Increased systemic and myocardial expression of neutrophil gelatinase-associated lipocalin in clinical and experimental heart failure

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Aims

Neutrophil gelatinase-associated lipocalin (NGAL or lipocalin-2) is a glycoprotein with bacteriostatic properties. Growing evidence suggests that NGAL may also be involved in cell survival, inflammation, and matrix degradation. We therefore aimed to investigate the role of NGAL in heart failure (HF).

Methods and results

Our main findings were (i) patients with acute post-myocardial infarction (MI) HF (n = 236) and chronic HF (n = 150) had elevated serum levels of NGAL (determined by enzyme immunoassay), significantly correlated with clinical and neurohormonal deterioration, (ii) in patients with HF following acute MI, elevated NGAL levels of at baseline were associated with adverse outcomes (median of 27 months follow-up), (iii) in a rat model of post-MI HF, NGAL/lipocalin-2 gene expression was increased in the non-ischaemic part of the left ventricle primarily located to cardiomyocytes, (iv) strong NGAL immunostaining was found in cardiomyocytes within the failing myocardium both in experimental and clinical HF, (v) interleukin-1β and agonists for toll-like receptors 2 and 4, representing components of the innate immune system, were potent inducers of NGAL/lipocalin-2 in isolated neonatal cardiomyocytes.

Conclusion

Our demonstration of enhanced systemic and myocardial NGAL expression in clinical and experimental HF further support a role for innate immune responses in the pathogenesis of HF.

Keywords

Heart failure • Lipocalin • Innate immunity • Matrix metalloproteinase

Introduction

Heart failure (HF) is a highly complex multi-step disorder in which a number of physiological systems participate.1 During the recent years, persistent inflammation has been suggested to play a pathogenic role in HF by influencing heart contractility, inducing matrix degradation and fibrosis, and promoting apoptosis, thereby contributing to myocardial remodelling.2 However, although several lines of evidence support the involvement of inflammation in the pathogenesis of HF, there are still issues to be clarified, including the identification and characterization of the different actors involved.

Neutrophil gelatinase-associated lipocalin (NGAL or lipocalin-2) is a 25 kDa glycoprotein, belonging to the lipocalin superfamily, whose members share a barrel-shaped tertiary structure with a hydrophobic pocket that can bind lipophilic molecules.3–5 NGAL is found to be constitutively synthesized during a narrow
window of maturation in the granulocyte precursors in the bone marrow, and is stored in specific granules of mature neutrophils in complex with gelatinase. NGAL may also be released by epithelial cells, renal tubular cells, and hepatocytes during inflammation or injury, and has been found to be expressed in endothelial cells, smooth muscle cells, and macrophages in atherosclerotic plaques. Recently, high NGAL levels were also demonstrated in adipocytes, potentially involved in obesity-associated insulin resistance.

NGAL possesses bacteriostatic properties by binding bacterial siderophores, thereby preventing bacteria from retrieving iron from this source. However, growing evidence suggests that NGAL may also be involved in other processes such as cell survival, inflammation, and matrix degradation. Through direct binding to matrix metalloproteinases (MMP)-9, NGAL inhibits its inactivation, leading to enhanced proteolytic activity with prolonged effects on collagen degradation. Based on its relation to inflammation and matrix degradation, we hypothesized a role for NGAL in HF. In the present study, we report increased expression of NGAL in experimental and clinical HF, with enhanced expression both systemically and within the failing myocardium.

**Methods**

**Patients with acute heart failure following myocardial infarction—longitudinal analyses**

The design and main results of the OPtimal Trial In Myocardial infarction with Angiotensin II Antagonist Losartan (OPTIMAAL) have previously been reported. Briefly, 5477 patients with acute myocardial infarction (MI) complicated with HF during the acute phase were randomly assigned and titrated to a target dose of losartan (50 mg daily) or captopril (50 mg tid) as tolerated. Median randomization time was 3 days after MI and patients were followed for a median of 2.7 years for mortality and morbidity endpoints. The present study was a prospectively designed sub-study of the main OPTIMAAL trial comprising 236 consecutive patients from six centres that was designed to analyse plasma/serum levels of inflammatory and anti-inflammatory mediators. Serum levels of N-terminal pro-brain natriuretic peptide (Nt-proBNP) and C-reactive protein levels were determined as previously reported.

