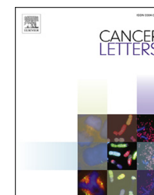
Cancer Letters 395 (2017) 20–30

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet



Original Article

Fibroblast-led cancer cell invasion is activated by epithelial–mesenchymal transition through platelet-derived growth factor BB secretion of lung adenocarcinoma

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A R T I C L E I N F O

Article history:
Received 9 August 2016
Received in revised form
18 February 2017
Accepted 22 February 2017

Keywords:
Epithelial–mesenchymal transition
Cancer-associated fibroblasts
Fibroblast-dependent cancer invasion
Extracellular matrix remodeling
Platelet-derived growth factor B

A B S T R A C T

Cancer-associated fibroblast (CAF)-dependent local invasion is the process by which cancer cells invade the extracellular matrix using tracks that have been physically remodeled by CAFs. In the present study, we investigated the process by which the epithelial–mesenchymal transition (EMT) of cancer cells affect CAF-dependent local invasion. Using an *in vitro* collagen invasion assay, we showed cancer cells undergoing EMT to promote the matrix-remodeling ability of CAFs and thereby enhance CAF-dependent local cancer cell invasion. Platelet-derived growth factor (PDGF)-BB secretion was significantly elevated in cancer cells undergoing EMT, and this induced an increase in the invasion ability of both CAFs and cancer cells. Conversely, knockdown of PDGF-B expression in cancer cells undergoing EMT, or treatment with a PDGF-receptor inhibitor, decreased the invasion ability of both CAFs and cancer cells. By analyzing the gene expression profiles of 442 patients with lung adenocarcinomas, we established that high expression of PDGF-B and presentation of mesenchymal-like tumors were significantly associated with a high rate of disease recurrence and poor patient prognosis. Thus, cancer cells undergoing EMT may accelerate their own ability to invade local tissues via PDGF-BB secretion to promote CAF matrix remodeling. Therefore, targeting PDGF signaling between cancer cells undergoing EMT and CAFs is a promising therapeutic target to inhibit cancer progression and improve patient prognosis.

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Introduction

Epithelial-mesenchymal transition (EMT) has been shown to play a critical role in promoting cancer cell invasion and metastasis, via a loss of cell–cell adhesion. Cancer cells undergoing EMT are characterized by decreased expression of proteins that enhance cell–cell contact (such as E-cadherin), increased expression of mesenchymal markers (including N-cadherin and vimentin), and high matrix metalloproteinase (MMP) activity [1,2]. During cancer progression, EMT enables cancer cells to detach from a given tumor, invade surrounding tissues, and intravasate into blood or lymphatic vessels [1–3]. EMT is also known to be induced by several

Abbreviations: CAFs, cancer-associated fibroblasts; ECM, extracellular matrix; EGF, epidermal growth factor; ELISA, enzyme-linked immunosorbent assay; EMT, epithelial–mesenchymal transition; FGF, fibroblast growth factor; MMPs, matrix metalloproteinases; NSCLC, non-small cell lung cancer; PDGF, platelet-derived growth factor; PDGFR, platelet-derived growth factor receptor; qRT-PCR, quantitative real-time reverse transcriptase-polymerase chain reaction; rhPDGF-BB, recombinant human platelet-derived growth factor BB; TGFβ1, transforming growth factor β1.

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http://dx.doi.org/10.1016/j.canlet.2017.02.026
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microenvironmental factors including growth factors secreted from stromal cells and extracellular matrix (ECM) components such as collagen I [4–7]. Cancer-associated fibroblasts (CAFs) are a major component of the tumor microenvironment, and secrete growth factors such as transforming growth factor β1 (TGFβ1), to promote EMT in cancer cells [4,8,9].

Moreover, cancer cells undergoing EMT are known to mediate immune cell activity during tumor progression. For example, EMT in cancer cells enhances metastasis by activating tumor-associated macrophages via GM-CSF [10], or by inducing regulatory T cells and impaired dendritic cells via TSP1 [11]. Cancer metastasis is accelerated by the interplay between cancer cells undergoing EMT and immune cells in the tumor microenvironment.

