Increased production of CXCL16 in experimental and clinical heart failure; a possible role in extracellular matrix remodeling

Christen Peder Dahl, MD1,2,7; Cathrine Husberg, PhD6,7; Lars Gullestad, MD, PhD2,7; Anne Wæhre, MD6,7; Jan Kristian Damås, MD, PhD1,3; Leif Erik Vinge, MD, PhD5; Alexandra V. Finsen, MD, PhD1,2,6,7; Thor Ueland, PhD1,4; Geir Florholmen, PhD6,7; Svend Aakhus, MD, PhD2; Bente Halvorsen, PhD1; Pål Aukrust, MD, PhD1,3; Erik Øie, MD, PhD1,2,7; Arne Yndestad, PhD1,7; Geir Christensen, MD, PhD6,7

1Research Institute for Internal Medicine, 2Department of Cardiology, 3Section of Clinical Immunology and Infectious Diseases, 4Section of Endocrinology, 5Institute for Surgical Research, Rikshospitalet University Hospital, University of Oslo, 6Institute for Experimental Medical Research, Ullevål University Hospital, 7Center for Heart Failure Research, University of Oslo, Oslo, Norway.

Short title: CXCL16 in heart failure

Word count: 5978

Addresses for correspondence:
Pål Aukrust, MD, PhD
Section of Clinical Immunology and Infectious Diseases, Rikshospitalet University Hospital, N-0027 Oslo, Norway
Phone: +47-23070000, Fax: +47-23070000, e-mail: pal.aukrust@rikshospitalet.no

Geir Christensen, MD, PhD
Institute for Experimental Medical Research, Ullevål University Hospital, Kirkeveien 166, N-0407 Oslo, Norway
Phone: +47-23016800, Fax: +47-23016799, e-mail: geir.christensen@medisin.uio.no
Abstract

Background: Inflammation has been implicated in the pathogenesis of heart failure (HF), but knowledge about production and role of inflammatory actors remain incomplete. Based on its role in vascular inflammation, vascular proliferation and matrix degradation we hypothesized a role for the chemokine CXCL16 in the pathogenesis of myocardial remodeling and development of HF.

Methods and Results: Our main findings were: (i) Patients with chronic HF (n=188) had raised plasma levels of CXCL16, which correlated with disease severity. (ii) Left ventricular (LV) tissue from patients with end-stage HF (n=8) showed enhanced CXCL16 levels compared to non-failing LV (n=6) as assessed by Western blotting. (iii) In mice with post-myocardial infarction HF, expression of CXCL16, as assessed by real-time RT-PCR, was increased in the infarcted and the non-infarcted areas of LV 3 and 7 days after coronary ligation, indicating early onset of CXCL16 production. Also, mice exposed to aortic banding had enhanced CXCL16 expression in LV, indicating that CXCL16 expression is not only related to ischemia. (iv) In vitro, CXCL16 promoted proliferation and impaired collagen synthesis in myocardial fibroblasts, and in cardiomyocytes and myocardial fibroblasts, CXCL16 increased MMP activity, primarily reflecting increased MMP-2 levels. (v) Using specific inhibitors, we showed that the effect of CXCL16 on fibroblasts involved activation of Jun N-terminal kinase.

Conclusion: We show enhanced myocardial CXCL16 expression in experimental and clinical HF. The effect of CXCL16 on cardiomyocytes and fibroblasts suggest a role for CXCL16 in matrix remodeling and ultimately also in the development of HF.

Key words: heart failure, inflammation, metalloproteinases, remodeling, CXCL16
Introduction

Chronic heart failure (HF) is a progressive disorder in which dysregulation of several physiological systems contributes to its pathogenesis. HF is, despite state-of-the-art treatment, characterized by high morbidity and mortality, indicating that important pathogenic mechanisms remain active and unmodified by the present treatment modalities. It has been suggested that persistent inflammation could represent one such unmodified mechanism, contributing to the myocardial remodeling process characterizing chronic HF. However, although both experimental and some clinical studies indicate a pathogenic role for inflammation in the development of myocardial failure, the role of cytokine dysregulation in human HF as well as the identification and characterization of the different inflammatory actors need to be further elucidated.

