Chemokines regulate small leucine-rich proteoglycans in the extracellular matrix of the pressure-overloaded right ventricle

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Waehre A, Vistnes M, Sjaastad I, Nygård S, Husberg C, Lunde IG, Aukrust P, Yndestad A, Vinge LE, Behmen D, Neukamm C, Brun H, Thaulow E, Christensen G. Chemokines regulate small leucine-rich proteoglycans in the extracellular matrix of the pressure-overloaded right ventricle. J Appl Physiol 112: 1372–1382, 2012. First published February 16, 2012; doi:10.1152/japplphysiol.01350.2011.—Chemokines have been suggested to play a role during development of left ventricular failure, but little is known about their role during right ventricular (RV) remodeling and dysfunction. We have previously shown that the chemokine (C-X-C motif) ligand 13 (CXCL13) regulates small leucine-rich proteoglycans (SLRPs). We hypothesized that chemokines are upregulated in the pressure-overloaded RV, and that they regulate SLRPs. Mice with RV pressure overload following pulmonary banding (PB) had a significant increase in RV weight and an increase in liver weight after 1 wk. Microarray analysis (Affymetrix) of RV tissue from mice with PB revealed that CXCL10, CXCL6, chemokine (C-X3-C motif) ligand 1 (CX3CL1), chemokine (C-C motif) ligand 5 (CCL5), CXCL16, and CCL2 were the most upregulated chemokines. Stimulation of cardiac fibroblasts with some of these chemokines showed that CXCL16 increased the expression of the four SLRPs: decorin, lumican, biglycan, and fibromodulin. CCL5 increased the same SLRPs, except decorin, whereas CX3CL1 increased the expression of decorin and lumican. CXCL16, CX3CL1, and CCL5 were also shown to increase the levels of glycosylated decorin and lumican in the medium after stimulation of fibroblasts. In the pressure-overloaded RV tissue, Western blotting revealed an increase in the total protein level of lumican and a glycosylated form of decorin with a higher molecular weight compared with control mice. Both mice with PB and patients with pulmonary stenosis had significantly increased circulating levels of CXCL16 compared with healthy controls measured by enzyme immunoassay. In conclusion, we have found that chemokines are upregulated in the pressure-overloaded RV and that CXCL16, CX3CL1, and CCL5 regulate expression and posttranslational modifications of SLRPs in cardiac fibroblasts. In the pressure-overloaded RV, protein levels of lumican were increased, and a glycosylated form of decorin with a high molecular weight appeared.

Right-sided heart failure (HF) is a major challenge in the increasing population of grown-ups with congenital heart defects, but few studies have focused on the molecular changes leading to right ventricular (RV) failure (29). While a role for inflammation in left ventricular (LV) failure has been sug-
cells were treated for 24 h with or without chemokines (200 ng/ml). CXCL10 inducible protein (IP)-10, CXCL6, chemokine (C-X3-C motif) ligand 1 (CX3CL1/fractalkine), chemokine (C motif) ligand 5 [CCL5/regulated upon activation, normal T cell expressed and presumably secreted (RANTES)], CXCL16, and CCL2/monocyte chemotactic protein 1 (MCP-1) (all from R&D Systems, Minneapolis, MN).

RNA isolation. Total RNA was extracted from LV and LV tissue and neonatal rat fibroblasts using RNaseasy Mini Kit from Qiagen (Qiagen mini kit, catalog no. 74104, Qiagen). Concentration and purity were measured using NanoDrop (ND-1000, Thermo Fisher Scientific, Wilmington, DE). RNA quality was characterized by measuring RNA integrity number on the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Reverse transcription reactions were performed with iScript Select cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA).

Gene expression profiling and microarray data analysis. Total RNA was isolated from the LV in sham-operated (n = 4) and PB (n = 5) wild-type mice. Preparation of cRNA and the subsequent steps leading to hybridization, washing, and scanning of Affymetrix Ge- nump mouse Genome 430A 2.0 Array (Affymetrix, Santa Clara, CA) were performed according to standard protocols (Affymetrix). Microarray data preprocessing was done using guanine cytosome robust multiarray average analysis (30). Differentially expressed genes were found using significance analysis of microarrays (26). In the analysis, we focused on chemokine coding genes, and multiple testing correction was performed as to account for testing all probes representing such genes. All data are MIAME compliant, and the following link has been created to allow review of the data in Gene Expression Omnibus (GSE30922): http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=zyxhmsmgccwbgdacc=GSE30922.

