Augmented Expression of Cardiotrophin-1 in Failing Human Hearts Is Accompanied by Diminished Glycoprotein 130 Receptor Protein Abundance

Oliver Zolk, MD; Leong L. Ng, MD; Russell J. O’Brien, MB; Michael Weyand, MD; Thomas Eschenhagen, MD

Background—Cardiotrophin-1 (CT-1), a member of the interleukin-6 superfamily, is a potent inducer of cardiomyocyte hypertrophy that prolongs myocyte survival. Although cardiac CT-1 gene expression is known to be upregulated in some animal models of congestive heart failure, the activation state of the CT-1 system in patients with congestive heart failure is unknown.

Methods and Results—This study was designed to determine left ventricular expression of CT-1 and its glycoprotein 130 (gp130)/leukemia inhibitory factor receptor complex in human end-stage heart failure due to ischemic and dilated cardiomyopathy. In addition, we investigated the activation state of signal transducer and activator of transcription 3 (STAT3), the downstream effector of gp130 signaling. In the failing left ventricular myocardium, expression levels of CT-1 mRNA and protein were significantly increased by 142% and 68%, respectively, compared with non-failing donor hearts. Immunohistochemistry confirmed the increased expression of CT-1 in cardiac myocytes. Although gp130 gene expression was increased by 91% (P < 0.001), gp130 protein abundance was significantly diminished by 34% in the failing myocardium. In contrast, leukemia inhibitory factor receptor and suppressor of cytokine signaling-3 protein concentrations were not changed. In addition, the ratio of activated tyrosine phosphorylated STAT3 to total STAT3 was not significantly altered in failing hearts compared with non-failing controls.

Conclusions—Our data suggest that gp130 receptor downregulation balances enhanced CT-1 expression in human heart failure and thereby inhibits excessive activation of the gp130 signaling pathway. (Circulation. 2002;106:1442-1446.)

Key Words: genes heart failure growth substances receptors signal transduction

Heart failure is the leading cause of mortality that ensues after the chronic activation of biomechanical stress pathways that results from various forms of cardiac injury.

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The myocardium first develops adaptive, compensatory hypertrophy, which ultimately leads to an irreversible decompensation in cardiac function. Although the molecular mechanisms that may promote or, conversely, inhibit the transition from compensatory hypertrophy to heart failure are unknown, recent evidence suggests an important role for cardiotrophin-1 (CT-1). CT-1 belongs to the interleukin-6 (IL-6) family of cytokines. CT-1 binds to the glycoprotein 130 (gp130)/leukemia inhibitory factor (LIF) receptor complex and activates both mitogen-activated protein kinase and janus kinase-signal transducer and activator of transcription (JAK-STAT) signaling pathways. Through these pathways, CT-1 induces hypertrophy and prolongs survival of cardiomyocytes. Excessive CT-1 signaling is efficiently attenuated by negative feedback mechanisms. These include gp130 receptor internalization and degradation and induction of intrinsic suppressor of cytokine signaling (SOCS) proteins, such as SOCS-3.

We and others have shown that patients with congestive heart failure have raised plasma levels of CT-1 that correspond to disease severity. Enhanced CT-1 secretion seems to be an early event that occurs before onset of left ventricular systolic dysfunction and therefore may have an impact on disease progression. The observation that CT-1 concentrations were significantly higher in the coronary sinus than in the aorta indicates that the heart is a prominent source of circulating CT-1 in humans. In the present study, we defined cardiac expression levels of CT-1 in donor hearts and explanted hearts from patients with end-stage heart failure. Moreover, we addressed the question of whether negative feedback mechanisms, such as a loss of gp130/LIF receptor

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From the Institut für Experimentelle und Klinische Pharmakologie und Toxikologie, Friedrich-Alexander-Universität Erlangen-Nürnberg, Erlangen, Germany (O.Z., T.E.); the Department of Medicine and Therapeutics, University of Leicester, Leicester, United Kingdom (L.L.N., R.J.O.); and Zentrum für Herzchirurgie, Friedrich-Alexander-Universität Erlangen-Nürnberg, Erlangen, Germany (M.W.).

Correspondence to Dr Oliver Zolk, Institut für Experimentelle und Klinische Pharmakologie und Toxikologie, Friedrich-Alexander-Universität Erlangen-Nürnberg, Fahrstr. 17, 91054 Erlangen, Germany. E-mail Zolk@pharmakologie.uni-erlangen.de

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were relevant in human heart failure.

**Methods**

**Human Cardiac Tissue**

Failing hearts were obtained from patients undergoing orthotopic heart transplantation because of end-stage heart failure (New York Heart Association functional class III to IV) that resulted from idiopathic dilated cardiomyopathy or ischemic cardiomyopathy (diagnosis made by coronary angiography). All patients gave written informed consent before surgery. Myocardial tissue from 8 non-failing donor hearts that could not be transplanted because of surgical reasons or blood group incompatibility were studied for comparison. Neither the donor patient histories nor 2-dimensional echocardiography had revealed signs of heart disease (Table).