CXCL16 is an exceptional chemokine since it is expressed as a multi-domain molecule consisting of a chemokine that is linked to a mucin-like stalk followed by a transmembrane segment and a short cytoplasmic tail, similar only to CX3CL1. As a transmembrane molecule on the surface of cells, CXCL16 can interact with its receptor CXCR6, promoting tissue-homing and activity of various leukocyte subsets. Within the vasculature CXCL16 has been located on endothelial cells, smooth muscle cells (SMC), and macrophages, and up-regulation has been found under inflammatory conditions such as atherosclerosis, consistent with a role in vascular inflammation. However, growing evidence from in vitro studies suggest that the function of CXCL16 is not exclusively limited to leukocyte recruitment. Thus, while the chemokine domain of CXCL16 can act as a classical chemoattractant for various leukocyte subsets, recent studies suggest that soluble CXCL16, that is shed from the cell surface by the metalloproteinase ADAM10, may induce proliferation of vascular SMC as well as matrix degradation.
myocardial remodeling process characterizing chronic HF. At present, there are no data on the production and possible effects of CXCL16 within the myocardium, but based on its combined role in vascular inflammation, vascular proliferation and matrix degradation we hypothesized that CXCL16 could be involved in the pathogenesis of myocardial remodeling and development of HF. In the present study our hypothesis was examined by different approaches including studies in clinical and experimental HF as well as in vitro experiments using cardiomyocytes and myocardial fibroblasts.

**Methods**

**HF patients**

One hundred and eighty-eight patients with stable HF for >6 months in New York Heart Association (NYHA) functional class II-IV were consecutively included in the study (Table 1). Blood samples (platelet-poor EDTA plasma) were collected and stored as previously described. Details are given in the online Data Supplement.

**Mouse models of experimental HF**

To study production of CXCL16 in the myocardium during HF development, we used mouse models with HF following aortic banding and myocardial infarction (MI). The mouse models allow measurements in the myocardium of CXCL16 gene expression and protein content at various time points during early stages of HF development. Moreover, using these models we were able to relate CXCL16 production more reliably to the HF etiologies ischemia and pressure overload. Details are given in the online Data Supplement.

**CXCL16 production in isolated cells following MI**

In a separate set of experiments, we investigated expression of CXCL16 in cardiomyocytes and non-cardiomyocytes isolated from rat hearts 7 days following MI. Details are given in the online Data Supplement.
Isolation and stimulation of neonatal cardiomyocytes and myocardial fibroblasts

Primary neonatal cardiomyocytes and fibroblasts were isolated from 1 – 3 days old Wistar rats (Taconic, Skensved, Denmark). Details are given in the online Data Supplement.

Protein synthesis assessed by $[^3H]$-leucine incorporation

Cardiomyocytes and myocardial fibroblasts were plated on 24-well culture dishes (Corning Int.), and after 24 hours with or without recombinant CXCL16 treatment (200 ng/ml, chemokine domain of mouse CXCL16; R&D Systems), the culture medium was supplemented with 5 μCi/ml $[^3H]$-leucine (American Radiolabel Chemicals, St. Louis, MO) and further incubated for 24 hours. Cardiomyocytes were fixed and washed in chilled 95% ethanol and solubilized in 0.2 mol/l NaOH as previously described. The total protein-associated radioactivity was measured in a TRI-CARB 2300 TR Scintillation Counter (Packard, Meriden, CT).

Cardiomyocyte measurements

Cardiomyocyte size was measured as previously described and analyzed with ImageJ downloaded at http://rsb.info.nih.gov/ij/.

Zymography

Gelatinolytic activity was detected in conditioned media from fibroblasts and cardiomyocytes after incubation for 24 and 48 hours, respectively. Details are given in the online Data Supplement.

Real-Time quantitative RT-PCR

Total RNA was extracted by using acid-phenol extraction in the presence of chaotropic salts (TRIzol, Invitrogen, San Diego, CA) and subsequent isopropanol-ethanol precipitation. All total RNA samples were subjected to DNase I treatment (RQ1 DNase; Promega, Madison, WI) and stored in RNA storage solution (Ambion, Austin, TX) at -80°C. Sequence-specific PCR primers for CXCL16, matrix metalloproteinase (MMP)-2, MMP-9, alpha-skeletal actin,
atrial natriuretic peptide (ANP), and brain natriuretic peptide (BNP) were designed with Primer Express software version 1.5 (Applied Biosystems, Foster City, CA). Primer sequences can be provided on request. Quantification of mRNA was performed with ABI Prism 7500 (Applied Biosystems). Gene expression of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used for normalization (Applied Biosystems).

**Immunohistochemistry**

Immunohistochemical analysis was performed on left ventricular (LV) myocardial tissue from human cardiac explants (n=2) and non-failing donor hearts (n=2) as previously described. Details are given in the online Data Supplement.

**Western blotting**

Western blotting was performed as described previously, with minor modifications. Details are given in the online Data Supplement.

A description of tissue sampling from human myocardium, multiplex suspension array, fibroblast proliferation, [3H]-proline incorporation, total MMP activity, enzyme immunoassay, ethics, and statistical analysis is given in the online Data Supplement.