Quantitative real-time polymerase chain reaction. Real-time polymerase chain reaction (RT-PCR) was carried out in a 7900 HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). Predesigned TaqMan assays (Applied Biosystems) were used to determine gene expression of biglycan (Rn00567229_m1 and Mm00455918_m1), fibromodulin (Rn00589918_m1 and Mm00491215_m1), lumican (Rn00579127_m1 and Mm01248292_m1), decorin (Rn01503161_m1 and Mm00514535_m1), atrial natriuretic peptide (Mm01255747_g1), cardiac muscle myosin heavy chain-7 (Mm00600555_m1), skeletal muscle actin-α1 (Mm00808218_g1), CXCL16 (Mm0469712_m1), CXCL1

| Table 1. Demographic and echocardiographic characteristics of the pulmonary stenosis patient group |

<table>
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<tr>
<th>Sex</th>
<th>Age, mo</th>
<th>PV Peak, m/s</th>
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<td>F</td>
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<tr>
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</tr>
<tr>
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<td>87</td>
</tr>
</tbody>
</table>

F, female; M, male; PV peak, pulmonary valve velocity; pro-BNP, terminal pro-brain natriuretic peptide.

MATERIALS AND METHODS

Ethics. All animal experiments were approved by the Norwegian Animal Research Committee (project no. 110) and confirmed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (National Institutes of Health publication no. 85–23, revised 1996). The part of the study that involved humans was approved by the Regional Committee for Research Ethics in Eastern Norway, Box 113, Blindern, 0318 Oslo, Norway (project no. 1.2006.3272) and conducted according to the ethical guidelines outlined in the Declaration of Helsinki for use of human tissue and subjects. Informed, written consent was obtained from the parents. The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written.

Experimental animal model. Mice were housed in M2 or M3 cages with Bee Kay bedding (Scanbur BK, Nittedal, Norway) in 55% humidity on a 12:12-h light-dark cycle with food pellets (RM1, 801151, Scanbur BK) and water ad libitum. All mice utilized in this study were male and wild-type C57BL/6 obtained from Taconic (Skensved, Denmark), weighing 20–30 g at the time of primary operation. After visualization of the trachea by a midcervical incision, the animals were intubated and connected to a rodent respirator (Minivent, Hugo Sachs Elektronik, Harvard Apparatus, March-Hugstetten, Germany; respiratory rate 150 strokes/min, 300 μl tidal volume) and ventilated with a mixture of 2% isoflurane and 98% oxygen. The recordings were assured by a peak flow velocity over the stenosis of >1–2 m/s, and a RV weight-to-tibial length (TL) ratio of >1.8 mg/mm was included.

Tissue sampling. One week after the primary operation, mice were euthanized by receiving a dose of analgesic (buprenorphine, 0.1 mg/kg) subcutaneously and allowed to recover. In vivo heart function was evaluated in PB mice 7 days after the primary operation by Doppler echocardiography and analyzed with a VIVID 7 echocardiograph (GE Vingmed Ultrasound, Horten, Norway), as previously described (9). During the examination, the animals were sedated by inhalation of 1–2% isoflurane and 98–99% oxygen. The recordings were analyzed with EchoPac PC 3x software (GE Healthcare Technologies, Oslo, Norway). Animals with a significant stenosis, measured by a peak flow velocity over the stenosis of >1.8 m/s, and a RV weight-to-tibial length (TL) ratio of >1.8 mg/mm were included.

