Original Article Hypoxia-induced fractalkine-stimulated endothelial/ pericyte dynamics in pulmonary hypertension

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Received November 5, 2015; Accepted December 30, 2015; Epub January 15, 2016; Published January 30, 2016

Abstract: Distal vascular remodeling is a hallmark of hypoxic pulmonary hypertension (PH). The capillary walls of the pulmonary non-muscular microvessels consist of endothelial cells and pericytes. Since hypoxia stimulates overproduction of a unique chemokine/fractalkine, CX3CL1 and pericytes functions as contractile elements within the microvasculature, this study is focused to identify the role of CX3CL1 and its sole receptor CX3CR1 axis in hypoxiainduced endothelial/pericyte dynamics in pulmonary pathophysiology. Hypoxia-induced release of CX3CL1 from mouse lung microvascular endothelial cell (MVEC) enhanced proliferation of pericytes. Treatments with CX3CL1 neutralizing antibody or CX3CR1 antagonist attenuated recombinant CX3CL1 (rCX3CL1)-mediated pericyte proliferation. Direct interaction of increasing levels of pericyte with MVEC attenuated MVEC function assessed by acetylcholine (Ach)-stimulated nitric oxide (NO)/cGMP levels. Hypoxia exposure increased pericyte expression in the lungs of wild-type (WT) but not in CX3CR1 knockout (KO) mice. Echocardiographic assessment of hypoxia-induced pulmonary hemodynamic changes indicated decreased pulmonary acceleration time (PAT) and PAT/ejection time (PET) in WT hypoxic as compared to WT normoxic mice. However, PAT and PAT/PET in CX3CR1 KO hypoxic and normoxic mice were comparable to WT normoxic mice. Assessment of RV free wall thickness indicated increases in hypoxic WT but not in CX3CR1 KO mice. These results indicate that hypoxia-induced elevation of CX3CL1 increased pericyte proliferation leading to MVEC dysfunction and altered pulmonary hemodynamics consistent with the pathophysiologic changes observed in the development of PH.

Keywords: Hypoxia, fractalkine, lung, endothelium, pericyte, hypertension

Introduction

Distal capillary muscularization is a hallmark of pulmonary hypertension (PH) caused by oxygen deficiency, a condition in patients with obstructive lung diseases and people living at highaltitude regions [1-4]. The capillaries of the pulmonary microvasculature consist of endothelial cells and pericytes [5]. Pericytes are located around endothelial cells in the walls of nonmuscular microvessels and functions as contractile element within the microvasculature [6-8]. Microvascular endothelial cells (MVEC) are exquisitely located in the gas exchange region and respond to alveolar hypoxia by overproduction of a unique chemokine/fractalkine, CX3CL1. The CX3CL1 receptor, CX3CR1 is expressed in lung cells and the CX3CL1/ CX3CR1 axis has been suggested to play a critical role in the pathophysiology of PH [9-12]. We recently reported that: i) elevated levels of CX3CL1 in peripheral and lung capillary of patients with PH, ii) hypoxia enhanced CX3CL1 release from human MVEC, iii) CX3CL1-stimulated smooth muscle cell proliferation, and iv) exposure to hypoxia induced pulmonary artery pressure elevation and vascular remodeling in WT but not in CX3CR1 KO mice [13]. As such, hypoxia-mediated proliferation of SMC in vascular remodeling observed in the development of PH has been well documented. Whether hypoxia-induced expression of CX3CL1 impact proliferation of pericyte and increased pericyte/ MVEC interaction leading to MVEC dysfunction remained to be determined.