Some previously published studies have demonstrated the existence of positive feedback loops between cancer cells and CAFs [12–15], such that CAFs enhance invasion and proliferation or self-renewal of cancer cells through secretion of CXCL6 or CCL2, respectively. Conversely, cancer cells produce various secreting factors such as epidermal growth factor (EGF) and fibroblast growth factor (FGF) that activate CAFs [12,13]. For example, Giannoni et al. demonstrated that MMPs secreted by CAFs induce EMT in prostate cancer cells, and that the corresponding secretion of IL-6 by cancer cells activates CAFs [14]. Similarly, Martin et al. reported that cancer-cell EMT activates fibroblasts via high expression of thrombospondin 2, and that conversely, the activated fibroblasts promote cancer cell proliferation [15]. Nevertheless, the mechanism by which cancer cells undergoing EMT activate CAFs during cancer invasion remains unclear.

Cancer cells invade the ECM via physical tracks that are remodeled by CAFs during CAF-dependent local invasion [16–18]. In this study, we used an *in vitro* CAF-dependent invasion assay to investigate the way in which cancer cells undergoing EMT facilitate the invasion ability of CAFs, and thereby promote cancer cell invasion [17,18].

Materials and methods

Cell culture

CAFs were obtained from surgically resected tumors of patients with lung adenocarcinoma, and cultured as previously described to establish four primary CAF cultures (Supplementary Table 1) [18–20]. All the specimens were collected after patients provided written informed consent, and specimen collection was approved by the Institutional Review Board of our institution. The human lung adenocarcinoma cell line, A549 (American Type Culture Collection, Manassas, VA), was cultured in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM/F-12, Sigma–Aldrich, St. Louis, MO) containing 10% FBS, 1% penicillin, and 1% streptomycin. The human lung adenocarcinoma cell lines, PC9 (Public Health England, London, UK) and H1975 (American Type Culture Collection, Manassas, VA), were cultured in RPMI-1640 Medium (Sigma–Aldrich) containing 10% FBS, 1% penicillin, and 1% streptomycin. All CAFs and cell lines were maintained at 37 °C, in an atmosphere containing 5% CO_2.

Quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR)

To measure CXCL12, EGFR, HBEGF, IGF1, PDGFB, VEGFA, and MUC5A mRNA levels, cells were suspended in 1 mL of TRIzol (ThermoFisher Scientific, Waltham, MA) and stored at −80 °C. Total RNA was purified from thawed samples using standard techniques, and cDNA was synthesized using the PrimeScript RT reagent Kit (Takara Bio, Shiga, Japan), according to the manufacturer's instructions. qRT-PCR was performed in a Smart Cycler System (Takara Bio) with SYBR Premix Ex Taq II (Takara Bio). Utilized primers are shown in Supplementary Table 2. To measure of MMP1, MMP2, MMP8, MMP9, and MMP13 mRNA levels, total RNA was purified using an RNeasy Plus Mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. cDNA was synthesized using Ready-To-Go You-Prime First-Strand Beads (GE Healthcare, Little Chalfont, UK) according to the manufacturer's instructions. qRT-PCR was performed using a StepOne Plus thermocycler (Applied Biosystems, Foster City, CA). TaqMan probes and primers for MMP1, MMP2, MMP8, MMP9, MMP13, and GAPDH were obtained as part of TaqMan Gene Expression assays (assay ID: Hs00899658_m1, Hs01548727_m1, Hs01029057_m1, Hs00234579_m1, Hs00942584_m1, and Hs03929097_g1, respectively).

Western blot analysis

Cells were lysed in RIPA buffer (Thermo Fisher Scientific) containing cOmplete and PhosSTOP (protease and phosphatase inhibitor cocktail tablets, respectively; Roche, Basel, Switzerland). Proteins were separated on a 7.5% or 12% SDS-polyacrylamide gel, and transferred to an Immobilon-P PVDF membrane (Millipore, Billerica, MA). Blots were incubated overnight (4 °C) with an anti-E-cadherin (#13-1700, Thermo Fisher Scientific), anti-N-cadherin (#33-3900, Thermo Fisher Scientific), anti-vimentin (# NCL-L-VIM-572, Leica Biosystems, Wetzlar, Germany), or anti-α-tubulin antibody (#2144, Cell Signaling Technology, Danvers, MA). After washing in TBS-T, the membranes incubated with anti-rabbit IgG, HRP-linked donkey whole antibody, or anti-mouse IgG, HRP-linked sheep whole antibody (GE Healthcare). ECL Western Blotting Detection Reagents (GE Healthcare) were used to develop the high-performance chemiluminescence film (GE Healthcare).

