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Cooperation between human fibrocytes and endothelial colony forming cells increases angiogenesis via CXCR4 pathway

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Abstract

Background—Fibrotic diseases of the lung are associated with a vascular remodeling process. Fibrocytes (Fy) are a distinct population of blood-borne cells that coexpress hematopoietic cell antigens and fibroblast markers, which have been shown to contribute to organ fibrosis. The purpose of this study was to test the hypothesis that Fy might cooperate with endothelial colony forming cells to induce angiogenesis.

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Methods/Results—We successfully isolated Fy from blood of idiopathic pulmonary fibrosis (IPF) patients, which were further characterized by flow cytometry, Reverse Transcriptase quantitative-PCR (RTQ-PCR), and confocal analysis. We investigated the interaction between Fy and cord blood derived endothelial colony forming cells (ECFC) angiogenic potential in vitro and in vivo in a Matrigel implant model. Compared to fibroblast culture media, secreted media from Fy increase ECFC proliferation and their differentiation ability via SDF-1/CXCR4 pathway. IPF-Fy co-implanted with human ECFC in a matrigel plug in immunodeficient mice formed functional microvascular beds, whereas fibroblasts did not. Evaluation of implants after 2 weeks revealed an extensive network of blood vessels containing erythrocytes. CXCR4 blockade significantly inhibited blood vessel formation in the implants. The clinical relevance of these data was confirmed by the high expression level of CXCR4 in vessels close to fibrotic areas in biopsy specimens from patients with IPF, in contrast to control lungs.

Conclusions—Circulating Fy might be contribute to the intense remodeling of the pulmonary vasculature in patients with IPF.

Keywords

Fibrocytes; endothelial progenitor cells; ECFC; pulmonary fibrosis; CXCR4

INTRODUCTION

Fibrocytes (Fy) are a distinct population of blood-borne cells that co-express hematopoietic cell antigens (CD45⁺) and fibroblast markers (collagen 1⁺). They are known to contribute to organ fibrosis, the complex result of several events that might follow tissue injury, i.e. inflammation, aberrant healing and angiogenesis. Fy are involved in vascular repair, intimal hyperplasia after revascularization (1), atherosclerosis (2), myxomatous valve disease (3) or transplant vasculopathy (4). They also contribute to lung fibrogenesis in several interstitial pneumonitis such as idiopathic pulmonary fibrosis (IPF) (5) and scleroderma (6). Attenuation of Fy trafficking in mouse models directly correlates with a reduction in pulmonary fibrosis (7, 8). Furthermore, circulating levels of Fy in patients with idiopathic pulmonary fibrosis may represent a surrogate biomarker of disease severity (5, 9, 10).

Organ fibrosis is always associated with an abnormal and heterogeneous vascular remodeling such as that observed in IPF, in which fibrotic areas exhibit a decreased vascular density in the vicinity of adjacent highly vascularized non-fibrotic tissues (11, 12). Mouse Fy have been described to promote angiogenesis by growth factors secretion (13, 14). However, no data exist on the potential interactions between human Fy isolated from IPF patients and endothelial cells or their progenitors nor their role in aberrant pulmonary vascularization. Endothelial progenitor cells in lung diseases have been proposed as contributors of these anomalies but the nature of the cells mediating proangiogenic effects remains unclear (15). Endothelial colony-forming cells (ECFC), (16, 17) also known as "late endothelial progenitor cells (EPCs)" are rare cells that can be isolated from circulating human blood. They develop after 2 to 3 weeks of culture and have the characteristics of precursors committed to an endothelial lineage. Using an immunodeficient mouse model of human cell implantation, ECFC, combined with human bone marrow-derived mesenchymal stem cells (MSCs), have been shown to form functional vascular connected with mouse

vessels (18). We previously described in IPF, involvement of ECFCs in sixty-four consecutive patients newly diagnosed and prospectively enrolled. All endothelial progenitor subtypes have been explored in this previous study and we found in IPF subjects with a low diffusing capacity of the lung for carbon monoxide ($DL_{CO} < 40\%$), that ECFC were higher compared to those with $DL_{CO} > 40\%$. ECFCs quantified in culture were the only endothelial progenitor subtypes negatively correlated with DL_{CO} . Moreover, we found in this previous study that ECFC from patients with exacerbation had a proliferative potential strongly increased. Thus, these data about DL_{CO} correlation and exacerbation allow us to think that ECFC are mobilized in exacerbation, and so have a major role in aberrant angiogenesis observed in IPF (19).