**Results**

**Plasma levels of CXCL16 in human HF**

To examine whether CXCL16 is up-regulated in human HF, we first measured the plasma levels of CXCL16 in HF patients (n=188) and in sex- and age-matched healthy individuals (n=20). As shown in Figure 1A, the patients with the most severe HF (i.e., NYHA class III/IV) had significantly raised CXCL16 levels as compared with healthy controls and patients in NYHA class II, with no difference between NYHA class III and IV (data not shown).
Moreover, in the patient group as a whole, we found that CXCL16 levels were negatively correlated with LV ejection fraction (LV-EF) \(r = -0.30, P<0.02\) and positively correlated with plasma levels of N-terminal pro-BNP (Nt-proBNP) \(r = 0.30, P<0.02\). Within NYHA class II, we found significantly raised CXCL16 levels in those with ischemic as compared to those with non-ischemic cardiomyopathy (Figure 1B). In contrast, no difference in CXCL16 levels was found when comparing these two etiological subgroups in NYHA class III and IV (Figure 1B).

**The expression of CXCL16 in failing human myocardium**

We then examined whether the human myocardium itself could produce CXCL16 during HF. By using immunohistochemistry, we found CXCL16 immunoreactivity in cardiomyocytes, vascular SMC, and endothelial cells (Figure 2). Immunostaining of its corresponding receptor CXCR6 was observed in the same types of cells and in addition, fairly strong CXCR6 immunostaining was seen in vimentin-positive fibroblasts (Figure 2). To obtain a more quantitative assessment of the production of CXCL16 and CXCR6 in the failing human myocardium, we analyzed the protein levels of these components in LV myocardial tissue by Western blotting. As depicted in Figure 3, protein expression of CXCL16 and CXCR6 were up-regulated in failing \(n=8\) as compared with non-failing \(n=6\) myocardium although the increase in CXCR6 did not reach statistical significance. The same pattern was seen in ischemic \(n=5\) and non-ischemic \(n=3\) cardiomyopathy (data not shown).

**Gene expression of CXCL16 in experimental HF**

Mouse models of experimental HF allow us to study the myocardial expression of CXCL16 at an early stage in the development of HF. We therefore next examined the expression of CXCL16 by means of real-time RT-PCR in two experimental mouse models of HF, i.e., post-MI HF and HF secondary to pressure overload (aortic banding), representing two important etiological subgroups of HF. As depicted in Figure 4A, we found that mice with post-MI HF
had markedly elevated levels of CXCL16 mRNA in both the ischemic and the non-ischemic region of the LV already 3 days following MI compared with the LV in the sham operated group. Increased expression was also found at 7 and 21 days after MI, with particularly high expression in the infarcted area (7 days, Figure 4A). Also in mice subjected to LV pressure overload, the level of CXCL16 mRNA was increased, indicating that enhanced expression of CXCL16 in the failing myocardium is not only related to ischemia (Figure 4B). While both mice with decompensated and compensated hypertrophy had significantly enhanced expression of CXCL16 as compared with the sham group, particularly high levels were found in those with overt HF (i.e., decompensated myocardial hypertrophy) (Figure 4B). While mRNA levels of CXCL16 in the non-ischemic part of LV (post-MI HF) was quite similar to the myocardial expression of CXCL16 in mice with HF secondary to aortic banding, the myocardial CXCL16 response seem to be markedly increased within the infarcted area in post MI HF rats, potentially reflecting the involvement of CXCL16 in repair processes (Figure 4A-B).

Gene expression of CXCL16 in cardiomyocytes and non-cardiomyocytes during experimental post-MI HF

To examine if the increased expression of CXCL16 during experimental HF was localized to cardiomyocytes or other myocardial cells, we examined the mRNA level of CXCL16 in a rat model of post-MI HF, allowing us to separate the non-ischemic LV from HF rats (n=7) and sham rats (n=7) into cardiomyocytes and non-cardiomyocytes (primarily consisting of fibroblasts) 7 days after the induction of MI or sham operation. As shown in Figure 5, both cardiomyocytes and non-cardiomyocytes showed increased expression of CXCL16 in non-ischemic LV in post-MI rats as compared with sham rats.

