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Dependence of fibroblast infiltration in tumor stroma on type IV collagen-initiated integrin signal through induction of platelet-derived growth factor

Sheng-Yi Chen [a], Jo-Shi Lin [b], Huan-Ching Lin [a], Yan-Shen Shan [c], Yu-Jung Cheng [d], Bei-Chang Yang [a,b,e,*]

[a] Institute of Basic Medical Sciences, National Cheng Kung University, Tainan 70101, Taiwan
[b] Department of Microbiology and Immunology, College of Medicine, National Cheng Kung University, Tainan 70101, Taiwan
[c] Department of Surgeon, College of Medicine, National Cheng Kung University, Tainan 70101, Taiwan
[d] Department of Physical Therapy, China Medical University, Taichung 40402, Taiwan
[e] Center for Gene Regulation and Signal Transduction Research, National Cheng Kung University, Tainan 70101, Taiwan

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ABSTRACT

Cancer-associated fibroblasts play a crucial role in accelerating tumor progression, but there is a knowledge gap regarding the chemotactic signal activated in a tumor microenvironment. In this study, the expression of type IV collagen was knocked down using a lentiviral-mediated short hairpin RNA strategy. Although there was no obvious effect on cell growth in vitro, silencing the *Col4-α1* gene decreased the tumorigenicity of B16F10 in C57BL/6 mice, which was accompanied by a reduction in the infiltration of alpha-smooth muscle actin-positive (α-SMA^{+}) fibroblasts. Silencing the *Col4-α1* gene or disrupting integrin engagement by blocking the antibody reduced the expression of platelet-derived growth factor A (PDGF-A), a potent chemotactic factor for fibroblasts. Furthermore, ectopic expression of the autoclustering integrin mutant significantly stimulated PDGF-A expression in murine B16F10 and human U118MG and Huh7 cells. PDGF-A-specific sh-RNA and neutralizing anti-PDGF-A antibody effectively inhibited the transwell migration of fibroblasts. Adding recombinant PDGF-A back to shCol cell-conditioned media restored the fibroblast-attraction ability indicating that PDGF-A is a major chemotactic factor for fibroblasts in the current study model. The integrin-associated PDGF-A production correlated with the activation of Src and ERK. High type IV collagen staining intensity colocalized with elevated PDGF-A expression was observed in tumor tissues obtained from hepatoma and glioma patients. The integrin signal pathway was activated by collagen engagement through Src and ERK, leading to enhanced PDGF-A production, which serves as a key regulator of fibroblast recruitment.

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1. Introduction

Solid tumors are surrounded by distinctive basement membrane-like structural barriers that physically separate them from other tissue compartments [1,2]. Extracellular matrix (ECM) molecules constituting the basement membrane contain laminins, collagen type IV, nidogens, and heparan sulfate proteoglycans [1,3,4]. Many tumors exhibit aberrant deposition of ECM as compared with that of their normal counterparts [5,6]. In particular, a positive correlation of type IV collagen expression with tumor malignancy has been documented in several clinical studies. Type IV collagen is a major component of the complex basement membrane zone, where it has an important molecular filtration function. This microenvironmental change is considered to be important for tumor progression and metastasis. For instance, distinct organizational and distribution patterns of type IV collagen around tumors have been associated with tumor progression in melanoma and colorectal carcinoma [7,8].

Accompanied with atypical matrix composition, various types of cells, including fibroblast, vascular, glial, smooth muscle, epithelial, fat, and immune cells, infiltrate into tumor stroma, a process which further contributes to the altered tissue architecture of tumor tissue [9]. Among these cells, cancer-associated fibroblasts play a crucial role in accelerating tumor progression through multiple mechanisms for cell viability, immune evasion, and epithelial–mesenchymal transition [10–12]. Despite a positive correlation of excessive type IV collagen deposition and altered stromal infiltration, there is a knowledge gap regarding the signal transduction pathways initiated by collagen deposition as they relate to the altered stromal infiltration.