**Patients with chronic heart failure—cross-sectional analysis**

Patients with stable HF (n = 150) for >4 months in NYHA functional class II–IV, on optimal cardiovascular treatment regimens, attending to Department of Cardiology at Rikshospitalet University Hospital, were consecutively included in the study (Table 1). Most of the patients were evaluated by standard right- and left-sided cardiac catheterization. Patients with acute coronary syndromes during the past 6 months and patients with significant concomitant disease (e.g. infection, malignancy, or autoimmune disorders) were not included. The underlying cause of HF was classified as ischaemic (n = 66) or idiopathic dilated cardiomyopathy (n = 84) based on disease history and coronary angiography. Control subjects were 20 sex- and age-matched healthy individuals. Serum samples were collected and stored as previously described. All parts of the study were approved by the local Ethical Committee. Informed written consent was obtained from all subjects.

<table>
<thead>
<tr>
<th>Aetiology (CAD/IDCM)</th>
<th>66/84</th>
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<tbody>
<tr>
<td>NYHA class (II/III/IV)</td>
<td>40/71/39</td>
</tr>
<tr>
<td>LV-EF (%)</td>
<td>32 ± 13</td>
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<tr>
<td>Nt-proBNP (pmol/L)</td>
<td>411 ± 472</td>
</tr>
</tbody>
</table>

Data are mean ± SD or number or percentage of subjects. CAD, coronary artery disease; IDCM, idiopathic dilated cardiomyopathy; LV-EF, left ventricular ejection fraction; ACE, angiotensin converting enzyme; HMG-CoA, hydroxymethylglutaryl coenzyme A.

**Tissue sampling from human myocardium**

Tissue aliquots from the failing myocardium were removed from still-beating hearts immediately on explantation from patients with end-stage HF [NYHA class III–IV, left ventricular ejection fraction (LV-EF) <35%] undergoing cardiac transplantation, snap-frozen in liquid nitrogen, and stored at −80°C until use. Control (non-failing) human LV tissue was obtained from sex- and age-matched subjects whose hearts were rejected as cardiac donors for surgical reasons. Myocardium from these subjects was kept in ice water for 1–4 h before tissue sampling was conducted as described earlier.

**Rat model of experimental heart failure**

Myocardial NGAL/lipocalin-2 expression was investigated in male Wistar rats 2, 7, 28, and 64 days after ligation of the left coronary artery with subsequent development of HF. Sham-operated rats underwent the same procedure except for ligation of the coronary artery. All MI animals included had a transmural infarction of the LV free wall, comprising 40–50% of the ventricular circumference as assessed by perimetry of LV tissue sections and LV end-diastolic pressure >15 mmHg (2 days post-MI: >10 mmHg). Assessment of haemodynamic function and tissue sampling procedures were performed as described previously.

**Isolation of cardiomyocytes, non-cardiomyocytes, and endothelial cells from rat hearts**

Cardiomyocytes, non-cardiomyocytes, and endothelial cells were isolated from LV of non-ischaemic myocardial tissue of rats euthanized 56 days after MI or sham operation by enzymatic digestion using...
collagenase (90 U/mL) as described previously.\textsuperscript{17,18} Immunohistochemical analyses showed that >95% of the cells in the cardiomyocyte fraction were identified as cardiomyocytes [monoclonal anti-rabbit sarcomeric actin antibody (alpha-Sr-1, Dako, Glostrup, Denmark)]. >90% of the cells in non-cardiomyocyte fraction were vimentin-positive fibroblasts [anti-human vimentin antibody (V9, Zymed, San Francisco, CA)], and >90% of the cells in the endothelial cell fraction were CD31-positive endothelial cells [mouse anti-rat CD31 antibody (FitzGerald)].

**Neonatal cardiomyocyte isolation and maintenance protocols**

Primary neonatal cardiomyocytes were isolated from 1–3 days old Wistar rats as previously described.\textsuperscript{19} The cells were treated with a panel of stimulants (interferon (IFN)$\gamma$ and interleukin (IL)-1$\beta$, both 10 ng/mL, R&D Systems, Minneapolis, MN; leukaemia inhibitory factor (ILF), 20 ng/mL, Chemicon Inc., Temecula, CA; tumour necrosis factor (TNF)$\alpha$, 10 ng/mL, BioSource International, Camarillo, CA; and endothen-1 (ET-1), 250 ng/mL; noradrenalin, 100 $\mu$M; lipopolysaccharide (LPS) from Escherichia coli 026:B6, 1 $\mu$g/mL; and a toll-like receptor (TLR)2 agonist (Pam$_2$Cys), 1 $\mu$g/mL, all from Sigma, St Louis, MO) for 24 h.