Isolation and stimulation of neonatal rat fibroblasts. Primary neonatal fibroblasts were isolated from 1- to 3-day-old Wistar rats (Taconic, Skensved, Denmark), as previously described (10).
Protein isolation and enzymatic digestion. Protein was extracted from RV and LV myocardial tissue samples. Homogenates were prepared in homogenization buffer, and SDS was added to a concentration of 1%. Protein concentration was measured using Micro BCA protein assay kit production no. 23235 (Thermo Fisher Scientific, Rockford, IL) and read on Victor 3 1420 Multilabel Counter (PerkinElmer, Waltham, MA). For the analysis of decorin and biglycan protein core, 40 μg protein were subjected to enzymatic treatment with both chondroitinase ABC (Sigma-Aldrich, St. Louis, MO) and PNGaseF in a protein deglycosylation mix (PNGase F, endo-α-N-acetylgalactosaminidase, neuraminidase, β1–4 galactosidase, and β-N-acetylgalactosaminidase; both from New England BioLaboratories, Beverly, MA). Eight microliters of chondroitinase ABC were added at 37°C overnight to remove chondroitin sulfate or dermatan sulfate side chains from the protein cores of decorin and biglycan. N-linked oligosaccharides attached to the decorin and biglycan core protein were enzymatically released using 4 μl of the deglycosylation enzyme mix containing PNGase F, according to the manufacturer’s instructions. Forty micrograms of proteins were digested with 8 μl keratanase II (Seikagaku Biobusiness, Tokyo, Japan) at 37°C overnight, to remove keratan sulfate (KS) side chains from the protein core of fibromodulin and lumican. This sample was further digested with 4 μl of the deglycosylation enzyme mix containing PNGase F. The reaction was terminated by boiling the samples for 10 min at 100°C.

Western blotting. For Western blotting of SLRPs, 5- to 20-μg samples were mixed with sample buffer and boiled for 5 min. Equal amounts of protein were run on Criterion Precast Gel 4–15% before transferred to polyvinylidene fluoride membranes (GE Healthcare Life Sciences, Uppsala, Sweden) by electrophoretic blotting. Nonspecific bindings to the membranes were blocked with 5% bovine serum albumin for 1 h at room temperature for decorin, lumican, and fibromodulin, while the biglycan membrane was blocked with 1% casein, followed by incubation with anti-fibromodulin (SC-33772; Santa Cruz Biotechnology, Santa Cruz, CA), anti-decorin (AF1060; R&D Systems, Minneapolis, MN), anti-biglycan (ab49701; Abcam, Cambridge, UK), or anti-lumican (AF2745; R&D) overnight at 4°C. The membranes were washed in Tris-buffered saline-Tween 20, followed by species-specific horseradish peroxidase-coupled secondary antibodies in 5% bovine serum albumin added for 1 h. After washing, the blots were developed using the enhanced chemiluminescence ECL Plus Western Blotting Detection System (GE Healthcare, Buckinghamshire, UK) and visualized in the Las-4000 mini from Fujifilm (Japan). Quantification of the protein bands and processing of immunoblots were performed using Image Quant. Membranes were reprobed with actin (SC-8432; Santa Cruz Biotechnology) as a protein loading control.

Patients and control subjects. Nine patients, four of them male [mean age 64 mo (2–186 mo)] were recruited after referral to a tertiary clinic for first-time or follow-up control of moderate to severe pulmonary stenosis (Table 1). All patients were evaluated by continuous Doppler echocardiography. Patients with a higher peak pulmonary valve velocity than 3 m/s were included. This cut-off value was chosen because it represents a gradient above which balloon valvuloplasty can be considered. The patients had a peak velocity across the pulmonary valve ranging from 3.0 to 5.5 m/s, with a mean of 3.7 m/s. All patients had a typical valvular pulmonary stenosis. There were no associated cardiac defects in eight patients, but one patient had a mild supravalvular aortic stenosis with a peak aortic valve velocity at 2.0 m/s. The same patient had Williams syndrome. Two patients had a pulmonary regurgitation classified as mild to moderate. None of the patients had clinical signs of RV failure. Four of the patients underwent right-sided catheterization, and RV pressure ranged from 34 to 63 mmHg, with a mean of 48

### Table 3. Significantly upregulated chemokines (>3.5-fold) in the right ventricle of pulmonary banded vs. sham-operated mice assessed by microarray