* Corresponding author at: Department of Microbiology and Immunology, College of Medicine, National Cheng Kung University, Tainan 70101, Taiwan. Tel.: +886 6 235 3535x5637; fax: +886 6 208 2705.
E-mail addresses: wilson1211983@gmail.com (S.-Y. Chen), dutyzoe@gmail.com (J.-S. Lin), michellin323@hotmail.com (H.-C. Lin), ysshan@mail.ncku.edu.tw (Y.-S. Shan), chengyu@mail.cmu.edu.tw (Y.-J. Cheng), y1357@mail.ncku.edu.tw (B.-C. Yang).

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Accumulating data show that tumor cells grown in high-density collagen matrices exhibit elevated activation of non-receptor focal adhesion kinase and small GTP binding protein, leading to increased cell proliferation and invasiveness [13]. Specifically, the signal initiated by integrin engagement with collagen confers high invasiveness of human pancreatic cells through extracellular signal-regulated kinase (ERK) activation [14]. Rac and ERK pathways have been shown to be required for fibroblast activation and migration [15,16]. These findings raise the possibility that aberrant collagen-associated signaling is involved in cancer-associated fibroblast recruitment. In this study, the molecular mechanism linking collagen deposition and fibroblast infiltration in tumors is elucidated. By knocking down the *Col4-α1* gene, integrin-associated signaling and platelet-derived growth factor A (PDGF-A) expressions in various tumor cell lines were impaired. In addition, the knockdown strategy drastically reduced the capacity of tumor cell lines to recruit myofibroblasts both in vitro and in vivo. Based on these findings, a mechanism for stromal cell infiltration initiated by the deposition of collagen is proposed.

2. Materials and methods

2.1. Antibodies and reagents

Rabbit antibodies (Abs) for type IV collagen and PDGF-A were obtained from Novus Biologicals LLC (Littleton, CO). Rabbit Abs specific for Src, phospho-Src, ERK, and phospho-ERK were obtained from Cell Signaling Technology (Beverly, MA). Mouse Abs for GAPDH and CD29 were purchased from BD Pharmingen (San Diego, CA). Mouse anti-human integrin β1-blocking Ab was obtained from Millipore (Billerica, MA). Purified NA/LE hamster anti-rat CD29-blocking Ab was purchased from BD Pharmingen. Mouse anti-mouse and human α-smooth muscle actin (SMA) antibodies were purchased from Sigma-Aldrich (St. Louis, MO). Recombinant PDGF-A and neutralizing anti-PDGF-A Ab were purchased from Sigma-Aldrich. Growth factor-reduced matrigel was purchased from BD Bioscience (Bedford, MA). Inhibitors for MEK (U0126) and Src family kinase (PP1) were purchased from Calbiochem (La Jolla, CA). Puromycin was purchased from Sigma-Aldrich. Short hairpin (sh) RNA interference pLKO.1-shColIV for type IV-α1 collagen and pLKO.1-shPDGF-A were obtained from the National RNAi Core Facility of Taiwan (Taipei, Taiwan). Plasmids encoding β1-integrin wild-type, constitutively active (G429N), and autoclustering (V737N) variants were kindly provided by Dr. Valerie M. Weaver (UCSF Helen Diller Family Comprehensive Cancer Center, USA).

2.2. Cell culture

Mouse melanoma cell line B16F10, mouse fibroblast cell line NIH3T3, human glioma cell line U118MG, and human fetal lung fibroblast cell line MRC-5 were obtained from the American Type Culture Collection (Manassas, VA). Human hepatocellular carcinoma cell line Huh-7 was obtained from the Japanese Collection of Research Bioresources Cell Bank (Tokyo, Japan). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 1% antibiotic–antimycotic. Viable cells were counted using a trypan blue dye exclusion assay.