Regulation of CXCL16 in neonatal rat cardiomyocytes

Given the increased expression of CXCL16 in cardiomyocytes during post-MI HF, we next
examined factors that might be responsible for varying regulation of CXCL16 in these cells using a well established neonatal rat cell system. The expression of CXCL16 was measured by means of real-time quantitative RT-PCR after stimulating cardiomyocytes for 24 hours with various stimuli with relevance to HF, such as the inflammatory cytokines tumor necrosis factor [TNF]α, interleukin [IL]-1β and interferon [IFN]-γ, mediators with potential hypertrophic effects such as leukemia inhibitory factor [LIF] and endothelin [ET]-1, neurohormones such as noradrenaline, and toll-like receptor (TLR)2 and TLR4 agonists.\textsuperscript{2,3} ET-1, noradrenaline, and the hypertrophic cytokine LIF did not regulate CXCL16 expression. In contrast, TNFα, IL-1β, and IFNγ as well as TLR2 and TLR4 activation induced increased CXCL16 mRNA levels, with a particular enhancing effect of IL-1β (online Data Supplement Figure 1).

**Effects of CXCL16 on neonatal rat cardiomyocytes and myocardial fibroblasts**

To study potential pathogenic consequences of the enhanced CXCL16 expression during development of HF, we examined effects of CXCL16 on neonatal rat cardiomyocytes and myocardial fibroblasts, both shown to express significant amounts of CXCR6 as assessed by western blotting (Figure 6A). In cardiomyocytes, CXCL16 promoted an increase in protein synthesis as assessed by a modest, but significant increase in [\textsuperscript{3}H]-leucine incorporation (Figure 6B). However, CXCL16 had no effect on size (area; \(1300 \pm 174 \ \mu m^2\) vs \(1475 \pm 307 \ \mu m^2\)) or expression of hypertrophy markers (BNP [\(1.05 \pm 0.05\) vs \(0.92 \pm 0.05\)], ANP [\(1.02 \pm 0.05\) vs \(0.98 \pm 0.04\)], alpha-skeletal actin [\(1.08 \pm 0.06\) vs \(1.13 \pm 0.02\)] in cardiomyocytes (data are given as CXCL16 stimulated and un-stimulated, respectively). In fibroblasts, CXCL16 markedly increased cell proliferation as assessed by [\textsuperscript{3}H]-thymidine incorporation (Figure 6C). To examine the signaling pathways involved in the effects of CXCL16 on myocardial fibroblasts, showing the most prominent cellular response to CXCL16, we examined the effect of CXCL16 on fibroblasts by multiplex suspension array technology that detects
phosphorylation of intracellular signaling molecules. CXCL16 induced enhanced phosphorylation of JNK and p38 MAPK, and to some degree also of ERK 1/2 in fibroblasts after 10 minutes of stimulation (Figure 7A-C). Moreover, blocking of JNK, but not of MAPK/ERK (data not shown), totally abolished the enhancing effect of CXCL16 on fibroblast proliferation (Figure 7D). There were no differences in cell toxicity between cell cultures with and without the JNK and MAPK/ERK blockers (LDH leakage assay, data not shown).

The effect of CXCL16 on MMP activity

Matrix degradation plays an important role in myocardial remodeling and we have previously shown enhanced expression of MMPs in CXCL16 stimulated vascular SMC. To elucidate if similar effects potentially could be seen within the myocardium, we examined MMP activity in myocardial fibroblasts and neonatal rat cardiomyocytes that had been exposed to CXCL16 for 24 and 48 hours, respectively. CXCL16 induced a significant increase in total MMP activity in both fibroblasts and cardiomyocytes (Figure 8A-B). The CXCL16-induced increase in total MMP activity was accompanied by an increase in MMP-2, but not in MMP-9, levels as assessed by EIA (Figure 8C-D), and in fibroblast, also by an increase in gelatinolytic activity (Figure 8E), suggesting that the CXCL16-mediated increase in MMP activity at least partly reflects increased MMP-2 levels. In cardiomyocytes, low or undetectable gelatinolytic activity was seen in both un-stimulated and CXCL16 stimulated cells. In contrast to the effect on protein levels, CXCL16 had no effect on mRNA levels of MMP-2 and MMP-9 in either fibroblasts or cardiomyocytes (data not shown), suggesting that the CXCL16-mediated induction of MMP activity, or at least MMP-2, is regulated at the post-transcriptional level.

CXCL16 down-regulates proline incorporation in myocardial fibroblasts

Our findings suggest that CXCL16 may influence myocardial extracellular matrix remodeling. To further elucidate these issues we examined the ability of CXCL16 to modulate
proline incorporation in myocardial fibroblasts as a marker of collagen synthesis in these cells. While CXCL16 had no significant effect on leucine incorporation, CXCL16 significantly down-regulated proline incorporation, suggesting an attenuating effect of CXCL16 on collagen synthesis in myocardial fibroblasts (online Data Supplement Figure II).