The aim of our study was to evaluate the potential interactions of Fy and endothelial progenitors ECFC in vitro and in vivo in a matrigel implant model. Additionally, we explored the relevance of our findings in human IPF lung specimens.

MATERIAL AND METHODS

Ethics statement

This study initially designed to assess the circulating endothelial compartment in IPF had obtained the approval from the Ethics committee of Ile de France II (registry number 2006-108). Animal studies have been done in animal facility of Paris Descartes University with agreement number C75-06-02. This study was completed between 2010 and April 2012. DM Smadja has a personal authorisation for academic French Veterinary Service since March 2007. For human Biopsies, this study had obtained the approval from the Ethics committee of Ile de France II (registry number 2006-108, Director of this committee is Prof. Christian Hervé). We confirm that written informed consent from donors was obtained for use of their tissue samples in research.

Circulating fibrocyte and endothelial progenitor cell isolation

Peripheral blood mononuclear cells (PBMC) were isolated from patients with idiopathic pulmonary fibrosis (IPF) defined according to the ATS/ERS consensus (20). PBMC were isolated using Ficoll density centrifugation. Cells were thereafter cultured in EGM2 growth medium containing Endothelial Basal Cell Medium (EBM), SingleQuot Kit (Lonza® Allendale, NJ, USA) supplemented with 5% foetal bovine serum (FBS). Typically, 5×10^6 PBMC were plated into each well of a 6-well plate. When adherent cells showing a mesenchymal phenotype appeared in culture, they were harvested by trypsinisation, replated and cultured in EBM supplemented with 20% FBS, penicillin and streptomycin. They were thereafter characterized as Fy using flow cytometry and RTQ-PCR (as detailed below). Normal human lung fibroblasts (NHLF) were commercially purchased (Lonza® Allendale, NJ, USA) and cultured on fibronectin-coated plates in EBM-2/20% FBS. Culture of ECFC from cord blood mononuclear cells has been described in details elsewhere (21). Briefly, ECFC isolated from cord blood obtained from consenting mothers with normal birth weight children were cultured in EGM2, as previously described (21, 22). When an ECFC colony became microscopically visible, cells were trypsinized and replated in six-well plates and thereafter in T25 and T75 flasks when confluent. As previously described, ECFCs express

endothelial markers (CD31, CD144) without any expression of leukocytes antigens CD14 or CD45 (supplemental Figure 1).

Flow cytometry

Cultured cells were detached with trypsin (Eurobio, Courtaboeuf, France), washed in PBS containing 10% FBS, resuspended in 50 µl of PBS-10% FBS, and incubated for 30 min at 4°C with human specific monoclonal antibodies (mAb), FITC conjugated anti-CD45 (Beckman Coulter) and PE-conjugated anti-CXCR4 (R&D Systems)). Isotype-matched mouse IgG1 and IgG2a mAbs from the same manufacturer were used as negative controls. Ten thousand events were acquired on a FACScan flow cytometer (21, 22) (Becton Dickinson, Le Pont-de-Claix, France). Data were analyzed using the FlowJo software.

Real-Time Quantitative Polymerase Chain Reaction (RTQ-PCR)

The theoretical and practical aspects of RTQ-PCR on the ABI Prism 7900 Sequence Detection System (Applied Biosystems, Villebon sur Yvette, France) are described in detail elsewhere (21). Briefly, we quantified transcripts of the TBP gene, which encodes the TATA box-binding protein (a component of the DNA-binding protein complex TFIID) as the endogenous RNA control, and target gene expression was normalized on the basis of TBP content of each sample and was subsequently normalized to a basal mRNA level with the equation: $N_{\text{target}} = 2^{-C_{\text{t sample}}}$ where C_{t} is the C_{t} value of the target gene minus the C_{t} value of the TBP gene. The results are presented as 'normalized mRNA levels', *i.e.*, the N_{target} value divided by the N_{target} value of the smallest quantifiable amount of target gene mRNA (*i.e.* target gene C_{t} value = 35). For each target gene, normalized mRNA levels reflect the variation of the corresponding mRNA content in ECFCs and/or fibrocytes between passage 3 and 6. Primers for TBP and the target genes (Table 1) were chosen with the assistance of Oligo 5.0 software (National Biosciences, Plymouth, MN, USA). To avoid amplifying contaminating genomic DNA, one of the two primers was placed at the junction between two exons. The thermal cycling conditions comprised an initial denaturation step at 95°C for 10 min. and 50 cycles at 95°C for 15 sec and 65°C for 1 min.