Pericytes are associated with arterioles, venules, and specifically capillaries where there are no SMCs [5]. The branching cytoplasmic processes of pericytes are partially encircle MVEC and thought to play a critical role in the regulation of microvascular function. As such, pericytes are essential components of the capillary vessel wall that plays a critical role in maintaining microvascular integrity in pathophysiologic conditions [5, 6]. For example, the role of pericytes in various mediator-stimulated pathogenesis of pulmonary fibrosis and PH has been recently reported [14, 15]. However, CX3CL1-mediated pericyte proliferation in the hypoxic microvasculature is unclear and remained the puzzling aspect of hypoxic PH. Hypoxic proliferation of pericytes can enhance distal vessel wall stiffness with profound impact on flow dynamics leading to RV dysfunction. In addition, currently there are no approved therapies available to treat proliferative component of PH pathobiology. At present nothing is known about hypoxia/CX3CL1-mediated pulmonary capillary pericyte growth in progression of PH. Here we examined the impacts of hypoxia-mediated MVEC released CX3CL1 on proliferation of pericytes, endothelial dysfunction due to increased interaction of pericytes with MVEC, and altered pulmonary hemodynamics using WT/CX3CR1 KO mice.

Materials and methods

Cell culture

Mouse pulmonary MVEC and pulmonary artery smooth muscle cells (SMC) were purchased from Cell Biologics (Chicago, IL) and propagated in monolayers as previously described [11, 13]. Pericytes were isolated from the outer 0.1 mm peripheral ages of the C57BL/6 mouse lungs using slight modifications of the previously described method [16]. The initial culture consisting of pericytes and possibly MVEC and SMC were placed in pericyte growth medium from PromoCell (Heidelberg, Germany) containing 1% platelet deficient serum to allow preferential growth of pericytes. The serum starvation stage is critical for growth depletion of contaminating MVEC and SMC. After second passage, a homogeneous pericyte cultures were obtained and maintained in 0.1% fetal bovine serum (FBS) for 96 to 120 hr. Pericytes were identified using mouse anti-NG2 antibody (Santa Cruz Biotechnology) marker. These pericytes remained undifferentiated up to 7 passages under our experimental conditions. MVEC, SMC, and pericytes were subculture < 5 passages and were used for all experiments.

Exposure of cells to hypoxia

Confluent monolayers of MVEC or pericytes in RPMI 1640 containing 1% FBS were exposed to gas mixture containing 3% oxygen/5% CO_2 /balanced N_2 or to room air containing 5% CO_2 (control or normoxia) at 37°C for 48 hr as previously described [13]. We used 48 hr hypoxia exposure timeline in this study based on our previous time-dependent observation that demonstrated maximum release of soluble CX3CL1 from human MVEC [13]. After exposure cells were used to measure CX3CR1 levels by qRT-PCR as previously described [13]. We also used MVEC/hypoxia conditioned medium to measure the levels of MVEC released CX3CL1 and its impact on pericyte proliferation.

Exposure of mice to hypoxia

Three-month old male C57BL/6 WT and CX3CR1 KO mice were obtained from Jackson Laboratories and from Dr. Jeffrey Harrison, University of Florida. The animal experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC). WT and CX3CR1 KO mice were exposed to hypoxia $(10\% O_{2})$ in a Coy Laboratory hypoxia exposure chamber equipped with auto purge, airlock, and automated live animal filtration and dehumidification systems or normoxia (room air) for 4 weeks as previously described [13, 17]. After exposure animals were used to assess: i) pericyte proliferation in lungs, and ii) non-invasive assessment of pulmonary hemodynamics and right ventricular (RV) hypertrophy by echocardiography.

Hypoxia-stimulated CX3CR1 expression in MVEC and pericytes

To determine the impact of hypoxia on CX3CR1 expression, mouse lung MVEC and pericytes were exposed to room air (normoxia) or hypoxia $(3\% O_2)$ for 48 hr. After exposure, total RNAs were extracted by using the Pure-link RNA purification system (Invitrogen). RNAs were converted to cDNA using a reverse transcription kit (Applied Biosystems). The relative steady state levels of CX3CR1 mRNA in MVEC and pericytes were measured using qRT-PCR as previously described [13].

