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BASIC AND TRANSLATIONAL—PANCREAS

Interleukin 35 Expression Correlates With Microvessel Density in Pancreatic Ductal Adenocarcinoma, Recruits Monocytes, and Promotes Growth and Angiogenesis of Xenograft Tumors in Mice

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BACKGROUND & AIMS: Cells of the monocyte lineage contribute to tumor angiogenesis. Interleukin 35 (IL35) is a member of the IL12 family produced by regulatory, but not effector, T cells. IL35 is a dimer comprising the IL12 alpha and IL27 beta chains, encoded by *IL12A* and *EBI3*, respectively. Expression of IL35 is increased in pancreatic ductal adenocarcinomas (PDACs) compared with normal pancreatic tissues, and promotes metastasis. We investigated the role of IL35 in monocyte-induced angiogenesis of PDAC in mice. **METHODS:** We measured levels of IL35 protein, microvessel density, and numbers of monocytes in 123 sequential PDAC tissues from patients who underwent surgery in China in 2010. We performed studies with the human PDAC cell lines CFPAC-1, BxPC-3, Panc-1, MIA-PaCa-2, and mouse PDAC cell line Pan02. Monocyte subsets were isolated by flow cytometry from human peripheral blood mononuclear cells. Fused human or mouse *IL12A* and *EBI3* genes were overexpressed in PDAC cells or knocked down using small hairpin RNAs. Cells were grown as xenograft tumors in SCID mice; some mice were given injections of an IL35-neutralizing antibody and tumor growth was monitored. We performed chemotaxis assays to measure the ability of IL35 to recruit monocytes. We analyzed mRNA sequences of 179 PDACs in the Cancer Genome Atlas to identify correlations between expression of *IL12A* and *EBI3* and monocyte markers. Monocytes incubated with IL35 or PDAC cell supernatants were analyzed in tube formation and endothelial migration assays. **RESULTS:** In PDAC samples from patients, levels of *IL35* mRNA and protein correlated with microvessel density and infiltration of monocyte lineage cells. In cells and mice with xenograft tumors, IL35 increased recruitment of monocytes into PDAC tumors, which required CCL5. Upon exposure to IL35, monocytes increased expression of genes whose products promote angiogenesis (CXCL1 and CXCL8). IL35 activated transcription of *CCL5*, *CXCL1*, and *CXCL8* by inducing GP130 signaling, via IL12RB2 and phosphorylation of STAT1 and STAT4. A combination of a neutralizing antibody against IL35 and gemcitabine significantly decreased monocyte infiltration, microvessel density, and volume of xenograft tumors grown from PDAC cells in mice. **CONCLUSIONS:** PDAC cells produce IL35 to recruit monocytes via CCL5 and induce macrophage to promote angiogenesis via expression of CXCL1 and CXCL8. IL35 signaling promotes angiogenesis and growth of xenograft tumors from PDAC cells in mice. IL35 might serve as a therapeutic target for patients with pancreatic cancer.

Keywords: Tumor Progression; Immune Cell Migration; Anti-tumor Immune Response; Infiltration.

Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive tumors worldwide and has a generally poor prognosis, with a 5-year survival rate of less than 5%.[1] Angiogenesis is crucial for the continued growth, invasion, and metastasis of PDAC and other solid tumors.[2] Although PDAC is generally hypovascular, foci of enhanced endothelial cell proliferation are extensively distributed in the tumor tissues. Blood vessel density and tumor vascular endothelial growth factor A (VEGF-A) levels have been positively correlated with PDAC progression.[3] Several studies have also demonstrated that anti-angiogenic therapy efficiently suppresses tumor growth in animal models of PDAC.[4]

Recently, monocyte lineage cells (MLCs) have emerged as critical contributors to tumor angiogenesis.[5] MLCs are recruited by chemoattractants, including CSF1, CCL2, CCL3, CCL5, and placenta growth factor, in the tumor microenvironment.[6] In response to signals from the microenvironment, MLCs differentiate into macrophages and adopt a pro-angiogenic phenotype.[5] MLCs have been shown to play important roles in tumor angiogenesis of PDAC and other cancers.[7] Furthermore, MLCs are believed to be responsible for the development of resistance to anti-VEGF therapy through activation of pro-angiogenic pathways.[8] This likely accounts for the failure of early clinical anti-angiogenic trials in PDAC patients.[9] Recently, therapies targeting monocyte-driven angiogenesis have been shown

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Abbreviations used in this paper: ChIP, chromatin immunoprecipitation; CM, conditioned medium; HUVEC, human umbilical vein endothelial cell; IHC, immunohistochemistry; MLC, monocyte lineage cell; MVD, microvessel density; PDAC, pancreatic ductal adenocarcinoma; SCID, severe combined immunodeficiency; SD, standard deviation; TCGA, The Cancer Genome Atlas; VEGF, vascular endothelial growth factor.

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EDITOR'S NOTES

BACKGROUND AND CONTEXT

VEGF-targeted therapy was effective in some tumors such as colorectal cancer, however, the overall outcomes are disappointing in pancreatic cancer. The mechanism is still not completely understood.