Assays for *in Vitro* Cellular Proliferation

We tested proliferation in presence of a blocking mAb against CXCR4. ECFC proliferation was assessed after seeding 10^4 cells on fibronectin-coated 24-well plates and culturing in growth medium [Endothelial Basal Cell Medium (EBM), SingleQuot Kit (Lonza, Allendale, NJ) without hydrocortisone, supplemented to 20% fetal bovine serum (FBS)] during 24 hours. At Day 1, medium was replaced with conditioned medium of Fibrocytes, Fibroblasts or EBM without growth factors and with 5%FBS, with or without blocking mAb against CXCR-4 or a control isotype mAb. Cell numbers at days 2, 3, 4, and 6 were determined by counting with a phase-contrast microscope and disposable hemocytometer (Digital Bio, Seoul, Korea).

Wrinkle assay

The capacity of circulating Fy recovered from patients with IPF (IPF-Fy) to contract and to potentially serve as perivascular cells was evaluated using a wrinkle assay as previously

described (23, 24). Briefly, eight microliters of silicone (Sigma) was pipetted onto glass coverslips and then thermally cross linked. A glow plasma discharge apparatus was used to generate a plasma discharge onto silicone-coated coverslips. The silicone surface was coated with 100 μ l of 0.1 mg/ml Type I collagen (BD Biosciences) in PBS. Approximately 4×10^3 IPF-Fy were seeded onto silicone-coated coverslips and cultured for 5 h prior analysis. Analysis of contractile force production was performed by observation with a phase contrast microscope of cells that induced deformation of silicone substratum (wrinkles).

Effects of IPF-Fy conditioned medium on ECFC angiogenic markers

Cocultures of IPF-Fy and human cord blood ECFC were set up using 6-wells plate Boyden chambers (Costar, Corning, Lowell, MA, USA) with 0.4- μ m pore-size filters to prevent cell migration. IPF-Fy were seeded in the upper chambers and ECFC in the lower ones. After 24 hours, medium from both chambers was recovered and replaced by EBM/0.5%FBS for 48h hours at 37°C. ECFC were thereafter trypsinized and analyzed. VEGF and its receptors, bFGF, SDF-1 and CXCR4 gene expression was assessed using RT-qPCR and CXCR4 protein expression using flow cytometry.

In vivo effects of IPF-Fy in a murine model of angiogenesis

1.5×10^6 ECFC were mixed up with 1.5×10^6 IPF-Fy then resuspended in 200 μ L phenol red-free Matrigel (BD Bioscience, San Jose, CA) on ice. The mixture was implanted on the backs of 6-week-old male athymic nu/nu mice (Massachusetts General Hospital, Boston, MA) by subcutaneous injection using a 25-gauge needle. Two implants were injected per mouse (one with a combination of ECFC and IPF-Fy and one in which the latter were replaced by NHDF serving as control). The implants were removed 10 days after xenografting, then fixed in 10% buffered formalin overnight, embedded in paraffin and sectioned. Hematoxylin and eosin (H&E)-stained 7- μ m-thick sections were examined for the presence of luminal structures containing red blood cells. Images were taken with a Nikon Eclipse TE300 (Nikon, Melville, NY) using Spot Advance 3.5.9 software (Diagnostic Instruments, Sterling Heights, MI) and a 10 $\times$ /0.45 objective lens. Microvessels were detected by the evaluation of H&E-stained sections taken from the middle part of the implants. The full area of each individual section was evaluated. Microvessels were identified and counted as luminal structures containing red blood cells. The area of each section was estimated by image analysis. Microvessel density was calculated by dividing the total number of red blood cell-filled microvessels by the area of each section (expressed as vessels/mm²) as previously described (18, 25, 26). The role of CXCR4 expressed by ECFC was tested through the incubation of these cells with a blocking anti-CXCR4 mAb prior to their combined injection with IPF-Fy into the mice. Each matrigel implant experiment has been done with, at least, 5 mice.

