Microarray 8 × 60K Ver.2.0 (Agilent Technologies, CA, USA). Data analysis was performed using the Feature Extraction software (Agilent Technologies).

Real-time quantitative reverse transcription-PCR

Real-time quantitative reverse transcription-PCR (qRT-PCR) was performed using iTaq Universal SYBR Green One-Step Kit and CFX96 Touch Real-Time PCR Detection systems (Bio-Rad Laboratories). The primers were purchased from Takara Bio (Kusatsu, Japan). Human GAPDH was used as the endogenous control gene. The following primers were used in this study: PLOD2, 5′-CAATTGCTCTATTGAGTCACCACGA-3′ (forward) and 5′-CTTCATGCAAATGTGTGAGTCTCC-3′ (reverse); GAPDH, 5′-GCACCGTCAA GGCTGAGAAC-3′ (forward) and 5′-TGGTGAAGACGCCAGTGGA-3′ (reverse).

Western blotting

Whole cell lysates were prepared in PRO-PREP solution (iNtRON Biotechnology, Seongnam, Korea) from subconfluent PSCs. The supernatants of cultured PSCs were concentrated using an Amicon Ultra-15 filter unit (Millipore, MA, USA). Proteins from cell cultured supernatant and cell lysate were fractioned by SDS–PAGE and transferred to a PVDF membrane (Bio-Rad Laboratories). The membrane was incubated overnight at 4 °C with the following antibodies: anti-PLOD2 (21214-1-AP; 1:1000; Proteintech), anti-HIF-1 α (610958; 1:1000; BD Biosciences, NJ, USA), anti-collagen I (sc-8783; 1:500; Santa Cruz Biotechnology, CA, USA), anti-fibronectin (ab6328; 1:5000; Abcam), anti-SMAα (ab32575; 1:5000; Abcam), anti-α-tubulin (05–829; 1:5000; Millipore). Then it was probed with horseradish-peroxidase-conjugated secondary antibodies (Cell Signaling Technology, MA, USA). Immunoblots were detected by enhanced chemiluminescence.

Immunohistochemical procedures and imaging

Tissue sections were cut at 4-µm thickness from paraffin-embedded material, deparaffinized in xylene, and rehydrated through a graded series of ethanol and water. Endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxide in methanol for 30 minutes. Antigen retrieval was achieved by microwaving sections in sodium citrate buffer at pH 6.0. Sections were incubated with rabbit anti-PLOD2 antibody (1:50; Proteintech) or mouse anti-α-SMA antibody (1:100; DAKO, Glostrup, Denmark) overnight at 4 °C.

Silencing of PLOD2 by small interfering RNA

Subconfluent PSCs were transfected with PLOD2-1 (sense, 5′-GGUUGUCA UGUUUACUGAATT-3′; antisense, 5′-UUCAGUAAACAUGAC AACCAG-3′) and PLOD2-2 (sense, 5′-CUAUUGAGUCACCACGAAATT-3′; antisense, 5′-UUUCGUGGUGACUCAA UAGAG-3′) small interfering RNA (siRNA) (Qiagen, Venlo, Netherlands) by electroporation using Nucleofector System (Amaxa Biosystems, Köln, Germany). To verify the specificity of the knockdown effects, we used nontargeting siRNA as a control (Qiagen). PLOD2-silenced cells were used for the following assays 48 hours post-transfection.

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