**Discussion**

In the present study, we demonstrate enhanced myocardial expression of CXCL16 in both experimental and clinical HF. In HF patients this increase was accompanied by elevated plasma levels of CXCL16, which significantly correlated with LV-EF and Nt-proBNP levels, both well-known markers for disease severity and predictors of outcome in HF. In a mouse model of post-MI HF, we found significantly increased gene expression of CXCL16 in both the ischemic and non-ischemic part of the LV as early as 3 days after MI. In a mouse model of pressure overload without ischemia (aortic banding), we also found markedly enhanced expression of CXCL16 in both compensated and particularly in decompensated hypertrophy. In the failing human myocardium, the up-regulation of CXCL16 was accompanied by significant immunostaining of its corresponding receptor CXCR6 in cardiomyocytes and in fibroblasts. Our *in vitro* experiments suggest that CXCL16 could induce enhanced protein synthesis and MMP activity in cardiomyocytes and promote a proliferating and MMP activating phenotype in myocardial fibroblasts, accompanied by inhibition of collagen synthesis. If similar CXCL16/CXCR6 interactions also are operating *in vivo* within the failing myocardium, it could contribute to the pathological myocardial remodeling characterizing the development of HF.

Previously, raised levels of soluble CXCL16 have been reported in various autoimmune disorders, and although some discrepancies exist, increased plasma levels have also been found in coronary artery disease (CAD). Here, we present novel evidence
that also HF patients are characterized by significantly raised plasma levels of CXCL16, with increasing levels according to disease severity as assessed by clinical, hemodynamic, and neurohormonal parameters. Whereas HF patients classified as NYHA II with CAD etiology had significantly elevated CXCL16 levels, those with dilated cardiomyopathy (DCM) had plasma levels of CXCL16 similar to healthy controls. These results may support earlier findings of increased CXCL16 levels in CAD, but our study also indicates that the raised plasma levels of CXCL16 in HF do not merely reflect accompanying atherosclerosis. Hence, in more severe HF, CXCL16 levels were markedly increased in both ischemic and non-ischemic cardiomyopathy, not only in plasma, but also within the failing myocardium. Based on the plasma data, showing no (DCM) or a modest (CAD) increase in NYHA class II, one could suggest that CXCL16 is not a pathogenic mediator at the early stage of HF. However, plasma levels may not necessarily reflect the myocardial expression of CXCL16, and our data from experimental HF may suggest an early up-regulation of CXCL16 within the failing myocardium. Nonetheless, future studies should more precisely address the role CXCL16 at the early stage of human HF.

Production of CXCL16 in myocardial tissue has to our knowledge not previously been demonstrated, but Yamauchi et al.\textsuperscript{24} showed CXCL16 expression in endothelial cells of the cardiac valves during murine development. On the other hand, CXCL16 expression in human cardiac valves was undetectable in the absence of valvular inflammation.\textsuperscript{24} Herein we show enhanced expression of CXCL16 accompanied by significant immunostaining of its corresponding receptor within the failing human myocardium. We further demonstrate markedly elevated levels of CXCL16 mRNA in two different experimental mouse models of HF. Mice with post-MI HF showed a significant and early increase in gene expression of CXCL16 in both the ischemic and the non-ischemic region of the LV. Moreover, the increase in myocardial CXCL16 expression in mice subjected to pressure overload shows that
atherosclerosis or ischemia is not necessary for the myocardial induction of CXCL16. Our findings may indicate that the failing myocardium itself also contributes to the increased systemic CXCL16 levels in HF, in particular in decompensated myocardial failure. However, while these findings clearly show that the failing myocardium produces and expresses CXCL16, the increased plasma levels of CXCL16 in chronic HF could also, at least partly, reflect enhanced release secondary to systemic inflammation and endothelial cell activation,

Inflammatory cytokines like TNFα and IFNγ have previously been found to enhance CXCL16 expression in endothelial cells, SMC, and macrophages, and we have recently shown that IL-1β has similar properties in endothelial cells and peripheral blood mononuclear cells. In the current study we show that these inflammatory cytokines as well as TLR2 and TLR4 agonists induce the expression of CXCL16 in neonatal rat cardiomyocytes, with particularly enhancing effects of IL-1β. There are several reports of increased levels of these inflammatory cytokines, including IL-1β during HF. TLR2 and TLR4 activation has also been linked to the development of myocardial failure. It is therefore conceivable that these inflammatory mediators could be operating within the failing myocardium, contributing to the enhanced myocardial expression of CXCL16 in overt HF, being part of the inflammatory network that could contribute to the progression of myocardial failure.