Expression of the SDF-1/CXCR4 pathway in the human IPF lung

Human IPF lung specimens were recovered after surgical biopsies performed for diagnostic purpose (n=6). Three mm thick paraffin embedded sections were stained with hematoxylin and eosin to evaluate parenchyma and vessel morphology. Subsequently, the expression of SDF-1 and CXCR4 was evaluated using immunohistochemistry. For this purpose, sections were stained with anti-human SDF-1 (dilution 1:60; monoclonal mouse IgG1, clone 79018;

R&D Systems, Lille, France) or anti-human CXCR4 antibody (dilution 1:100; monoclonal mouse IgG2B Clone 44716; R&D Systems). Biotinylated anti-mouse IgG (Abcam, Paris, France, reference ab64255) was used for primary antibody detection and Vector Red alkaline phosphatase substrate (Vector, Philadelphia, reference Sk-5100) was the chromogen. Tissues obtained after lung resection for a localized tumor served as controls.

Statistical analysis

Data were expressed as means $\pm$ SEM. Single comparisons were performed using two-tailed Student's unpaired t-tests with StatView software (Cary, NC 27513, USA). Values of $p < 0.05$ were considered significant.

RESULTS

Fibrocyte (Fy) characterization

Adherent spindle-shaped cells resembling Fy were recovered during culture of PBMC from 3 subjects with IPF, addressed during an acute exacerbation of their disease. IPF-Fy were assessed over time in culture for expression of key Fy markers. The cells were positive for hematopoietic CD45 and CXCR4 antigens during the first two passages (Figure 1A and B). CD45 gradually decreased after passage 3 and fully disappeared after 4 weeks of culture. Fibrocytes expressed the smooth muscle actin (SMA) protein as shown by confocal analysis (Figure 1C). Compared to fibroblasts, these cells expressed high levels of Collagen 1 and fibronectin mRNA transcripts (Figure 1D) as well as a 100-fold higher expression of interleukin 8 (IL-8), Fibroblast specific protein 1 (FSP-1) and Twist transcripts. Altogether, the combined expression of these phenotypic, transcriptional and protein markers allowed us to confirm a Fy phenotype.

IPF- Fy conditioned media modulates SDF1-CXCR4 pathway on ECFC

ECFC were exposed for 24 h to conditioned media (CM) from IPF-Fy or from fibroblasts as control. Neither IPF-Fy nor fibroblast-CM significantly modified VEGF, VEGF receptors or bFGF expression (Table 2). However, both IPF-Fy and fibroblast CM strongly influenced SDF1/CXCR4 pathway expression on ECFC as shown by an increased expression of SDF1 transcripts ($p=0.03$ and 0.001 for IPF-Fy and fibroblasts, respectively). In addition, both CM led to an increase in ECFC CXCR4 expression at 24h but this effect was particularly dramatic for IPF-Fy 20-fold increase, $p<0.0001$, compared to 4-fold increase for fibroblast, ($p=0.03$) (Table 2 and supplemental Figure 2). These findings were confirmed at the protein level by flow cytometry. As shown in Figure 2, the relatively low baseline expression of CXCR4 in ECFC was increased after exposure to IPF-Fy-CM (Figure 2B and D) in contrast to VEGFR2 (KDR) or CD31 (Figure 2A and C).

IPF-Fy can cooperate *in vivo* with ECFC to form vessels through CXCR4

Vascular formation results from a finely tuned balance between at least proliferation and differentiation.

Thus, we tested the effect of CM from Fy and Fibroblast to activate ECFC *in vitro*. We have explored Ki67 expression and proliferation by cell counting. For Ki-67 expression, we

incubated conditioned medium of Fibrocytes, Fibroblasts or EBM without growth factors and with 5%FBS after a 16 hours of serum and growth factor deprivation. We quantified Ki-67 level by RTQ-PCR 24 hours after beginning of incubation. As shown in Figure 3A, Fy induces a significant increase in Ki-67 expression (* $p=0.01$) while no modification of Ki-67 expression is found with conditionnel medium from fibroblast ($p=0.97$). As shown in Figure 3, CM from fibrocytes also increases ECFC proliferation quantified by cell counting (Figure 3B). Incubation with CXCR-4 blocking mAb does not modify the increased proliferation observed with CM from fibrocytes.