MVEC/hypoxia conditioned medium-stimulated pericyte proliferation

To determine whether MVEC/hypoxia-stimulated CX3CL1 release is associated with pericyte proliferation, the cultures of serum-starved MVEC in RPMI 1640 containing 1% FBS were exposed to room air (normoxia) or hypoxia (3% O₂) for 48 hr. After exposure, CX3CL1 mRNA and CX3CL1 protein levels in the medium were measured by using qTR-PCR with β -2microglobulin primers in a multiplexed reaction and the quantitative sandwich immunoassay and anti-CX3CL1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), respectively. as previously described [13]. MVEC normoxia/ hypoxia conditioned medium (5 ml) each were used to stimulate pericytes for 24 hr with or without the presence of CX3CL1 neutralizing CX3CL1 antibody (5 µg/ml) or CX3CR1 antagonist (10 µg/ml) obtained from Dr. Karim Dorgham (INSERM, Paris, France). Soluble CX3CL1-stimulated pericyte proliferation was using 4-[13-(4-lodophenyl)-2-(4assessed nitrophenyl)-2H-5 tetrazoliol-1,3-benzene disulfonate (WST-1) assay as previously described [13].

Recombinant CX3CL1 (rCX3CL1)-mediated pericyte proliferation

To assess the direct impact of rCX3CL1 on pericyte proliferation, [methyl-[³H] thymidine incorporation assay as an index of DNA synthesis was used. Pericytes (10^5 per well) were serum starved for 24 hr. Cells were then incubated in fresh pericyte medium (PromoCell) containing 0.25 µCi [methyl-³H] thymidine per well for 12 hr. Cells were pretreated with or without (control) the presence of anti-CX3CL1 antibody (5 µg/ml) or CX3CR1 antagonist (10μ g/ml) for 30 min prior to addition of rCX3CL1 (2 ng/ml). Cells were then washed and dissolved in 0.25 M NaOH. The hydrolysate was used to count methyl-[³H] thymidine incorporation and protein content as previously described [18].

Impact of pericyte/MVEC interaction on MVEC function

To determine the impact on increasing levels of pericytes on MVEC function, we used multiple co-culture models using the Transwell system as previously described [19]. In brief, MVEC or pericytes alone (each of 10,000 cells/well) or MVEC/pericyte 1:1 (10,000:10,000 cells/well) or 1:4 (10,000:40,000 cells/well) were seeded on microporous surface of the removable upper chamber (Transwell) and SMCs were cultured independently in the lower chamber of a separate Transwell unit. Transwell upper units containing MVEC or pericytes alone or MVEC/pericyte were used in four groups: group 1 = MVECalone, group 2 = pericytes alone, group 3 = MVEC/pericytes 1:1, and group MVEC/pericytes 1:4 were placed on lower unit containing SMC. The Transwell units containing both chambers that prevents physical contact with cells in upper and lower units were stimulated by adding 5 µM acethylcholine (Ach) or RPMI 1640 (control) in the upper chamber with or without the presence of NO synthase inhibitor L-NAME (100 µM) for 60 min at 37°C. After incubation the cGMP levels in SMC were measured using a cGMP enzyme immunoassay system kit (Amersham) as previously described [19]. To determine the impact of medium from pericytes on MVEC function, in some experiments, Transwell unit containing MVEC alone were incubated for 1 hr with medium (5 ml) from pericytes or RPMI 1640 (control) then MVEC and SMC Transwell units were used to determine the effects of Ach-stimulation of MVEC on cGMP levels in SMC as described above.

Immunostaining of lung pericytes

After normoxia/hypoxia exposure lungs from WT and CX3CR1 KO mice were isolated and embedded with OCT solution and snap frozen. The cryosections (7 mm) of OCT-embedded lungs were used for immuno staining with anti-NG2 (1:100 dilution, TRITC-red, Santa Cruz Biotechnology) primary antibody and counter stained with DAPI (blue, Life Technologies) and anti-aSM actin (1:100 dilution, FITC-green, Molecular Probe) antibodies as previously described [13]. The images were taken using EVOS fl (Advanced Microscopy Group) inverted immunofluorescence microscope. Quantitative analysis of NG2 immuno stained pericytes was performed on five separate areas of each image from three separate animals in each group and an average number of pericyte expression was determined.

Hemodynamic measurements by echocardiography

To determine whether CX3CL1/CX3CR1 axis plays a critical role in hypoxia-induced modula-



Figure 1. Hypoxia increased CX3CR1 expression in MVEC and pericytes. Mouse lung MVEC and pericytes were exposed to room air (normoxia, N) or hypoxia (3% O_2 , H) for 48 hr. After exposure, CX3CR1 mRNA levels were measured using qRT-PCR as described in methods. *P < 0.05 vs. N. Values are means ± SE, n = 4 in each group.