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>Synonym</th>
<th>Fold Change</th>
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<tbody>
<tr>
<td>CXCL10</td>
<td>Chemokine (C-X-C motif) ligand 10</td>
<td>IP-10</td>
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IP-10, inducible protein 10; RANTES, regulated upon activation, normal T cell expressed and presumably secreted; MCP-1, monocyte chemotactic protein 1. P < 0.05, after multiple testing correction.
mmHg. The mean value of NH₂-terminal pro-brain natriuretic peptide levels was 28 pmol/l (1.9–87 pmol/l) in the pulmonal stenosis patients, and 6 pmol/l (1–29 pmol/l) in healthy controls (P < 0.05). Signs of infection at clinical examination and/or serum levels of C-reactive protein >10 mg/l were exclusion criteria. Healthy children of hospital staff (n = 4), and a group of children with nevus flammeus without additional chronic illness (n = 5) served as control subjects. All controls [mean age 53 mo (12–146 mo)] had C-reactive protein levels <10 mg/l.

Plasma sampling from humans. Blood samples were collected from the antecubital vein of all of the patients and controls. Blood was collected into sterile ethylene diamine tetra-acetic acid tubes that were immediately placed on melting ice and centrifuged at 2,500 g for 25 min. Plasma samples were frozen at −80°C.

Enzyme immunoassays. Plasma levels of CXCL10, CXCL6, CX3CL1, CCL5, CXCL16, and CCL2 in humans were measured by enzyme immunoassay obtained from R&D systems. Serum levels of CXCL16 were measured by enzyme immunoassay obtained from R&D systems. All analyses were performed in duplicate blinded to clinical information.

Statistical analysis. Data are expressed as group means ± SE. Range is presented for the patient group. For comparison of two groups, the nonparametric Mann-Whitney U-test was employed. Differences between more than two groups were determined by one-way ANOVA with post hoc Tukey test. All tests were employed using a 5% significance level. Statistical analysis was performed using GraphPad Prism 5.

RESULTS

RV hypertrophy following pressure overload due to PB. One week after PB, echocardiographic assessment showed an average peak flow velocity over the stenosis of 2.4 m/s (Table 2). Hypertrophy of the RV was confirmed by an 84% increase in RV weight-to-TL ratio in mice following PB compared with sham-operated mice (Table 2), with no significant change in LV weight-to-TL ratio or lung weight-to-TL ratio. Mice in the PB group showed signs of right-sided HF with an increase in liver weight-to-TL ratio (Table 2). In PB mice, LV diameter in diastole was significantly lower, they had evidence of septal flattening, M-mode-derived LV fractional shortening was increased, but cardiac output was significantly reduced compared with sham-operated mice (Table 2). RVs from mice following PB exhibited a marked increased mRNA levels of markers of cardiac hypertrophy and failure (i.e., atrial natriuretic peptide, cardiac muscle myosin heavy chain-7, and skeletal muscle actin-1) (4) compared with sham-operated mice (Fig. 1).

Microarray analysis identified altered expression of genes encoding chemokines in the pressure-overloaded RV. To examine a potential role for chemokines in matrix remodeling following pressure overload in the RV, we first performed microarray analysis (Affymetrix) of myocardial tissue from PB and sham-operated mice 1 wk after PB to identify the most

Fig. 2. The effects of chemokine C-X-C motif ligand 10 (CXCL10), CXCL6, chemokine (C-X3-C motif) ligand 1 (CX3CL1), chemokine (C-C motif) ligand 5 (CCL5), CXCL16, and CCL2 (200 ng/ml for all) on gene expression of decorin (A), lumican (B), fibromodulin (C), and biglycan (D) (n = 4–6) in myocardial fibroblasts after 24 h of stimulation normalized to GAPDH. The results are means ± SE. *P < 0.05, **P < 0.01, ***P < 0.001 vs. unstimulated fibroblasts.
upregulated chemokines. As shown in Table 3, PB induced a marked upregulation of several chemokines in the RV, including CXC chemokines (i.e., CXCL10/IP-10, CXCL6, and CXCL16), CC chemokines (i.e., CCL2/MCP-1), and the C-X3-C chemokine CX3CL1/fractalkine, with a fold increase, compared with sham-operated mice, ranging from 3.6 (CCL2) to 7.6 (CXCL10).