We also tested impact of fibrocytes on ECFC differentiation in matrigel implant model *in vivo*. We co-implanted IPF-Fy in a 1:1 ratio with ECFC, suspended in Matrigel, in nu/nu mice for 10 days. Implants that contained the combination of the 2 cell types presented numerous functional vessels containing erythrocytes (Figure 4A, left panel). In contrast, implants with ECFC and normal fibroblasts instead of IPF-Fy were devoid of vessels (Figure 4A, right panel and Figure 4B). Vessels formed with Fy and ECFC are from human origin since vessels are positive for specific human CD31 (Figure 4C). Fy alone were also tested in matrigel implant model with a cell density of 3.10^6 cell per plug of 200 μ l. Fy alone induced vessel formation in Matrigel implants (supplemental Figure 3, left panel). We found that these Fy alone induced vessel formation with a significant increase of microvascular density in contrast to fibroblasts or ECFC alone (supplemental Figure 4). As shown in supplemental Figure 3 (middle and right panel) none of these vessels are positive for human CD31 (supplemental Figure 3, middle panel), in contrast to Fy combined with ECFC (Figure 4C).

To confirm the potential of IPF-Fy to serve as perivascular cells, a normally contractile cell, we tested their contractile activity by their ability to form wrinkles *in vitro* on silicon-based substrata. As shown in Figure 4C, IPF-Fy exhibited a strong ability to form wrinkles in these conditions whereas fibroblasts and ECFC did not. The *in vivo* relevance of our above-cited findings on the modulation of SDF1/CXCR4 pathway in ECFC in the presence of Fy-CM was tested through the incubation of ECFC with a blocking anti-CXCR4 mAb prior to their combined injection with IPF-Fy into the mice (Figure 5A).

The vessel forming ability of ECFC was reduced, as shown by an average 60% decreased microvessel density ($p < 0.0001$, Figure 5B).

SDF-1 and CXCR4 expression in the human IPF lung

Human IPF lung specimens ($n=6$) were stained with hematoxylin and eosin coloration to evaluate lung parenchyma and vessel morphology. Subsequently the appropriate antibodies were used to assess the expression of SDF-1 and CXCR4. In fibrotic parenchymal areas, pulmonary arteries of the muscular type ($<500 \mu$ m) displayed pronounced remodeling with intimal fibrotic thickening and moderate medial hypertrophy. Also, adventitial broadening with collagen deposition and multiplication of Vasa vasorum was frequently observed. Arterial remodeling was commonly associated with perivascular inflammatory infiltrate. SDF-1 and CXCR4 were both highly expressed and localized in endothelial cells of pulmonary arteries and of Vasa vasorum (Figure 6B for SDF-1, Figure 6D and E for CXCR4). This was observed only in IPF lungs while the SDF1 and CXCR4 expression in

control lungs was mild or absent (Figure 6A for SDF-1 and 6C for CXCR4). In addition, CXCR4 was detected on perivascular inflammatory cells, mainly on lymphocytes. Also, so called fibroblastic foci, a histologic feature typically found in lungs from patients with usual interstitial pneumonia, displayed numerous CXCR4+ fibroblasts (Figure 6F).

DISCUSSION

We have shown in this study that circulating Fibrocytes (Fy) isolated from patients with IPF act synergistically with ECFC to form vessels in a Matrigel implant model, part of this effect resulting from an up-regulation by IFP-Fy of CXCR4 expression on ECFC. In addition, we have shown that human IPF lung vessels, particularly those present in the vicinity of fibroblasts foci, express high levels of SDF-1 and CXCR4.

As a whole, these data suggest a key role for Fy in IPF-associated aberrant vascular remodeling. In some cardiovascular settings such as the adverse ventricular remodeling seen in ischemic cardiomyopathy (27–29), intimal hyperplasia after revascularization (1) and atherosclerosis (2), Fy have been shown to play an important role. In all these settings, inflammatory, fibrotic and proliferative factors stimulate other resident vascular wall cell proliferation, supporting the hypothesis that Fy might contribute both directly and indirectly to vascular remodeling processes. Fy are circulating mesenchymal like-cell able to traffic from bone marrow to the lung to mediate pulmonary fibrosis (7). They are an indicator of poor prognostic in IPF (9). Fy have been described to have angiogenic properties *in vitro* (14) and *in vivo* especially in diabetic mice (13, 14). In our study, Fy were isolated from 3 patients that were all in acute exacerbation phase. No Fy were obtained from patients with a stable IPF. We have shown here that IPF-Fy have an inherent ability to increase the number of functional microvessels *in vivo* when co-implanted with ECFC. The presence of human endothelial cell-lined lumens containing erythrocytes throughout the implants indicated not only a process of vasculogenesis mediated by these 2 cell types but also the formation of functional anastomoses with the host circulatory system. Fy alone induced newly formed vessels in matrigel implant, however none of these vessels are positive for human CD31 in contrast to Fy combined with ECFC. This result is in line with our hypothesis of two different proangiogenic mechanism induced by Fy that are first, a paracrine effect (recruitment of mouse vessels) but also on human ECFC when co-implanted in plug, as demonstrated by the SDF-1/CXCR-4 involvement after conditioned media (CM) ; and second, a perivascular cell when co-implanted with human ECFC. Moreover, the absence of human CD31 positive labelling in implants with Fy alone exclude an endothelial differentiation potential as previously described in another vasculogenic mesenchymal subtype when implanted alone: hemangioma stem cells (30, 31). *In vitro*, Fy were shown to differentiate into SMA-positive cells with a contractile phenotype, indicating that they had achieved a smooth muscle phenotype. In the pulmonary context, a role for Fy has been suggested in pulmonary arterial hypertension (PAH) (32). Experimental models of PAH have shown that pulmonary adventitial remodeling was due to a robust recruitment of non-resident, bone marrow-derived cells which can produce collagen and express α -actin of smooth muscle cells, evoking Fy (33). Our results showing that Fy might enhance angiogenesis through their differentiation into perivascular cells are in agreement with this observation.