Cell growth was determined using the MTT assay. About 2×10^3 cells in 100 μl of medium were seeded into wells of a 96-well plate and incubated for the indicated time. At the end of incubation, 10 μl of 5 mg/ml MTT solution was added into each well. After incubation for another 4 h, the purple crystal sediment was dissolved in 150 μl DMSO and read at 540 nm in an ELISA reader. The absorbance value was used to represent the cell number.

2.3. Lentivirus production and cell transduction

To knock down the expression of type IV-α1 collagen and PDGF-A, tumor cells were transduced with lentivirus encoding shRNA for the *Col4-α1* and *pdgf-a* genes, respectively. The packaging plasmid (psPAX2) and envelope plasmid (pMD2.G) were kindly provided by Dr. Y. L. Lin (Centre National de la Recherche Scientifique, France). To produce lentivirus, 15 μg of the transfer plasmid, 9 μg of the packaging plasmid (psPAX2), and 6 μg of the envelope plasmid (pMD2.G) were cotransfected into 293T cells using a calcium phosphate method. After 24 h of transfection, the cells were cultured in 15 ml of fresh serum-free medium for another 48 h. Culture medium with virions was collected, filtered through a 0.45-μm filter, and further cleaned using centrifugation. For transduction, tumor cells were infected with appropriate amounts of virus suspension containing polybrene (Sigma-Aldrich) for 16 h. Alternatively, plasmid DNA was directly delivered into the cells using lipofection at a ratio of 2 μg DNA/5 μl Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's protocol.

2.4. Quantitative real-time PCR

RNA was isolated using a High Pure RNA Isolation Kit (Roche Molecular Biochemicals, Mannheim, Germany) and treated with DNase to eliminate genomic DNA contamination. Quantitative real-time PCR was conducted using SYBR Green PCR MasterMix according to the manufacturer's instructions. 50 ng of template cDNA in SYBR® Green master mixes in total 20 μl volume was used for PCR reaction and run on a StepOnePlus™ Real-Time PCR System in triplicate (Applied Biosystems, Foster City, CA, USA). To normalize readings, we used GAPDH as internal control for each run. The real time PCR conditions were 95 °C for 15 s, 60 °C for 60 s for 40 cycles. The primers used for amplification are listed in Table 1. Data was analyzed by StepOne Software v2.3 (Applied Biosystems).

2.5. Reverse-transcription polymerase chain reaction

Total RNA was reverse-transcribed into cDNA with Oligo(dT)10–18 primers and MMLV reverse transcriptase (Invitrogen). Supplementary Table S1 summarizes the primer sequences for the target genes used for the polymerase chain reaction (PCR) in this study. PCR products were separated by 2% TAE agarose electrophoresis and made visible by ethidium bromide staining.

2.6. Western blot analysis

Total cell proteins were extracted with a buffer containing 250 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM

Table 1
Quantitative real-time PCR oligonucleotide primers used in this study.

Gene	Primer sequences (5′ → 3′)	mRNA target sequence (NCBI)
Human		
Col4-α1	Forward primer: ACTCTTTTGTGATGCACACCA Reverse primer: AAGCTGTAAGCGTTTGCGTA	NM_001845.4
PDGF-A	Forward primer: CCCTGCCCATTCGGAGGAAGAG Reverse primer: AAGTTGGCGGACGTGGGGTCGA	XM_005249770.1
GAPDH	Forward primer: TTCCAGGAGCGAGATCCCT Reverse primer: CACCCATGACGAACATGGG	NM_001289746.1
Mouse		
Col4-α1	Forward primer: GCTCTGGCTGTGGAAAATGT Reverse primer: CTTGCATCCCGGGAAATC	XM_006508693.1
PDGF-A	Forward primer: CTCTTGGAGATAGACTCCGTAGG Reverse primer: ACTTCTCTTCCTGCGAATGG	XM_006504658.1
GAPDH	Forward primer: AAGGTCATCCCAGAGCTGAA Reverse primer: CTGCTTCACCACCTTCTTGA	NM_001289726.1

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