CXCL16, CX3CL1, and CCL5 stimulate the expression of SLRPs. In our laboratory’s previous studies in mice deficient of the chemokine receptor CXCR5, we showed that CXCR5 activation could be involved in myocardial remodeling by regulating SLRP expression in fibroblasts (28). We hypothesized that the ability to modulate SLRP expression also could be a feature of other chemokines. To elucidate possible pathogenic consequences of the increased chemokine levels in RV following PB, we, therefore, stimulated cardiac neonatal rat fibroblasts with the chemokines found to be upregulated during PB and measured their effect on the gene expression of the SLRPs decorin, lumican, fibromodulin, and biglycan. While CXCL16 (all four SLRPs), CCL5 (lumican, fibromodulin, and biglycan), and CX3CL1 (decorin and lumican) enhanced the expression of these SLRPs, CXCL10 (all four SLRPs) and CXCL6 (fibromodulin and biglycan) downregulated the expression of these regulators of the collagen network (Fig. 2).

Regulation of mRNA levels of SLRPs following PB. Our findings so far suggest a complex interaction between chemokines and SLRPs within myocardial fibroblast, promoting both enhancing and downregulatory effects on their expression. To further examine the relevance of these findings to matrix regulation during PB, we next examined the regulation of SLRPs in the RV following PB. As shown in Fig. 3, PB induced a marked increase in mRNA levels of lumican, biglycan, and fibromodulin, but not of decorin, in the RV compared with sham-operated.

Increased protein levels of lumican and a glycosylated form of decorin in pressure-overloaded RV. To further examine the regulation of SLRPs following PB, we measured protein levels of the same four members of the SLRP family. As depicted in Fig. 4A, Western blots of decorin from RV and LV of sham-operated and PB mice revealed a band at 40 kDa corresponding to the core protein. Sham-operated mice did also have a broad band at 75 kDa in both RV and LV. The LV in PB mice did also have this 75-kDa band, while the RV of PB mice had a higher molecular mass band of decorin at 100 kDa. These data are consistent with identification of decorin with a longer GAG chain in the RV after PB due to posttranslational modifications. As shown in Fig. 4B, both the 75- and the 100-kDa bands were digested by chondroitin sulfatase ABC, known to degrade chondroitin sulfate and dermatan sulfate GAGs as found in decorin, and by PNGase F, known to remove N-linked oligosaccharides, leaving decorin as a major band at 40 kDa in all groups (2, 3, 8). Quantification of this core protein band at 40 kDa after enzymatic digestion revealed no significant differences in the protein level between PB and sham-operated mice in either RV or LV.

As shown in Fig. 5A, Western blot analysis of lumican resulted in the typical proteoglycan smear at 60–90 kDa in RV and LV in both sham-operated and PB mice, characteristic of lumican with KS chains (20). After digestion with keratanase II (to remove KS) and PNGase F (to remove N-linked oligosaccharides) the 60- to 90-kDa glycosylated band disappeared, whereas a 40-kDa band, corresponding to the core protein, was evident in both groups (Fig. 5B). Quantification of this band showed a twofold increase in the RV after PB. In the LV, PB induced no increase in the protein level of lumican. In contrast to the upregulation of lumican and posttranslational modulation of decorin in RV following PB, we observed no differences in the protein level of fibromodulin and biglycan in the RV or LV after PB. For both of these SLRPs, Western blot analysis revealed a major band at 67 kDa, indicating a population with N-linked oligosaccharides, and a small subpopulation of free core protein at 40 kDa in RV and LV for both sham-operated and PB mice (3). Quantification of the relative protein level at the 67-kDa band (1.00 ± 0.06 vs. 0.89 ± 0.12 for the RV, and 0.97 ± 0.03 vs. 0.99 ± 0.11 for the LV) and the relative protein level at the 40-kDa band of fibromodulin (1.00 ± 0.12 vs. 0.86 ± 0.10 for the RV, and 1.07 ± 0.03 vs. 0.98 ± 0.09 for the LV) revealed no differences between sham-operated and PB mice. Quantification of the relative protein level at the 67-kDa band (1.00 ± 0.12 vs. 0.91 ± 0.10 for the RV, and 1.10 ± 0.12 vs. 1.12 ± 0.10 for the LV) and the relative protein level at the 40-kDa band for biglycan (1.00 ± 0.12 vs. 0.87 ± 0.16 for the RV, and 0.97 ± 0.16 vs. 0.89 ± 0.03 for the LV) revealed no differences between sham-operated and PB mice.