We have extensively characterized the Fy population in our study. As expected, IPF-Fy expressed a variety of mesenchymal markers including collagen-1 and the leukocyte marker CD45 (Figure 1) (34) and gradually lost hematopoietic markers in culture, as reported by others (13). In addition, when compared to control fibroblasts, they overexpressed IL-8 (Figure 1), suggesting a potential role in progenitor subtypes recruitment to the lung (35, 36). However, this high level of IL-8 could not interfere with ECFC induced angiogenic process since we have previously described that ECFC do not express IL-8 receptors (35). Furthermore, we found that Fy, in contrast to normal lung fibroblasts, expressed high levels of FSP1 and Twist (Figure 1). These mediators have been implicated in the control of motility, migration or survival of epithelial, endothelial or fibroblast lineages and to contribute to epithelial-mesenchymal transition (37, 38). They have been shown to be increased in lung fibrotic areas (38–40). Their expression by IPF-Fy in our study provides additional support for the involvement of Fy in the lung fibrogenesis in human IPF. Furthermore, Twist has been correlated to tumor angiogenic processes and vascular development; it also reflects the metastatic state and/or aggressiveness in tumoral angiogenesis (41). The recruitment to the lung of Fy overexpressing Twist during IPF might participate to the abnormal angiogenic processes observed in this disease (11).

One of the most potent angiogenic factors is SDF-1; it acts by increasing endothelial progenitor and Fy mobilization, trafficking, differentiation or adhesion (21, 42–47). IPF-Fy increased SDF-1 and CXCR4 expression, which in turn induced an enhancement of ECFC differentiation. As shown in Figure 3, Fy induces a significant increase in ECFC proliferation in contrast to fibroblast-CM. Using blocking mAb against CXCR-4, we did not modify proliferation induced by CM from Fy. This absence of CXCR4 blocking on ECFC proliferation is in accordance to previous data showing that, the proangiogenic activity of SDF-1, does not include an effect on cell proliferation (21, 48). SDF1/CXR-4 has been involved in differentiation potential of ECFC and that's why we tested blocking mAb in matrigel implant. Altogether, our data suggest that SDF-1 and CXCR-4 overexpression results from transcriptional upregulation directly influences vascular tube formation in matrigel implant by affecting differentiation potential more than proliferation, confirming previous studies on CXCR4 implication in angiogenesis. The *in vivo* relevance of our present observations is supported by the efficacy of mAbs against CXCR4 in reducing vessel formation in the cell/Matrigel implants and by the marked expression of CXCR4 by strongly remodeled pulmonary vessels present in human IPF lungs. Exact mechanisms through which Fy increases CXCR4 need to be determined. Even without this knowledge, Fy, by locally activating the SDF-1/CXCR4 pathway, are likely to promote both Fy and endothelial progenitor recruitment to the lung, thereby participating in the amplification of the dramatic fibrotic and vascular changes observed in IP. CXCR4 function in ECFC has been already proposed in activated states (21, 44) whereas its basal expression is very low and or undetectable in most ECFC. However, ECFC-CXCR4 expression is regulated by different polymorphisms (43) and seems to correlate to angiogenic phenotype when it is highly expressed. CXCR4 has also been correlated to ECFC pseudo tubes differentiation while no increased proliferation is observed after its specific activation with SDF-1 (21, 44). In this study, increased levels of CXCR4 in ECFC after co-culture with Fy confirm the CXCR4 implication in activated conditions. Furthermore, its blockade with mAb 12G5 *in*