**Quantitative real-time RT–PCR**

Total RNA from rat myocardium and neonatal rat cardiomyocytes was extracted using TRIzol (Invitrogen, San Diego, CA) and subsequent DNase I treatment (RQI DNase; Promega, Madison, WI), and stored in RNA storage solution (Ambion, Austin, TX) at −80°C. cDNA was synthesized using High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). Quantification of gene expression was performed using the ABI Prism 7500 (Applied Biosystems), Power SYBR Green Master Mix (Applied Biosystems), and sequence-specific PCR primers for lipocalin-2 (forward primer: 5'-GATTGTCAGCTTTGCCAAGT-3', reverse primer: 5'-CATTGGTCGGTGAAACAG-3'). Gene expression of the housekeeping gene GAPDH was used for normalization.

**Immunohistochemistry**

Immunohistochemical analysis was performed on LV myocardial tissue from human cardiac explants and donor hearts and from hearts of HF and sham-operated rats as previously described.\textsuperscript{16} The NGAL expression was detected by affinity-purified polyclonal goat anti-human NGAL and rabbit anti-mouse NGAL IgG (both from Santa Cruz Biotechnology, CA). The primary antibodies were followed by biotinylated anti-goat or anti-rabbit IgG (Vector Laboratories, Burlingame, CA). The immunoreactivities were amplified by the avidin–biotinylated anti-goat or anti-rabbit IgG (Vector Laboratories, Burlingame, CA). The primary antibodies were followed by biotin–peroxidase system (Pierce Chemical, Rockford, IL). The sections were counterstained with haematoxylin. Omission of the primary antibody or use of specific blocking peptides (Santa Cruz Biotechnology) served as negative controls.

**Enzyme-linked immunoassay**

Serum levels of NGAL were measured by enzyme-linked immunoassay (ELISA) as described in detail elsewhere.\textsuperscript{20} The detection limit of the assay was 0.06 ng/mL and the intra- and interassay coefficient of variation was <6%. Neutrophil gelatinase-associated lipocalin may be found in complex with MMP-9, but there was no cross-reactivity with MMP-9 in the current ELISA.

**Statistical analyses**

For comparisons of two groups of individuals, the Mann–Whitney U test was used, while the Kruskal–Wallis test was used a priori when more than two groups were compared. Within groups, differences were analysed by the Wilcoxon rank-sum test. Relationships between variables were analysed by the Spearman rank test. Associations between NGAL concentrations and cardiovascular events (morbidity and mortality) were analysed by Kaplan–Meier survival analysis and the log rank test was used to compare event rates in relation to NGAL levels when appropriate. Probability values are two sided, with $P < 0.05$ being considered statistically significant.

**Results**

**Serum levels of neutrophil gelatinase-associated lipocalin in heart failure following acute myocardial infarction**

In the OPTIMAAL trial (n = 236), there was a significant decrease in serum levels of NGAL during follow-up, with no significant differences between the two treatment groups [losartan (n = 119) and captopril (n = 117)] (Figure 1A). When investigating baseline characteristics in relation to NGAL concentrations, significantly elevated levels were found in patients with NYHA class III (vs. NYHA II) (Figure 1B and C). Moreover, within the patient group as a whole, we found a significant correlation between NGAL and Nt-proBNP levels ($r = 0.15, P = 0.03$). Furthermore, NGAL and NYHA functional class were significantly correlated also during longitudinal testing. Since NYHA fluctuated within one individual during the investigation, we looked at the median NYHA for each patient (Figure 1B) as well as the last registered NYHA classification in the study period (Figure 1C). Regardless, patients in NYHA III had markedly higher NGAL levels at all time-points. Further, we looked at NGAL levels in relation to the composite endpoint [non-fatal myocardial infarction (n = 40); cardiovascular death (n = 26), total mortality death (n = 32), and stroke (n = 10)], and as shown in Figure 1D, those with the composite endpoint had higher levels of NGAL as compared with the other HF patients. Also, we tested the predictive value of high baseline NGAL levels (dichotomized or in quartiles) in relation to the composite endpoint. Figure 1E shows the Kaplan–Meier plot indicating a trend towards higher incidence of the composite endpoint in patients with high NGAL serum levels (i.e. above median). No significant associations were found with cardiovascular or total mortality alone.

In the patient group as a whole, NGAL was significantly correlated with total leucocyte counts at baseline ($r = 0.35, P < 0.001$) and after 1 month ($r = 0.28, P < 0.001$), but importantly, high NGAL levels at baseline were associated with composite endpoint also after correcting for total leucocyte counts.