Cardiomyocyte hypertrophy and an imbalanced regulation of the extracellular matrix are important features of myocardial remodeling during development of HF. In the present study we show that CXCL16 increases protein synthesis in neonatal rat cardiomyocytes, but no increase was found in the expression of hypertrophic markers or cardiomyocyte size. On the other hand, CXCL16 markedly increased MMP activity in both cardiomyocytes and fibroblasts. MMPs are a family of proteolytic enzymes that are regulated by inflammatory signals to mediate changes in extracellular matrix. Enhanced MMP activity, and in particular increased MMP-2 and MMP-9 activity, has been recognized to play an important role in
myocardial remodeling contributing to the development of myocardial failure.\textsuperscript{30,31} CXCL16 has previously been shown to promote MMP activity in vascular SMC and prostate cancer cell lines.\textsuperscript{6,32} In the current study, we show that CXCL16 is an inducer of MMP activity, at least partly reflecting increased MMP-2 levels, in both myocardial fibroblasts and cardiomyocytes, potentially promoting extracellular matrix remodeling. The ability of CXCL16 to attenuate proline incorporation in myocardial fibroblasts, potentially reflecting impaired collagen synthesis, may further support such a notion. Since we found fairly strong CXCR6 immunoreactivity in these cell types within the failing human myocardium, it is tempting to hypothesize that CXCL16/CXCR6 interaction also could be operating in clinical HF to alter the extracellular matrix via enhancement of MMP activity and down-regulation of collagen synthesis.

CXCL16 also induced proliferation of myocardial fibroblasts which could be blocked by inhibition of JNK. MAPK and ERK pathways have been implicated in CXCL16-mediated cell recruitment to rheumatoid arthritis synovial tissue and the CXCL16-mediated differentiation of glial precursor cells.\textsuperscript{33} In our study, we confirmed that CXCL16 stimulation induces phosphorylation of p38 MAPK and ERK 1/2, but importantly, in neonatal myocardial fibroblasts, CXCL16 also induced JNK activation, and only JNK inhibition abolished the CXCL16-induced proliferation of these cells. JNK activation seems to play an important role in myocardial remodeling,\textsuperscript{34,35} and our data suggest that CXCL16 acts, at least partly, through this signaling pathway to alter fibroblast function and thus potentially also extracellular matrix.

In the present study, we show increased expression of CXCL16 in both clinical and experimental HF, and in patients with overt HF, LV myocardial tissue showed enhanced protein levels of CXCL16 as well as significant immunoreactivity for its corresponding receptor CXCR6 in cardiomyocytes and fibroblasts. Our \textit{in vitro} experiments demonstrated
that CXCL16 stimulated MMP activity in cardiomyocytes and fibroblasts, and attenuated proline incorporation as a marker of collagen synthesis in fibroblasts. Although future studies, including the use of a rodent transgenic/null model, are needed to further establish a role for CXCL16 in the development of HF, our findings showing a combined effect on cardiomyocytes and myocardial fibroblasts may suggest a role for CXCL16 in extracellular matrix remodeling and ultimately also in the development of HF.

Acknowledgements

We thank Bjørg Austbø, Heidi Kvaløy, Dina Behmen, and Ellen Lund Sagen for excellent technical assistance.

Funding sources

This study was supported by the Norwegian Council on Cardiovascular Diseases, University of Oslo, Helse Sør-Øst, and Rikshospitalet University Hospital.

Disclosures

There are no conflicts of interest for any of the authors.
References


17. Øie E, Vinge LE, Tønnessen T, Grøgaard HK, Kjekshus H, Christensen G, Smiseth OA, Attramadal H. Transient, isopeptide-specific induction of myocardial endothelin-1...


Figure legends

Figure 1. Plasma levels of CXCL16 in chronic HF. Panel A shows plasma levels of CXCL16 in 20 healthy controls and in 54 patients with moderate (NYHA II) and 134 patients with severe (NYHA III [n=81] + IV [n=53]) HF. Panel B shows plasma levels of CXCL16 in relation to etiology of HF in NYHA class II (16 with CAD and 37 with DCM) and NYHA class III+IV (64 with CAD and 65 with DCM). Data are median and interquartile range. *P<0.05 versus controls; †††P<0.001 versus NYHA II; ##P<0.01 versus CAD.

Figure 2. The cellular localization of CXCL16 and CXCR6 in human myocardium. Representative photomicrographs demonstrating anti-CXCL16 (A), anti-CXCR6 (B), and anti-vimentin (C) immunostaining of a failing explanted human heart. CXCL16 and CXCR6 immunoreactivity was seen in cardiomyocytes and in vascular smooth muscle and endothelial cells. CXCR6 immunoreactivity was also seen in fibroblast-like cells similar to vimentin-positive cells in panel C (arrows). All sections were counterstained with hematoxylin. Magnification ×200.