vivo in the cell/Matrigel implant model, strongly argues for its important role in building vessels. SDF-1 has also been described recently to modulate endothelial cells in pulmonary vascular disease by a CXCR7 dependant mechanism (49). Thus our data emphasize SDF-1 implication in pulmonary vascular remodeling process. Relevance of our results about involvement of Fy, their interaction with endothelial progenitors and SDF-1 pathway in angiogenic disorders observed in IPF need to be confirmed in a preclinical model of fibrosis as bleomycin model in nude mice with injection of human Fy and/or fibroblasts with and without ECFC.

Our study may have clinical implications in the development of new therapeutic strategies for this devastating fibrotic disease. Indeed, it has been recently demonstrated that blocking Fy recruitment with a mAb anti-SDF1 may improve experimental IPF in animal models (7). Thus, AMD3100, which disrupts SDF-1/CXCR4 binding, or the peptidic CXCR4 inhibitor TF14016 developed for cancer (50) might limit Fy recruitment and IPF-associated angiogenesis.

In conclusion, in addition to contributing to local fibrogenesis in human IPF, Fy could differentiate into perivascular cells, stimulate proliferation and differentiation of other progenitor cells within the lung and be key actors in the aberrant angiogenesis associated to this disease. They might actively participate in the trafficking of endothelial progenitors from bone marrow, tissue parenchyma or the blood vessel wall. Activation of the SDF-1/CXCR4 pathway may amplify cell recruitment into the lung and be a trigger for abnormal angiogenesis independently from hypoxia. Fy might therefore be considered not only as fibrotic agents but also as a source of abnormal angiogenesis into the lung by recruiting and inducing endothelial progenitor differentiation. Our data suggest that Fy might be a novel target for innovative therapies in the field of fibrosis and their angiogenic related-disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Characterization of cultured human fibrocytes for IPF patients

A- Flow cytometric analysis of CD45 expression of cultured IPF-Fy. Green lines: cells labeled with FITC-conjugated anti-CD45. Red lines: cells labeled with FITC-conjugated isotype-matched control antibodies.

B- Flow cytometric analysis of CXCR4 expression of cultured IPF-Fy. Green lines: cells labeled with FITC-conjugated anti-CXCR4. Red lines: cells labeled with FITC-conjugated isotype-matched control antibodies.

C- Confocal analysis of smooth muscle actin in cultured IPF-Fy

D- RTQ-PCR analysis of Collagen 1, fibronectin, FSP-1, twist and IL-8 in ECFC, IPF-Fy and fibroblasts. mRNA levels normalized to TBP mRNA levels and to sample with lowest quantifiable level (*i.e.* 1 on the left ordinate, corresponding to a C_t value of 35). Values above 100 represent strong gene expression. Mean and SEM values of three different samples shown at each point.

Figure 2. Coculture of IPF-Fy with ECFC increases ECFC-CXCR4 expression

Cocultures of IPF-Fy and ECFC were set up using 6-wells plate Boyden chambers with 0.4- μ m pore-size filters to prevent cell migration. IPF-Fy were seeded in the upper chambers and ECFC in the lower ones. After 48h of coculture, ECFC protein expression was realized using flow cytometry. VEGFR2 (KDR) and CD31 expression were comparable in ECFC co-cultivated with IPF-Fy (A and C) in contrast to ECFC-CXCR4 expression (B and D) whose expression increased after the coculture.

Figure 3. Human IPF-Fy increase Ki-67 expression and proliferative potential of human ECFC
 A- mRNA level of Ki-67 have been quantified after 24 hours of incubation with conditioned medium from Fy or Fibroblast.
 B- Proliferation of ECFC cultured with conditioned media from fibrocytes with or without blocking mAb against CXCR-4 and fibroblast over 6 days evaluated by counting cells

Figure 4. Human IPF-Fy exhibit increased vasculogenic potential compared to fibroblasts

A- Cord blood ECFC co-injected with IPF-Fy or fibroblasts. Representative photographs of Matrigel explants at day10 after injection with corresponding histological sections stained with H&E.

B- Quantification of microvessel density (MVD) as microvessels/mm², Scale bar = 20 μm. The arrows on different stained sections point to lumens filled with red blood cells – i.e. perfused vessels. Data are mean ± SEM.