**Serum levels of neutrophil gelatinase-associated lipocalin in patients with chronic heart failure**

To further characterize NGAL levels during HF, we examined serum concentrations of NGAL in a population of patients with chronic HF (n = 150). As depicted in Figure 2A, patients with chronic HF had
significantly elevated NGAL levels compared with control subjects \((n=20)\), with the highest levels in NYHA class III and IV. A similar pattern with raised NGAL levels was seen in both ischaemic \((n=66)\) and idiopathic \((n=84)\) cardiomyopathy, with no difference between these aetiological groups of HF (data not shown). As in post-MI HF, NGAL was significantly correlated with Nt-proBNP levels \((r=0.37, P<0.001)\), but not with LV-EF, cardiac index, and LV end-systolic and LV end-diastolic dimension (data not shown). Furthermore, within the HF group as a whole, NGAL levels were significantly correlated with total leucocyte counts \((r=0.22, P=0.006)\) and CRP \((r=0.43, P<0.001)\), indicating a relation to inflammatory processes. Finally, NGAL seems to be a sensitive marker of renal failure,\(^{21}\) and we found a significant correlation between NGAL levels and serum creatinine \((r=0.55, P<0.001)\). However, also HF patients with serum creatinine within normal limits \((<120 \mu\text{mol/L}; n=120)\) had raised NGAL concentrations \((109.5 \pm 3.6 \text{ vs. } 80.3 \pm 3.3 \text{ ng/mL}, P<0.001)\), suggesting that the high NGAL levels in chronic HF do not merely reflect impaired renal function.

**Neutrophil gelatinase-associated lipocalin expression in experimental heart failure**

We then examined the myocardial expression of NGAL/lipocalin-2 by real-time RT–PCR in an experimental rat model of post-MI HF.
Rats with cardiac failure had significantly raised NGAL/lipocalin-2 expression in the non-ischaemic area of the LV 2, 7, 28, and 64 days after the induction of MI, representing the development from acute to a chronic stage of HF in this model (Figure 3A). Moreover, further sub-analyses of the non-ischaemic part of LV 56 days after MI (n = 7) or sham operation (n = 7) showed that the up-regulation of NGAL/lipocalin-2 in HF rats was restricted to cardiomyocytes (Figure 3B).

Myocardial immunostaining of neutrophil gelatinase-associated lipocalin in human and experimental heart failure

The cellular localization of NGAL/lipocalin-2 in myocardial failure was analysed by immunohistochemistry in the failing (n = 2) and non-failing (n = 2) human myocardium as well as in heart from sham-operated (n = 2) and post-MI HF (7 days after MI, n = 2; 56 days after MI, n = 2) rats. As shown in Figure 4, the strongest NGAL/lipocalin-2 immunostaining was found in cardiomyocytes within the failing myocardium in both human and experimental HF, with some immunoreactivity also in vascular smooth muscle cells and endothelial cells. In the granulation tissue of the ischaemic area and especially at the border zone between the ischaemic and the non-ischaemic region in post-MI rats, anti-NGAL/lipocalin-2 immunostaining was found in microvascular endothelial cells and in some of the infiltrating cells potentially representing granulocytes.

Regulation of neutrophil gelatinase-associated lipocalin/lipocalin-2 in neonatal rat cardiomyocytes

Our findings so far suggest enhanced expression of NGAL/lipocalin-2 in cardiomyocytes during HF. We therefore examined the effect of various stimuli with relevance to HF [i.e. cytokines (TNFα, LIF, IL-1β, IFNγ), neurohormones (norepinephrine and ET-1), and microbial antigens (TLR2 and TLR4 agonists)] on NGAL/lipocalin-2 expression in neonatal cardiomyocytes.

Discussion

In the present study, we demonstrate that patients with acute post-MI HF as well as those with chronic HF, irrespective of aetiology, have elevated serum levels of NGAL, significantly correlated with the degree of clinical and neurohormonal deterioration. Enhanced NGAL levels were also seen during experimental HF in a rat model of post-MI HF, with increased gene expression of NGAL/lipocalin-2 in the failing myocardium, primarily located to cardiomyocytes. Finally, we showed that IL-1β and TLR2 and TLR4 agonists are potent stimuli for NGAL/lipocalin-2 gene expression in isolated cardiomyocytes, indicating a role for innate immune responses in the regulation of myocardial NGAL/lipocalin-2 levels. Based on its ability to enhance the matrix degrading capacity of MMP-9, it is tempting to hypothesize that NGAL is
not only a marker of HF, but also a mediator in this disorder, potentially involved in myocardial remodelling.