Figure 3. The protein levels of CXCL16 and CXCR6 in failing and non-failing human myocardium. Protein levels of CXCL16 (A) and CXCR6 (B) in LV from failing (HF, n=8) and non-failing (Normal, n=6) myocardium as assessed by Western blotting. The upper panels show representative blots from two patients (HF) and two donor controls (N). Data are median and interquartile range. *P<0.05 versus non-failing myocardium (Normal).

Figure 4. The expression of CXCL16 in experimental HF. Panel A shows the gene expression of CXCL16 in the non-ischemic region and the ischemic region of the LV in post-MI HF mice (n=8) as compared with the expression in LV of sham-operated mice (n=7) 3, 7
and 21 days (d) after MI or sham operation. Panel B shows the gene expression of CXCL16 in sham-operated mice (n=7) and mice subjected to chronic pressure overload (aortic banding) with compensated (n=7) and decompensated (n=7) hypertrophy 21 days after intervention. Gene expression was quantified by real-time quantitative RT-PCR and is presented relative to GAPDH expression. Data are median and interquartile range. **P<0.01 and ***P<0.001 versus sham animals; ††P<0.01 versus compensated hypertrophy.

**Figure 5. Gene expression of CXCL16 in cardiomyocytes and non-cardiomyocytes during experimental post-MI HF.** The figure shows the expression of CXCL16 in cardiomyocytes (CM) and non-cardiomyocytes (NCM), primarily reflecting fibroblasts in the non-ischemic LV from post-MI HF rats (n=7) and sham rats (n=7) 7 days after the induction of MI or sham operation. Gene expression was quantified by real-time quantitative RT-PCR and is presented relative to GAPDH expression. Data are median and interquartile range. **P<0.01 versus sham animals.

**Figure 6. Effects of CXCL16 on neonatal rat cardiomyocytes and myocardial fibroblasts.** Panel A shows the expression of CXCR6 in neonatal rat cardiomyocytes and fibroblasts as assessed by Western blotting. Panel B and C show the effect of CXCL16 (200 ng/ml, chemokine domain) on protein synthesis in cardiomyocytes (B, n=16) and on proliferation (C, n=8) in myocardial fibroblasts after 24 hours of stimulation. Protein synthesis and fibroblast proliferation were assessed by [3H]-leucine and [3H]-thymidine incorporation, respectively. Data are median and interquartile range. *P<0.05 and **P<0.01 versus control cells.
Figure 7. The effect of CXCL16 on intracellular signaling pathways in neonatal rat myocardial fibroblasts. Myocardial fibroblasts were exposed to CXCL16 (200 ng/ml, chemokine domain) for 10 and 20 minutes (min) and the phosphorylation of ERK 1/2 (A), JNK (B), and p38 MAPK (C) were assessed by multiplex suspension array technology (n=6). Panel D shows the effect of blocking JNK activation with SP600125 (100 μM) on the CXCL16-mediated induction of fibroblast proliferation (n=6). Data are median and interquartile range. *P<0.05 and **P<0.01 versus control cells; #P<0.05 versus CXCL16 activated cells without JNK blocker.

Figure 8. The effect of CXCL16 on MMP activity. The figure shows the effect of CXCL16 (200 ng/ml, chemokine domain) on total MMP activity (panel A [n=4] and B [n=6]) and MMP-2 levels (panel C [n=8] and D [n=6]) in myocardial fibroblasts (left panels) and neonatal rat cardiomyocytes (right panels) that had been exposed to CXCL16 for 24 and 48 hours, respectively. Panel E shows gelatinolytic MMP-2 activity (pro-MMP-2 and two active processed MMP-2 forms) in un-stimulated (unstim.) and CXCL16 stimulated fibroblasts that had been cultured for 24 hours, as assessed by zymography (n=3). Purified pro-MMP-2 is included as a positive control (left column, control). Total MMP bioactivity was measured as relative units (r.u, see Methods). MMP-2 levels in panel C and D are assessed by EIA. Data are median and interquartile range. *P<0.05 and #P=0.07 versus un-stimulated cells (Control).
Table 1. Clinical and hemodynamic characteristics of the HF population