CXCL16, CX3CL1, and CCL5 increase the level of glycosylated decorin and lumican. Our findings suggest that PB is accompanied by altered posttranslational modification of decorin and by altered expression of lumican (protein levels) in the RV. To further characterize the interaction between chemokines and SLRPs in the RV following PB, we examined the ability of the three chemokines that enhanced the expression of these two SLRPs at the mRNA levels (i.e., CXCL16, CX3CL1,
and CCL5) to enhance the protein levels of decorin and lumican. As shown in Fig. 6A, CXCL16 and CX3CL1, but not CCL5, significantly increased the protein levels of glycosylated decorin at 75 kDa in the medium of neonatal rat fibroblast after culturing for 24 h. However, we did not see a 100-kDa band of decorin as was found in the RV following PB. The protein level of glycosylated lumican (60–90 kDa) was also significantly increased in the medium following stimulation of fibroblasts with CXCL16, CX3CL1, and CCL5 for 24 h (Fig. 6B).

Expression of CXCL16, CX3CL1, and CCL5 within the murine hearts. To further characterize the regulation of these chemokines in RV following PB, we analyzed mRNA expression by quantitative RT-PCR, not only in the RV, but also in the LV after PB. We confirmed the data obtained with microarray in RV tissue and found significantly enhanced myocardial expression of CXCL16, CX3CL1, and CCL5 in the RV of mice that underwent PB compared with sham-operated mice (Fig. 7, A–C). In contrast, no increase in mRNA levels of these chemokines was found in the LV following PB, suggesting that the upregulation of these chemokines within the myocardium is directly related to RV pressure overload. To further characterize the regulation of these chemokines within the myocardium, we analyzed the expression of these chemokines within the myocardium in endothelial cells, cardiomyocytes, and myocardial fibroblasts, separately, in sham-operated mice. While the expression of these chemokines in cardiomyocytes was rather low, CXCL16 was highly expressed in fibroblasts and endothelial cells (Fig. 7D), CX3CL1 had the highest expression in endothelial cells (Fig. 7E), and CCL5 was highly expressed in fibroblasts (Fig. 7F). Although we have no data on the cellular localization of these chemokines during PB, the strong expression of these chemokines, and in particular CXCL16 and CX3CL1, in myocardial fibro-

Fig. 4. Protein levels of decorin in the RV and left ventricle (LV) of mice 7 days after PB (n = 7) compared with sham (n = 7). The data are shown as representative immunoblots and means ± SE (bar graphs). The immunoblots show samples without (A) and with (B) chondroitinase ABC and PNGase F treatment. The bar graphs in A show relative protein level of glycosylated decorin at 75 and 100 kDa, as well as the core protein at 40 kDa. The bar graph in B shows the relative protein level of the core protein at 40 kDa after enzymatic digestion. All protein levels are normalized to actin. ***P < 0.001 vs. RV sham group.
blasts may be relevant to our findings of enhancing effects of these chemokines on the expression decorin and lumican in fibroblasts in vitro.

**mRNA levels of leukocyte cell-surface gene markers in the pressure-overloaded RV.** Chemokines are known to play an important role in leukocyte infiltration into inflamed tissue, and activation of these cells could also potentially modulate SLRP expression. We, therefore, next examined the expression of leukocyte infiltration into RV and LV following PB by examining mRNA levels of CD45R, CD3, CD4, and CD8 as a pan-marker of leukocytes, marker of T cells, and markers of CD4+ and CD8+ T cells, respectively. As shown in Fig. 8, PB induced an upregulation of CD45R (A), CD3 (B), CD4 (C), and CD8 (D) in the RV, with a 2.5- to 3.5-fold increase compared to the sham group.  