C- Immunohistochemical staining for human CD31 in implants after 10 days (scale bars, 50 μm).

D- Wrinkle-forming cells are observed in IPF-Fy (left panel) but not with fibroblasts (middle panel) or ECFC (right panel).

Figure 5. Blocking mAb against CXCR4 decreases in vivo vasculogenic potential of IPF-Fy co-implanted with ECFC

A- Representative photographs of Matrigel explants at day10 after injection of IPF-Fy and ECFC with control mAb or blocking CXCR4, with corresponding sections stained for H&E.

B- Quantification of total microvessel density (MVD) as microvessels/mm², Scale bar = 20 μm. The arrows on different stained sections point to lumens filled with red blood cells – i.e. perfused vessels. Data are mean ± SEM.

Figure 6. Immunohistochemistry analysis of SDF-1 and CXCR4 in pulmonary biopsy of IPF patients

A- In lungs from control patients, a mild SDF-1 expression is observed in endothelial cells of pulmonary arteries.

B- In lungs from patients with idiopathic pulmonary Fibrosis (IPF), SDF-1 expression is observed in endothelial cells of pulmonary arteries.

C- CXCR4 is not expressed in endothelial cells of pulmonary arteries from control patients.

D and E- CXCR4 is expressed in endothelial cells of remodeled pulmonary arteries from patients with IPF.

F- CXCR4 is also expressed in fibroblast foci from patients with IPF.

Scale bar = 50 μ m

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Table 1

Oligonucleotide used for real-time quantitative RT-PCR

	Upper Primer	Lower primer	PCR product size (bp)
VEGF-A	5' CTT GCC TTG CTG CTC TAC CTC C 3'	5' CAT CCA TGA ACT TCA CCA CTT CGT 3'	103
VEGFR1	5' ATC ATT CCG AAG CAA GGT GTG AC 3'	5' TCC TTC TAT TAT TGC CAT GCG CT 3'	122
VEGFR2	5' TGG GAA CCG GAA CCT CAC TAT C 3'	5' GTC TTT TCC TGG GCA CCT TCT ATT 3'	132
bFGF	5' AGA AGA GCG ACC CTC ACA TCA A 3'	5' GGT AAC GGT TAG CAC ACA CTC CTT 3'	87
SDF-1	5' - GCC CGT CAG CCT GAG CTA CA 3'	5' - TTC TTC AGC CGG GCT ACA ATC T - 3'	129
CXCR4	5' ACCATGGAGGGGATCAGTATATACA 3'	5' ACAGGGTTCCTTCATGGAGTCA 3'	87
KI67	5' ATT GAA CCT GCG GAA GAG CTG A '	5' GGA GCG CAG GGA TAT TCC CTT A 3'	105
COL1A1	5' CCT CCG GCT CCT GCT CCT CTT 3'	5' GGC AGT TCT TGG TCT CGT CAC A 3'	227
FN1	5' TGT TGT CAC CAC TCT GGA GAA TGT 3'	5' GAA GCC AGT GAT CGT CTC AGT CTT 3'	186
FSP1/S100A4	5' CTC GGG CAA AGA GGG TGA CAA 3'	5'GCT TCA TCT GTC CTT TTC CCC AA 3'	101
TWIST	5' CGC CCC GCT CTT CTC CTC T 3'	5' TGG ACA CGT CCT GCA TCA TCT CT 3'	75
IL8	5' CAC CGG AAG GAA CCA TCT CAC TGT 3'	5' TCC TTG GCA AAA CTG CAC CTT CA 3'	112

Table 2

Effect of conditioned medium (CM) from fibroblasts or IPF-Fy on the mRNA levels of angiogenic mediators produced by ECFC.

	CM-Fibroblast	CM-Fibrocytes
VEGF-A	1,9 ± 0,2	0,7 ± 0,1
VEGFR1	1,6 ± 0,04	1,9 ± 0,2
VEGFR2	1,6 ± 0,2	1,8 ± 0,2
bFGF	0,8 ± 0,1	0,5 ± 0,1
SDF-1	5,4 ± 0,1 [*]	2,9 ± 0,4 [*]
CXCR4	4,2 ± 1,4 [*]	19,6 ± 3 [*]

ECFC were stimulated with CM for 24 hours after 16 hours of serum and growth-factor deprivation. mRNAs were measured by RTQ-PCR and normalized to TBP mRNA (mean±SEM, n=3).

^{*} $P < 0.05$ compared with unstimulated cells.