Figure 2. MVEC/hypoxia conditioned medium stimulates pericyte proliferation. Mouse lung MVEC were exposed to room air (normoxia, N) or hypoxia (3% O₂, H) for 48 hr. After exposure, medium levels of CX3CL1 mRNA and protein were measured. MVEC-normoxia/ hypoxia conditioned medium (5 ml) each were used to stimulate pericytes for 24 hr as described in methods. *P < 0.05 vs. N; **P < 0.05 vs. H. Values are means \pm SE, n = 4 in each group.

tion of pulmonary hemodynamics, WT and CX3CR1 KO mice were expose to normoxia or hypoxia $(10\% O_2)$ as described in methods.

After exposure, animals were anesthetized using isofluorane (2%) inhalation. After shaving the chest and application of the ultrasound transmission gel, transthoracic closed-chest 2-D, M-mode, and pulsed-wave Doppler echocardiography was performed using Vevo 2100 imaging system (visualsonics) as previously described (20). Echocardiography was performed using 55 MHz MicroScan transducer and Vevo 2100 Imaging System (Visualsonics). Doppler tracings were recorded and pulmonary acceleration time (PAT) and PAT/ejection time (PET) were measured. M-mode and 2-dimensional (2-D) applications were used to measure RV free wall thickness during end diastole using right parasternal long-axis view.

Statistical analysis

Significance of the MVEC/hypoxia-mediated release of CX3CL1 and rCX3CL1 on pericyte proliferation, pericyte/MVEC function, and pulmonary hemodynamic variables were determined by analysis of standard errors and using *t*-test/ANOVA. *P* value of < 0.05 was considered statistically significant.

Results

Hypoxia increases CX3CR1 expression in MVEC and pericytes

We previously reported that hypoxia increases release of CX3CL1 from human MVEC [13]. Since CX3CL1 is processed by its sole receptor CX3CR1 and CX3CL1/CX3CR1 axis is linked with inflammatory pathophysiology, we determine the impact of hypoxia on CX3CR1 expression in MVEC and pericytes. MVEC and pericytes were exposed to room air (normoxia) or hypoxia (3% O_2) for 48 hr as described in methods. As shown in **Figure 1**, hypoxia exposure significantly elevated CX3CR1 expression in MVEC and pericytes.

MVEC/hypoxia conditioned medium enhances pericyte proliferation

To assess whether MVEC/hypoxia-stimulation CX3CL1 release is associated with pericyte proliferation, CX3CL1 levels in MVEC/hypoxia conditioned medium were determine and conditioned medium was used to stimulate pericytes as described in methods. Relative levels of CX3CL1 mRNA in normoxic and hypoxic medi-



Figure 3. Recombinant CX3CL1 (rCX3CL1)-mediated proliferation of pericytes was attenuated by CX3CL1 neutralizing antibody (Ab) or by CX3CR1 antagonist (Ag). The effects of rCX3CL1 (2 ng/ml) stimulation of pericyte proliferation were measured with and without (control) the presence or absence of anti CX3CL1 neutralizing Ab (5 µg/ml) or CX3CR1 Ag (10 µg/ml) for 12 hr. Pericyte proliferation was assessed by [methyl-3H] thymidine incorporation as an index of DNA synthesis. Cells (10⁵ per well) were serum deprived for 24 hr. Cells were the incubated in freshpericyte medium (PromoCell) containing 0.25 µCi [methyl-³H] thymidine per well. Cells were pretreated with or without anti CX3CL1 Ab or CX3CR1 Ag for 30 min prior to addition of rCX3CL1. *P < 0.05 vs control; **P < 0.05 vs. rCX3CL1 or CX3CR1 receptor Ag. Values are means \pm SE, n = 6 in each group.