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**Fig. 5.** Protein levels of lumican in the RV and LV of mice 7 days after PB (n = 7) compared with sham (n = 7). The data are shown as representative immunoblots and means ± SE (bar graphs). The immunoblots show samples without (A) and with (B) keratanase II and PNGase F treatment. The bar graph in A shows the relative protein level of glycosylated lumican at 60–90 kDa and in B shows the relative protein level of the core protein at 40 kDa after enzymatic digestion. All protein levels are normalized to actin. *P < 0.05 and **P < 0.01 vs. RV sham group.

**Fig. 6.** The effect of CXCL16, CX3CL1, and CCL5 (200 ng/ml for all) stimulation on the protein level of decorin (n = 4; A) and lumican (n = 4; B) in the medium of neonatal cardiac rat fibroblasts after 24-h stimulation. The results are means ± SE. *P < 0.05 and **P < 0.01 vs. unstimulated fibroblasts.
with sham-operated mice. In contrast, none of these markers was increased in the LV following PB (Fig. 8).

Circulating levels of selected chemokines in patients with pulmonary stenosis. To relate our findings to clinical RV failure, we measured plasma levels of the chemokines that were upregulated in RV following PB in pulmonary stenosis patients \((n = 9)\). Interestingly, these patients had significantly raised plasma levels of CXCL16, but none of the other measured chemokines, compared with age- and sex-matched healthy controls \((n = 9)\) (Fig. 9, A–F). CXCL16 had enhancing effects of both decorin and lumican levels in myocardial fibroblasts, and, notably, plasma levels of CXCL16 were also markedly upregulated in PB compared with sham-operated mice (Fig. 9G). However, owing to shortage of sample volumes, plasma levels of CCL5 and CXC3CL1 were not analyzed in the mice model.

**DISCUSSION**

Despite observations of enhanced levels of chemokines in human HF, the role of chemokines in altering cardiac structure and function in right-sided HF has not been established. The present work demonstrates that chemokines may be involved in RV remodeling by regulating SLRPs. The key results of this study were as follows: 1) increased expression of several chemokines in RV following PB; 2) enhancing effect of CXCL16, CX3CL1, and CCL5 on mRNA levels of several SLRPs in myocardial fibroblasts; 3) increased posttranslation modification of decorin and enhanced protein levels of glycosylated lumican in RV following PB; 4) enhancing effects of CXCL16, CX3CL1, and CCL5 (only lumican) on protein levels of glycosylated decorin and lumican in myocardial fibroblasts; and 5) increased plasma levels of CXCL16 in clinical (pulmonary stenosis) and experimental (PB) RV failure. Our findings suggest a link between chemokines, and in particular CXCL16 and matrix remodeling, in RV following pressure overload, potentially involving regulation of SLRPs and, in particular, decorin and lumican.

To our knowledge, this is the first report of increased levels of chemokines in the pressure-overloaded RV. Elevated myocardial and circulating levels of CCL2 \((19)\), CXCL16 \((5)\), and CX3CL1 \((11)\) have previously been shown in LV HF patients. Moreover, we have shown that CXCL9 is increased in the circulation of PB mice \((27)\), whereas CXCL16 \((5)\) and CCL2 (MCP-1) \((24)\) have been reported to be upregulated in experimental pressure-overloaded LV. In this study, we clearly show enhanced cardiac expression of chemokines in right-sided HF,
potentially linked to altered expression of SLRPs, suggesting a role for these inflammatory mediators in the development and progression of right-sided myocardial remodeling during pressure overload.

We have recently suggested that chemokines regulate the expression of SLRPs produced by myocardial fibroblasts, demonstrated by induction of SLRP expression following CXCL13 stimulation (28). In the present report, we show that CXCL16, CX3CL1, and CCL5 were able to enhance the expression of SLRPs in vitro in myocardial fibroblast, suggesting an association between these chemokines and the regulation of the ECM quality within the myocardium. Thus, CXCL16, CX3CL1, and CCL5 were shown to enhance the expression of lumican in myocardial fibroblasts, both at mRNA and protein level, with increased levels of glycosylated lumican in the medium after stimulation of fibroblasts. As for decorin, the regulation seems to be more complex. Thus we found alterations compatible with a glycosylated form of decorin with a higher molecular weight in the RV after PB. By stimulating rat fibroblast, we found that both CXCL16 and CX3CL1 increased the protein level of glycosylated decorin measured in the fibroblast medium after stimulation. However, these chemokines did not induce elongation of the decorin GAG chain after stimulation, potentially reflecting differences between studies in fibroblast cell culture in vitro and the more complex situation in vivo where different cells and mediators are operating in a complex network.