**Northern Blot Analysis**

For Northern blot analysis, polymerase chain reaction (PCR)-derived 608 bp and 996 bp fragments of human GAPDH cDNA and human gp130 cDNA, respectively, were used to generate specific probes.

**Noncompetitive Immunoluminometric Assay for CT-1**

The methodology for assay of CT-1 has been described previously. Approximately 100 mg of cardiac tissue was homogenized in 400 μL of buffer (consisting of 150 mmol/L NaCl, 50 mmol/L Tris pH 7.4, 1 mmol/L EDTA, 1 mmol/L PMSF, 1 μg/mL aprotinin, 1% Triton X-100 [Sigma]). After centrifugation, 20 μL of the extracts was assayed for CT-1 with standards in the range 1 to 20 fmol per well. Within and between assay coefficients of variation were 6.2% and 10.3%, respectively. CT-1 levels were determined by an investigator who was blinded to patient details. Each CT-1 value represents the

<table>
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<th>Patient</th>
<th>#</th>
<th>Age, y</th>
<th>Sex</th>
<th>Diagnosis</th>
<th>NYHA Class</th>
<th>LVEF, %</th>
<th>CI (L per min/m²)</th>
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<td>IV</td>
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<td>52</td>
<td>M</td>
<td>NF (ICB)</td>
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<td>5</td>
<td>19</td>
<td>M</td>
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<tr>
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<td>36</td>
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<td>M</td>
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LVEF indicates left ventricular ejection fraction; CI, cardiac index; ICB, intracerebral bleeding; SAB, subarachnoidal bleeding; CIC, cerebral ischemia; HT, head trauma; DCM, idiopathic dilated cardiomyopathy; ICM, ischemic cardiomyopathy; NF, non-failing donor; A, angiotensin-converting enzyme inhibitors or angiotensin II receptor antagonists; B, β-blockers; C, calcium channel blockers; D, diuretics; G, cardiac glycosides; N, nitrates; R, antiarrhythmics (except β-AR blockers); O, dopamine/dobutamine; n.d., unknown; and NYHA, New York Heart Association.

protein or induction of SOCS-3, were relevant in human heart failure.
mean of duplicate measurements and is expressed as pmol/mg protein.

**Real-Time Reverse Transcriptase-PCR**

Total RNA (2 μg) was reverse-transcribed with Moloney murine leukemia virus reverse transcriptase, and cDNA was subsequently amplified with the TaqMan system (Prism 7700, PE Biosystems). Primers and probes for CT-1 and GAPDH were designed to cross an intron/exon boundary (CT-1 forward 5'-CACTTGGAGGCCAAGATCC-3', reverse 5'-TCTCCCTGGAGCTGCACAT-3', probe FAM-5'-TACAGACACACACGCTTGGCAGCT-3'; GAPDH forward 5'-CTGCACACCAACTGCTTAG-3', reverse 5'-GTCTCTGGGTGCAGTGT-3', probe FAM-5'-ATGGAGCAGTGCATGAGGC-3', TAMRA; GAPDH forward 5'-CTCTGGGTGCAGTGT-3', probe FAM-5'-ATGGAGCAGTGCATGAGGC-3', TAMRA-3'), thereby eliminating the possibility of chromosomal DNA artifacts in the PCR. The level of GAPDH in each sample was used to normalize for the variability in RNA quantity or differences in the efficiency of the reverse transcriptase (RT)-reaction.

**Western Blot Analysis**

Proteins from human myocardium were extracted in 50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L PMSF, 1 μg/mL aprotinin, and 1% Triton X-100, (for quantification of phosphoproteins, phosphatase inhibitors 1 mmol/L NaVO3 and 1 mmol/L NaF were included) and separated by 10% SDS-PAGE. Membranes were blocked with 5% nonfat dried milk or 5% bovine serum albumin before incubation with polyclonal antibodies against human gp130, LIF receptor (LIFR) (Santa Cruz Biotechnology), SOCS-3 (Zymed), STAT3, and phospho-Tyr705-STAT3 (Cell Signaling), followed by horseradish peroxidase-conjugated anti-rabbit immunoglobulin G. Proteins were visualized using the enhanced chemiluminescence detection system (Amersham). The blots were also probed with a polyclonal casequestrin antibody (Affinity Bioreagents) as a control for loading.

**Immunohistochemistry**

Immunohistochemistry was performed on cryostat sections (10 μm) with the Rabbit ABC Staining Kit (Santa Cruz Biotechnology) according to the manufacturer’s protocol. The primary antibody, reactive to amino acids 186 to 199 (SRTEGDLGQLLPGG) of the CT-1 sequence, was affinity purified on a Sepharose CL4B protein A column before using the immunoglobulin G fraction. Preabsorption of the antibody with excess immobilized CT-1 was found to eliminate the immunostaining.

**Statistical Analysis**

Data are presented as mean±SEM. Box plots show the median and 25th and 75th percentiles. Statistical analysis was performed using Student’s t test. A value of P<0.05 was considered statistically significant.