Raised plasma levels of NGAL have been detected during various forms of bacterial infections and in autoimmune disorders. Earlier studies have also shown that NGAL is increased in patients with symptomatic cardiovascular disease, significantly correlated with risk factors of atherosclerosis, and has been found to predict mortality in patients experiencing from cerebral ischaemia. Here, we report increased serum levels of NGAL in both acute and chronic HF, significantly correlated with disease severity as assessed by clinical and neurohormonal parameters.

Neutrophil gelatinase-associated lipocalin was originally isolated as a neutrophil-derived protein, and these cells have been found to represent an important source for circulating NGAL during inflammation. Although we have no data on neutrophil counts, our demonstration of a significant correlation between NGAL levels and leucocyte counts in acute post-MI HF and chronic HF, may further support such a notion. Several reports suggest a pathogenic role for neutrophils in myocardial reperfusion injury. These cells have also been implicated in the pathogenesis of chronic HF, possibly related to their ability to release large amount of myeloperoxidase, an enzyme that may increase
oxidative stress and activate MMPs.28–30 Our current findings give further support to a role of neutrophil activation during acute and chronic HF, and NGAL release from neutrophils may represent a hitherto unrecognized part of the inflammatory responses during HF. However, future studies should analyse the correlation between NGAL and neutrophil counts, and not only total leucocyte counts, and should also more directly examine the release of NGAL from neutrophils in HF patients and healthy controls.

NGAL is not solely a product of activated neutrophils. Hence, epithelial cells, renal tubular cells, and hepatocytes have all been found to release NGAL during inflammation or injury.3 Recently, Hemdahl et al.8 demonstrated increased myocardial expression of lipocalin-2 in apoE−/− x LDLR−/− mice exposed to hypoxia, with particularly high levels in those with MI secondary to hypoxic stress. Herein we extend these findings by demonstrating increased gene expression of NGAL/lipocalin-2 in the non-ischaemic parts of LV in post-MI HF rats. Moreover, our data also indicate that cardiomyocytes, and not fibroblasts or endothelial cells, represent the most important cellular source for this increase. Our demonstration of strong immunostaining of NGAL in cardiomyocytes within the failing explanted myocardium supports a relevance of this finding to clinical HF. Also, our in vitro experiments suggest that TLR2 and TLR4 activation and in particular IL-1β, strongly induce NGAL/lipocalin-2 expression in neonatal cardiomyocytes. All these mediators are integral parts of the innate immune system that is activated in the heart upon stress and injury, such as MI, and also seems to be involved in the pathogenesis of chronic HF.31 Although some of the NGAL expression within the failing myocardium could reflect contribution from infiltrating granulocytes, our findings in the present study suggest that also cardiomyocytes may be an important cellular source of NGAL/lipocalin-2 during HF. These findings further link innate immune responses to HF through direct participation of the failing myocardium involving both afferent and efferent pathways. However, although we show strong NGAL immunostaining within the failing myocardium, the relative contribution of the failing heart to serum levels of NGAL is at present unclear.

Ventricular remodelling, ultimately leading to development and progression of HF, is a highly complex process, that not only involves changes in cardiomyocytes, but also important alterations of extracellular matrix, and MMPs are important mediators in this process. Neutrophil gelatinase-associated lipocalin specifically form a complex with MMP-9, inhibiting its autodegradation, leading to prolonged and enhanced activity of this MMP.11,12 Importantly, the activity of MMP-9 is increased in the failing myocardium, and studies have demonstrated a direct role of this protease in LV dilatation during ventricular remodelling after MI as well as in the myocardial contractile dysfunction during chronic HF.32 Thus, although we have no own data on the potential NGAL/lipocalin-2-mediated effects within the failing myocardium, it is not inconceivable that the elevated myocardial NGAL/lipocalin-2 expression may contribute to the enhanced MMP-9 activity observed during HF, potentially promoting extracellular matrix remodelling. The involvement of NGAL in cell differentiation and apoptosis may further suggest a role for this mediator in myocardial remodelling.10

The present study has some limitations such as a relatively low number of patients and no information on neutrophil counts. Based on the lack of placebo group in the OPTIMAAL trial, forthcoming studies will have to examine the ability of traditional cardiovascular drugs, including neurohormonal antagonists, to modulate NGAL levels. Nonetheless, our demonstration of enhanced systemic and myocardial NGAL expression in experimental and clinical HF, further support a role for innate immune responses in the pathogenesis of HF. Future studies will have to determine the potential adaptive and maladaptive effect of NGAL within the failing myocardium.

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**Conflict of interest:** none declared.

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