NEW FINDINGS

The researchers show that tumor-derived IL-35 promotes tumor angiogenesis and tumor growth in pancreatic ductal adenocarcinoma.

LIMITATIONS

This study did not use the patient-derived xenograft mice model to assess the IL35-targeted therapy.

IMPACT

This study indicates that IL35 might serve as a promising therapeutic target for patients with pancreatic cancer.

to decrease the pro-angiogenic factors in tumor growth and spread.[10] Notably, in a recent clinical trial, a CCR2 inhibitor (targeting macrophages) in combination with FOLFIRINOX chemotherapy showed strong anti-tumor effects in PDAC patients.[11] Although MLCs have recently emerged as attractive therapeutic targets for cancer treatment, the mechanism by which these cells participate in tumor angiogenesis is still not completely understood.

IL35 is a new member of the IL12 family of cytokines originally identified in Tregs, consisting of an EBV-induced gene 3 (EBI3) subunit and an IL12A (also named P35) subunit.[12] In contrast to other pro-inflammatory members of the IL12 family, IL35 exhibits strong immunosuppressive effects comparable with those of IL10 and TGFB1.[13] IL35 binds to 3 receptor dimers composed of IL12RB2 and GP130, including IL12RB2:GP130, IL12RB2: IL12RB2, and GP130: GP130.[14] Following engagement of the IL35 receptors, IL35 activates the JAK-STAT signaling pathway to initiate the transcription of downstream genes.[15]

We recently reported that IL35 overexpression in PDAC promotes tumor metastasis by facilitating the adhesion of PDAC cells to the endothelium.[16] Here, we investigate the role of PDAC-derived IL35 in monocyte recruitment and PDAC angiogenesis.

Methods

Cell Culture and Human Sample Collection

The human PDAC cell lines CFPAC-1, BxPC-3, and Panc-1 were obtained from the Committee of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China), and MIA-PaCa-2 was obtained from the American Type Culture Collection. The murine pancreatic cancer cell line Panc02 was a gift from Prof SY Yang. The cell lines were obtained in 2013 and authenticated in August 2014 using short tandem repeat analysis. Mycoplasma contamination was excluded in these cell lines. These cells were cultured in Dulbecco's modified eagle medium, RPMI-1640, or Iscove's modified Dulbecco's medium with 10% fetal bovine serum at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Human peripheral blood mononuclear cells were isolated from the peripheral blood of healthy donors by Ficoll density gradient centrifugation. Monocyte subsets were separated using a Monocyte Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) and then isolated by flow cytometric sorting (labeled with anti-CD14-PE and anti-CD16-FITC antibodies). The cells were cultured in RPMI-1640 medium with 10% fetal bovine serum.

A total of 123 sequential PDAC tissues were collected from patients who received radical surgery in 2010 in the Tianjin Medical University Cancer Institute and Hospital (Tianjin, China). The use of these specimens and patient information was approved by the Ethics Committee of Tianjin Medical University Cancer Institute and Hospital. All patients provided written consent for the use of their specimens and information for future investigations according to the ethics committee.

Tumor Model

All animal studies were approved by the Ethics Committee of Tianjin Medical University Cancer Institute and Hospital and conducted by skilled experimenters under an approved protocol in accordance with the principles and procedures outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Four-week-old female severe combined immunodeficiency (SCID) mice were maintained in a barrier facility on high-efficiency particulate air (HEPA)-filtered racks. Tumor cells were harvested by trypsinization, washed in phosphate-buffered saline and resuspended at 1×10^7 cells/mL in Matrigel. A total of 1×10^6 cells were subcutaneously or orthotopically injected to each SCID mouse to develop tumors. Tumor size was measured weekly. On the 7th day, when the long axis of the tumor was approximately 5 mm, Clophosome (clodronate liposomes; FormuMax Scientific, Palo Alto, CA) (200 μL), gemcitabine (15 mg/kg) or IgG control, anti-CCL5 (25 μg), anti-CXCL1 (20 μg), and anti-IL12A (25 μg) antibodies were intravenously injected into the corresponding mice twice a week. The tumors were harvested 5 or 6 weeks later. Each group had 6 mice.

Tube Formation Assays

Human umbilical vein endothelial cells (HUVECs) were isolated from fresh human umbilical cords by digestion with 0.1% collagenase II and cultured in Endothelial Cell Growth Medium (Lonza, Basel, Switzerland). For preparation of conditioned medium (CM), the indicated cells were seeded in a T75 tissue culture flask and grown to 30%–40% confluence (depending on the growth rate of the cell lines). The growth medium was then replaced with serum-free medium for 24 hours, and the supernatants were harvested when the cells reached 60%–80% confluence. The CM was diluted 2 times with total medium (Dulbecco's modified eagle medium with 10% fetal bovine serum) and then used to suspend the HUVECs to 1×10^5/mL. A 96-well plate was coated with growth factor-reduced Matrigel, and 100 μL HUVEC suspension was dispensed into each well of the coated 96-well plate. The plate was incubated at 37°C with 5% CO_2 for 4 hours. The plates were stained with calcein AM (Dojindo Molecular Technologies, Tokyo, Japan) and visualized using fluorescence microscopy. The capillary networks were imaged. The results of tube formation assays were assessed by a blinded observer. The tube