Figure 4. Direct interaction pericytes with MVEC attenuates MVEC function. MVEC or pericytes alone or MVEC/pericytes 1:1 or 1:4 were seeded on microporous surface of the removable upper chamber (Transwell) and SMC were cultured independently in the lower chamber of a separate Transwell unit. Transwell upper units containing MVEC or pericyte alone or MVEC/pericyte were used in four groups: group 1

= MVEC alone, group 2 = pericyte alone, group 3 = MVEC/pericyte 1:1, and group MVEC/pericyte 1:4 were placed on lower unit containing SMC. The Transwell units containing both chambers that prevents physical contact with cells in upper and lower units were stimulated by adding 5 μ M acethylcholine (Ach) or RPMI 1640 (control) in the upper chamber with or without the presence of NO synthase inhibitor L-NAME (100 μ M) for 60 min at 37 °C. After incubation the cGMP levels in SMC were measured using a cGMP enzyme immunoassay system kit (Amersham) as described in methods. Data represent means ± SE, n = 3 in each group. *P < 0.05 vs. control and **P < 0.05 vs. Ach in respective groups.

um were 0.04 ± 0.003 and 1.1 ± 0.08 , respectively. CX3CL1 protein levels in normoxic and hypoxic medium were 0.07 ± 0.01 and 0.25 ± 0.07 (ng/ml), respectively. As shown in **Figure 2**, exposure to MVEC/hypoxia conditioned medium significantly increased proliferation of pericytes that was attenuated by CX3CL1 neutralizing antibody.

MVEC released CX3CL1-stimulated pericyte proliferation was mimicked by rCX3CL1 and attenuated by CX3CL1 neutralizing antibody or CX3CR1 antagonist

Since capillary pericytes express CX3CR, we confirmed whether rCX3CL1 mimic the effects of MVEC released soluble CX3CL1 on pericyte proliferation. Pericytes were stimulated with rCX3CL1 (2 ng/ml) with or without the presence of anti-CX3CL1 antibody or CX3CR1 antagonist and proliferation was measured by monitoring thymidine incorporation as described in methods. As shown in **Figure 3**, rCX3CL1-stimulated proliferation of pericyte that was attenuated by treatment with anti-CX3CL1 antibody and by CX3CR1 antagonist.

Direct interaction of pericyte but not medium from pericytes with MVEC attenuates MVECmediated NO release and cGMP level in SMC

The increasing interaction of proliferative pericytes with MVEC may lead to dysfunctional microvasculature. To determine the impact of increasing levels of pericytes with MVEC and its function, we used multiple Co-culture models using Transwell system. We also examined the effects medium from pericytes on MVEC function as described in methods. As shown in **Figure 4**, the direct interaction of increasing levels of pericytes with MVEC resulted in





WT but not in CX3CR1 KO mice. WT and CX3CR1 KO mice were exposed to normoxia (N) or hypoxia (H, 10% O₂ for 4 weeks). Lungs were embedded with OCT solution and snap frozen. The cryosections (7 mm) of OCT-embedded lungs were used for immuno staining with anti-NG2 (1:100 dilution, TRITC-red, Santa Cruz Biotechnology) primary antibody and counter stained with DAPI (blue, Life Technologies) and anti- α SM actin (1:100 dilution, FITC-green, Molecular Probe) antibodies as described in methods. The images were taken using EVOS fl (Advanced Microscopy Group) inverted immunofluorescence microscope (A). Quantitave analysis of NG2⁺ pericytes was performed as described in methods (B). Representative images (40×) shown are from three animals in each group. An average number of pericytes from three separate animals are shown in B. Data represent means ± SE, n = 3 in each group. *P < 0.5 vs. WT-N; **P < 0.05 vs. WT-N or CX3CR1 KO-N.