The possible role of chemokines in the pathogenesis of HF has, at least in part, been attributed to their ability to promote leukocyte infiltration in failing myocardium. In the present study, we found increased expression of CD45R, CD3, CD4, and CD8 in the RV after PB, indicating increased infiltration of leukocytes. One could speculate that, in addition to the direct effects of chemokines on fibroblasts inducing SLRP expression and posttranslational modification, activated leukocytes may potentially also modulate SLRP expression in vivo.

Although there are increasing amounts of data on SLRPs in general, their cardiac function is not well studied. To our knowledge, our laboratory’s previous report (28) was the first to demonstrate an increase in the protein level of decorin and lumican in LV following aorta banding. Our findings in the present study suggest that upregulation of these SLRPs is not restricted to pressure overload in LV, but seems also to be a characteristic of myocardial remodeling following RV pressure overload. We do not know at which level SLRPs exactly act in the fibril assembly. However, Kalamajski and Oldberg (15) suggest that SLRPs, such as lumican, may regulate the intermolecular cross-linking of collagen. It is tempting to speculate that the need for lumican for cross-link formation and collagen assembly in the heart is increased in the pressure-overload model. As for decorin, our findings may suggest altered glycosylation with the expression of a higher molecular weight form after PB, presumably reflecting elongation of the GAG chain. ECM structure depends both on the collagen-regulating...
activity of SLRPs mediated by the core protein, and by the GAG chains, which are thought to maintain interfibrillar space (7, 16). Kuwabe and coworkers showed elongated decorin GAG chain, which extended across enlarged gaps between collagen fibrils in healing skin (16). It is possible that modulation of the GAG chain of decorin is important in cardiac remodeling by organizing the distance of collagen fibrils.

Our studies in patients with pulmonary stenosis suggest that our findings in experimental PB may have relevance to clinical RV pressure overload. We showed enhanced circulating protein level of CXCL16 in patients with pulmonary stenosis, as well as in the murine model of PB. These findings are in accordance with a previous study showing that CXCL16 was upregulated in the circulation of grown-up patients with chronic HF, as well as in the LV from both mice with myocardial infarction and LV pressure overload (5). The present study group of children is relatively small and heterogeneous with respect to age and severity of pulmonary stenosis, which also might be reflected in the variable brain natriuretic peptide levels. Thus other chemokines than CXCL16 may be significantly altered if larger and more homogenous groups of patients are studied. Such studies could also include sampling from coronary sinus to potentially assess the myocardial contribution to the systemic chemokine levels.

In summary, our findings suggest a link between chemokines, such as CXCL16, CXC3CL1, and CCL5, and certain SLRPs (i.e., decorin and lumican), potentially contributing to myocardial remodeling during RV pressure overload. It is possible that SLRPs and chemokines, both important regulators of ECM in inflammatory processes, are involved in the dynamic process of ECM remodeling, potentially representing a missing link between inflammation and matrix remodeling during RV HF. A better understanding of the complex role of SLRPs in the normal and failing myocardium may facilitate the development of targeted anti-remodeling strategies. However, further studies are needed to elucidate these issues. Such studies could include larger clinical studies in patients with RV overload, as well as studies in genetically modified animal models.

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Fig. 9. Plasma levels of CXCL16 (A), CX3CL1 (B), CCL2 (C), CCL5 (D), CXCL6 (E), and CXCL10 (F) in 9 patients with PS and 9 age-matched and sex-matched healthy controls (CTR). Values are means ± SE. *P < 0.05 vs. CTR. G: serum levels of CXCL16 in sham (n = 7) and PB (n = 7) groups. The results are means ± SE. *P < 0.05 vs. sham.
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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

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