Lentiviral vectors

Oligonucleotides were chemically synthesized for the PDGF-B short hairpin RNA (shRNA) experiments (Supplementary Table 3). To create entry clones, the top and bottom strands of each oligonucleotide were annealed and ligated into pENTR4-H1 (RIKEN BioResource Center, Tsukuba, Japan). Thereafter, LR recombination reactions were performed between the entry clones and CS-RfA-EG (RIKEN BioResource Center) using Gateway LR Clonase (Thermo Fisher Scientific). Lentiviruses were produced using HEK293T cells transfected with PCAG-HIV, pCMV-VSV-G-RSV-Rev, and either a PDGF-B shRNA or mRFP (CSII-CMV-mRFP1; RIKEN BioResource Center). Transfection was achieved using the LipofectAMINE 2000 reagent according to the manufacturer's instructions. Vector-containing medium was filtered through a 0.45-μm filter, and 8 μg/mL of polybrene (Santa Cruz Biotechnology, Dallas, TX) was added to facilitate target-cell transduction. Stable transformants of mRFP-infected cancer cells were selected using a FACSAria II cell sorter (BD Biosciences, Franklin Lakes, NJ).

EMT induction of cancer cells

We induced EMT in cancer cells as described by Kasai et al. [21]. Confluent cell cultures were maintained in serum-free DMEM/F-12 or RPMI-1640 containing 0.1% BSA for 24 h prior to stimulation with recombinant human TGF-β1 (PEPROTECH, Rocky Hill, NJ). Thereafter, cells were stimulated with 5 ng/mL of TGF-β1 in serum free 0.1% BSA medium for 48 h. We used a cDNA microarray to compare the expression profiles of EMT-induced A549 cancer cells after 72 h of treatment with TGF-β1 with those of control cancer cells (GSE17708 dataset) [22].

Trypan blue proliferation assays

Trypan blue proliferation assays were performed on the four primary CAF cultures, whereby half of the total medium was replaced with medium conditioned by A549 cancer cells. Two days after seeding in 60 mm plastic dishes (density 2×10^5 cells), cells were detached using trypsin-EDTA, and blocked with serum-supplemented culture medium. After addition of equal volumes of 0.4% trypan blue (Thermo Fisher Scientific, Waltham, MA) to cell aliquots, trypan blue-excluding cells were counted.

Enzyme-linked immunosorbent assay (ELISA)

To analyze the release of PDGF-BB, cancer cells were maintained in serum-free DMEM/F-12 or RPMI-1640 for 24 h after EMT induction. The levels of PDGF-BB in the cell culture supernatants were quantified using a human PDGF-BB ELISA kit (eBioscience, San Diego, CA) according to the manufacturer's instructions.

Measurement of matrix metalloproteinase activity

MMP activity was measured in the CAF supernatant 24 h after stimulation with a conditioned cancer cell medium, using the SensoLyte 520 Generic MMP assay kit (AnaSpec, Fremont, CA). This kit can simultaneously detect the activities of MMPs 1, 2, 7, 8, 9, 12, 13, and 14. Briefly, CAF supernatant, maintained in serum-free MEM alpha for 24 h after stimulation with a conditioned cancer cell medium, was incubated with the 5-FAM/QXL 520 FRET peptide at 37 °C for 1 h in a 96-well plate, and the resulting fluorescence intensity was measured at a wavelength of 490/520 nm (excitation/emission). 4-Aminophenylmercuric acetate-treated samples were used as positive controls. The performance of the kit (which was used within its linear range), was validated in preliminary studies using recombinant active MMP-1 (data not shown).

Collagen invasion assay

A mixed cell population of CAFs and cancer cells was plated in collagen-coated (0.3% type I collagen gel; Nitta Gelatin, Osaka, Japan) 96-well plates (Essen ImageLock; Essen BioScience, Ann Arbor, MI) at a density of 5×10^4 cells/well (2.5×10^4 cells each of CAFs and cancer cells). After an incubation period of 1 h, a wound was made in the cell layer using the 96-well WoundMaker (Essen BioScience), and the cells were embedded in type I collagen gel. Fresh medium was added, and scratched field images then obtained using the IncuCyte Live-Cell Imaging System. Time-lapse fluorescence and phase-contrast images were obtained at 3-h intervals for 48 h. Each data element was measured in at least eight fields (magnification ×10) in two

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