Figure 6. CX3CR1 deficiency attenuates hypoxia-induced pulmonary arterial hypertension and RV hypertrophy. Effects of hypoxia exposure on pulmonary hemodynamics and RV hypertrophy in WT and CX3CR1 KO mice were assessed by echocardiography. WT and CX3CR1 KO mice were exposed to normoxia (N) or hypoxia (H) for 4 weeks. Transthoracic closed-chest echocardiography was performed using 55 MHz Micro Scan transducer and Vevo 2100 Imaging System (Visualsonics). Doppler tracings were recorded and PAT and PET were measured. M-mode and 2-dimensional (2-D) applications were used to measure RV free wall thickness during end diastole using right parasternal long-axis view. Representative images of the pulse-wave Doppler of pulmonary flow and M-mode measurements of RV free wall thickness (marked by arrows) WT-N/Hand KO-N/H are shown in top three panels and quantitative values for PAT, PAT/PET, and RV free wall thickness are shown in the bottom panels, respectively. Data represent means \pm SE, n = 3 mice in eachgroup. *P < 0.05 vs. WT-N in each panel, **P < 0.05 vs. WT-H in each panel.

reduced NO release by MVEC with consequent reduction in cGMP levels in SMC. Achstimulated effects were abolished by L-NAME indicating that inhibition of eNOS activity and thus NO production. When MVEC were incubated with medium from pericytes, Ach-stimulated effects on cGMP levels in SMC were: 10.6 \pm 1.8; 33.2 \pm 4.6; 12.2 \pm 2.9; and 30.9 \pm 3.2 pmol/mg protein in MVEC/RPMI 1640, MVEC/ RPMI 1640/Ach, MVEC/pericyte medium, and MVEC/pericyte medium/Ach, respectively. This suggests that increasing direct interaction of pericytes but not pericyte medium with MVEC leads to MVEC dysfunction.

Hypoxia increased pericyte expression in WT but not in CX3CR1 KO mice

Since hypoxia increases MVEC release of CX3CL1 and CX3CL1/rCX3CL1-stimulation enhanced pericyte proliferation in vitro, we examined whether exposure to hypoxia increas-

es pericyte expression in WT and CX3CR1 KO mice lungs. WT and CX3CR1 KO mice were exposed to normoxia (N) or hypoxia (H, 10% O for 4 weeks). Lungs were embedded with OCT solution and snap frozen. The cryosections (7 mm) of OCT-embedded lungs were used for immuno staining with anti-NG2 antibody as described in methods. As shown in Figure 5A, hypoxia induced expression of NG2⁺ cells, a pericyte specific marker, in the lungs of WT but not in CX3CR1 KO mice. Figure 5B shows quantitative analysis of pericyte expression in WT and CX3Cr1 KO mice exposed to normoxia or hypoxia. As previously reported hypoxia also increased a SM actin expression in the lungs of WT mice (13). However, α -SM actin expression in hypoxia exposed CX3CR1 KO mice was comparable to that with WT normoxic mice.

Hypoxia altered pulmonary hemodynamics in WT but not in CX3CR1 KO mice

To examine the role CX3CL1/CX3CR1 axis in modulation of pulmonary hemodynamics, we used normoxia and hypoxia exposed WT and CX3CR1 KO mice to perform echocardiography using Vevo 2100 imaging system as described in methods. Figure 6 shows representative images of noninvasively recorded pulsed-wave Doppler of pulmonary flow, 2D parasternal short-axis view obtained at the level of the aortic valve, and estimated RV free wall thickness in WT and CX3CR1 KO normoxia/hypoxia exposed mice (top three panels). Pulmonary acceleration time (PAT) and PAT/ejection time (PET) were significantly decreased in WT-hypoxic as compared to WT-normoxic mice. However, PAT and PAT/PET in CX3CR1 KO-normoxic and hypoxic mice were comparable to WT-normoxic mice. Assessments of RV free wall thickness indicate significant increase in WT but not in CX3CR1 KO mice expose to hypoxia (bottom panels).

Discussion

The results of our study demonstrate, for the first time, that hypoxia-stimulation of lung MVEC resulted in over production of CX3CL1 that: i) triggered pericyte proliferation vivo and in vitro, ii) increased pericyte/MVEC interaction leading to MVEC dysfunction, iii) altered pulmonary hemodynamics and RV hypertrophy, and iv) CX3CR1 deficiency, or treatments with CX3CL1 neutralizing antibody or with CX3CR1 antago-

nist attenuated or abolished CX3CL1-mediated modulations. These observations support the notion that CX3CL1/CX3CR1 axis contributes to hypoxia-induced phathophysiologic changes observed in the development of PH.