<table>
<thead>
<tr>
<th></th>
<th>HF patients (n=188)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Age [year]</td>
<td>56±13</td>
<td></td>
</tr>
<tr>
<td>Gender [male/female]</td>
<td>148/40</td>
<td></td>
</tr>
<tr>
<td>Etiology [CAD/DCM/Others] [%]</td>
<td>43/54/3</td>
<td></td>
</tr>
<tr>
<td>NYHA class [II/III/IV] [%]</td>
<td>27/43/30</td>
<td></td>
</tr>
<tr>
<td>History [%]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Hypertension</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Previous myocardial infarction</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td>Biochemical values</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Creatinine [μmol/L]</td>
<td>101±46</td>
<td></td>
</tr>
<tr>
<td>Nt-proBNP [pmol/L]</td>
<td>437±547</td>
<td></td>
</tr>
<tr>
<td>Hemodynamics</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LV-EF [%]</td>
<td>30±12</td>
<td></td>
</tr>
<tr>
<td>PCW, mmHg</td>
<td>18±8</td>
<td></td>
</tr>
<tr>
<td>Cardiac index, L · min⁻¹ · m⁻²</td>
<td>2.1±0.6</td>
<td></td>
</tr>
<tr>
<td>Medication [%]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACE inhibitor</td>
<td>66</td>
<td></td>
</tr>
<tr>
<td>ARB</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>β-blocker</td>
<td>77</td>
<td></td>
</tr>
<tr>
<td>Diuretics</td>
<td>68</td>
<td></td>
</tr>
<tr>
<td>Aldosterone antagonist</td>
<td>41</td>
<td></td>
</tr>
<tr>
<td>Digitoxin</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>Statins</td>
<td>42</td>
<td></td>
</tr>
<tr>
<td>Warfarin</td>
<td>45</td>
<td></td>
</tr>
</tbody>
</table>

Data are presented as the mean ± SD or number or percentage of subjects. PCW, pulmonary capillary wedge pressure; ACE, angiotensin converting enzyme; ARB, angiotensin II receptor blocker.
Figure 1

A

CXCL16 [ng/mL]

0 100 200 300 400 500 600 700

Controls NYHA II NYHA III+IV

* †††

B

CXCL16 [ng/mL]

0 100 200 300 400 500 600 700

CAD NYHA II DCM CAD NYHA III+IV DCM

##
Figure 3

A

CXCL16

β-tubulin

N    HF    N    HF

0     50     100    150    200    250

CXCL16 protein level (% of normal)

Normal (n=6)  HF (n=8)

B

CXCR6

β-tubulin

N    HF    N    HF

0     50     100    150    200    250

CXCR6 protein level (% of normal)

Normal (n=6)  HF (n=8)
Figure 4

A

Sham
MI - Non-ischemic
MI - Ischemic

CXCL16:GAPDH mRNA

3d 7d 21d

** ***

B

Sham Compensated Decompensated

CXCL16:GAPDH mRNA

Hypertrophy

* * * * * * * * * * * * * * * * *
Figure 5

![Graph showing CXCL16:GAPDH mRNA levels in CM-Sham, CM-MI, NCM-Sham, and NCM-MI groups. The graph includes error bars and indicates significant differences with ** symbol.]
Figure 7

A. pERK1/2

B. pJNK

C. p-p38 MAPK

D. 3H Thymidine incorp [dpm/mg]
Figure 8

**A** Fibroblasts

- Total MMP activity [r.f.u.]
- Control and CXCL16 conditions

**B** Cardiomyocytes

- Total MMP activity [r.f.u.]
- Control and CXCL16 conditions

**C** Fibroblasts

- MMP-2 [ng/ml]
- Control and CXCL16 conditions

**D** Cardiomyocytes

- MMP-2 [ng/ml]
- Control and CXCL16 conditions

**E** Fibroblasts

- Pro-MMP-2 (92 kDa)
- MMP-2 (72 kDa)
- MMP-2 (62 kDa)

**F** Gelatinolytic MMP-2 activity [U/L]

- Control and CXCL16 conditions

* Indicates statistical significance.
Increased Production of CXCL16 in Experimental and Clinical Heart Failure: A Possible Role in Extracellular Matrix Remodeling

Christen Peder Dahl, Cathrine Husberg, Lars Gullestad, Anne Wæhre, Jan Kristian Damås, Leif Erik Vinge, Alexandra Finsen, Thor Ueland, Geir Florholmen, Svend Aakhus, Bente Halvorsen, Pål Aukrust, Erik Øie, Arne Yndestad and Geir Christensen

*Circ Heart Fail.* published online September 22, 2009;

*Circulation: Heart Failure* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Copyright © 2009 American Heart Association, Inc. All rights reserved.

Print ISSN: 1941-3289. Online ISSN: 1941-3297

The online version of this article, along with updated information and services, is located on the World Wide Web at:

http://circheartfailure.ahajournals.org/content/early/2009/09/22/CIRCHEARTFAILURE.108.821074

**Permissions:** Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Circulation: Heart Failure* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

**Reprints:** Information about reprints can be found online at:

http://www.lww.com/reprints

**Subscriptions:** Information about subscribing to *Circulation: Heart Failure* is online at:

http://circheartfailure.ahajournals.org//subscriptions/