**Results**

**CT-1 Expression Is Increased in Human Heart Failure**

The protein and mRNA levels of CT-1 were analyzed in left ventricular (LV) myocardium from donor hearts and in failing LV myocardium from patients with dilated or ischemic cardiomyopathy (Figure 1). Real-time RT-PCR analysis revealed that the ratio of CT-1 mRNA/GAPDH mRNA normalized to non-failing controls increased from 1.0±0.23 to 2.1±0.2 (P<0.01) in failing hearts. CT-1 protein was detectable by immunohistochemistry in failing hearts, as shown in Figure 2. CT-1 immunoreactivity was predominantly observed in the cytoplasm of cardiomyocytes, but was also seen in non-myocyte cells, such as vascular smooth muscle cells. To quantify CT-1 concentrations in the left ventricular myocardium more accurately, immunoluminometric analyses of LV tissue extracts were performed. Augmented CT-1 transcript expression was paralleled by increased CT-1 protein concentrations in the failing myocardium (Figure 1), which were raised from 7.9±0.8 pmol/mg (n=8) to 13.2±0.8 pmol/mg (n=23, P<0.001) in hearts from ischemic cardiomyopathy and dilated cardiomyopathy patients.

**LIF Receptor, gp130, and SOCS-3 Expression in Human Heart Failure**

We examined expression levels of major CT-1 signaling molecules (Figure 2). Northern blot analysis revealed that gp130 gene expression was increased by 91% (gp130 mRNA/
GAPDH mRNA \(0.78 \pm 0.03, n=16\), versus \(0.41 \pm 0.05, n=8, P<0.001\). Gp130 protein abundance, however, was diminished significantly by 34% \((35.0 \pm 1.9, n=21, \text{versus} 52.9 \pm 2.6, n=8, P<0.001)\). In contrast, LIF receptor \((9.4 \pm 0.5, n=21, \text{versus} 9.6 \pm 1.1, n=8)\) and SOCS-3 protein concentrations \((16.4 \pm 1.6, n=21, \text{versus} 12.4 \pm 2.5, n=8)\) were not significantly changed. The gp130/SOCS-3 protein ratio was significantly decreased by 53% (Figure 2C).

**Activation State of STAT3**

To assess whether downregulation of the gp130 receptor subunit in failing hearts may change activation levels of downstream effector molecules, we determined the phosphorylation state of STAT3 (Figure 2D). The ratio of activated tyrosine phosphorylated STAT3 (p-Tyr-STAT3) to total STAT3 was not significantly changed in failing LV myocardium compared with non-failing hearts \((0.69 \pm 0.09, n=21, \text{versus} 0.44 \pm 0.10, n=8)\). Preliminary experiments demonstrated that storage of human heart tissue on ice during transport to the laboratory led to dephosphorylation of STAT3 by less than 2.5%. Thus, we could exclude rapid dephosphorylation of STAT3 after explantation of the hearts, which could have affected our results.

**Discussion**

This study demonstrates for the first time that CT-1 gene expression and protein content are increased in failing human left ventricular myocardium. On the basis of the potent growth-promoting properties of CT-1 in vitro, this finding supports a potential role for CT-1 as a local paracrine/endocrine factor promoting cardiac hypertrophy via activation of gp130.

Gp130-induced hypertrophy in vitro is characterized by increased addition of sarcomeric units in series rather than in parallel, which has been linked to ventricular dilatation. Conversely, transgenic studies suggest that gp130 signaling may play an important role in the prevention of ventricular dilatation. Mice with a conditional heart-specific knock-out of gp130 present normal cardiac structure and function, but during acute pressure overload, the hearts of these mice display rapid onset of dilated cardiomyopathy and induction of myocyte apoptosis. The protective action of CT-1 seems to be mediated through JAK-STAT signaling, as demonstrated in transgenic mice. Cardiac-specific overexpression of STAT3 provides protection against doxorubicin-induced cardiomyopathy, thus resulting in an improved survival rate by preventing progression of heart failure. Altogether, these results suggest that preserved or even enhanced gp130/STAT3 signaling might delay onset of cardiac failure.

Does augmented CT-1, however, cause an activation of the gp130/STAT3 signaling in human end-stage heart failure? Gp130 protein was markedly decreased in the failing myocardium, despite augmented transcript expression. This was paralleled by an increase in the SOCS-3/gp130 protein ratio, which suggests an inactivation of the gp130 signaling pathway. Containment of gp130 signaling has been demonstrated for other IL-6 type cytokines like oncostatin (OSM), LIF, and IL-6. This seems to be directed by 2 distinct mechanisms, the induction of factors, such as SOCS proteins, that attenuate functions of the cytoplasmic domain of gp130, and ligand-induced gp130 internalization and degradation, which is compensated, at least in part, by enhanced gp130 transcription. The latter mechanism seems to be predominant in human heart failure. Steady state activation levels of STAT3,
the downstream effector of gp130 signaling mediating myocyte hypertrophy and survival, was unchanged in failing ventricles compared with donor hearts. This finding suggests that gp130 receptor downregulation balances enhanced CT-1 in human heart failure and thereby inhibits excessive activation of the gp130 signaling pathway.

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