Chronic hypoxia is known to modulate lung microvascular structure and function observed in PH by multiple mechanisms including increased expression of endothelial-derived CX3CL1 [13, 21]. We recently reported that human lung MVEC responded to alveolar oxygen deficiency by over production of CX3CL1 leading to CX3CL1-mediated MSC proliferation and pulmonary artery pressure elevation in hypoxic mice [13]. Pericytes share a common basal lamina with MVEC and serve many functions including contraction and under stress or injury state pericytes can proliferate, modulate inflammatory events, and alter mechanical integrity of vessel wall [22-24]. Our results demonstrated expression of CX3CR1, a sole receptor for CX3CL1, in pericytes and that hypoxic MVEC-conditioned medium induced pericyte proliferation that was mimicked by rCX3CL1. These observations indicate that the MVEC released CX3CL1 alone or its interaction with pericyte CX3CR1 is critical in hypoxia-induced pericyte proliferation. In fact, using CX3CL1 neutralizing antibody or blocking CX3CR1 by antagonist diminished CX3CL1-stimulated proliferation of pericytes. This observation supports the fact that CX3CL1/CX3CR1 axis contributes to hypoxic-stimulation of pericyte proliferation critical in the regulation of lung microvascular function.

The functional role of mediators including CX3CL1-stimulated proliferating pericytes on lung vascular endothelium has not been fully elucidated. The location of pericytes in lung microvasculature and its interaction with underlying endothelium may be critical in the regulation of contractile function, pulmonary hemodynamics, and vascular remodeling. Our results demonstrate that the hypoxia increases pericyte expression in vitro as well as in the lungs of WT but not CX3CR1 deficient mice. Although the impact of increasing expression of pericyte on MVEC function and pulmonary hemodynamics have not been previously reported, our findings demonstrate direct interaction of pericytes, but not pericyte medium, leads to endothelial dysfunction assessed by monitoring diminished levels of cGMP in SMC

using multiple co-culture models. Vascular endothelium-derived NO and increased cGMP production is critical to maintain sustained vasorelaxation in pulmonary circulation. The precise nature of molecular events involved in MVEC/pericyte interaction that leads to reduce NO/cGMP levels remains to be determined. However, our results are consistent with one of the critical mechanisms associated in the development of PH involving reduced cGMPlinked with the regulation of vascular resistance and vascular remodeling [19, 25]. These findings indicate dual actions of CX3CL1-stimulated pericyte proliferation: i) muscularization of lung microvasculature, and ii) contractile function via MVEC/pericyte interaction to promote MVEC dysfunction.

The results of the present study indicate that hypoxia-induced modulation of pulmonary hemodynamics and RV free wall thickness assessed by noninvasive echocardiography measures in WT but not in CX3CR1 deficient mice. These observations further confirms our previous report demonstrating CX3CL1-mediated proliferation of SMC and significantly lower levels of right ventricular systolic pressure (RSVP), RV hypertrophy, and vascular remodeling in CX3CR1 deficient than those in the WT mice using invasive measures [13]. Collectively these studies provide direct evidence regarding the role of CX3CL1/CX3CR1 axis in hypoxiainduced modulation of pulmonary hemodynamics and vascular remodeling consistent with pathophysiologic changes observed in the development of PH. Since hypoxia/CX3CL1 response was reversed by CX3CR1 deficiency or by use of pharmacologic targeting using CX3CR1 antagonist, the overall implications of understanding the novel role of CX3CL1/ CX3CR1-mediated proliferation of pericytes and SMC in lung capillaries leading to the progression of PH can be of considerable value for the development of therapeutic strategy that will prevent vascular remodeling, improve pulmonary hemodynamics and RV function with an enormous impact for treatment of patients with PH.

Acknowledgements

The authors thank Mr. Bert Herrera and Ms Lin Ai for their technical assistance in tissue culture and animal studies. This work was supported by NIH grant HL085133 and by Merit Review award from the Biomedical Laboratory Research & Development Service of the VA Office of Research and Development Award # I01BX007080 to JMP.

Disclosure of conflict of interest

None.

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