Elevated Levels of Activin A in Heart Failure
Potential Role in Myocardial Remodeling

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Background—Although modulation of inflammatory processes has been suggested as a new treatment modality in heart failure (HF), our knowledge about abnormalities in the cytokine network during HF is still limited. On the basis of previous cDNA array study examining peripheral blood mononuclear cells from HF patients, we hypothesized a role for activin A, a member of the transforming growth factor (TGF)-β superfamily, in the pathogenesis of HF.

Methods and Results—This study had 4 main and novel findings. First, serum levels of activin A were significantly elevated in patients with HF (n=86) compared with healthy control subjects (n=20), with increasing levels according to disease severity as assessed by clinical, hemodynamic, and neurohormonal parameters. Second, compared with control subjects, HF patients, as determined by real-time quantitative reverse transcriptase polymer chain reaction, also had markedly increased gene expression of the activin A subunit activin β3 in T cells but not in monocytes. Third, in a rat model of HF, we demonstrated a concerted induction of the gene expression of activin β3 and activin receptors IA, IB, IIA, and IIB after myocardial infarction. Immunohistochemical analysis localized activin A solely to cardiomyocytes. Finally, activin A markedly increased gene expression of mediators involved in infarction healing and myocardial remodeling (ie, atrial natriuretic peptide, brain natriuretic peptide, matrix metalloproteinase-9, tissue inhibitor of metalloproteinase-1, transforming growth factor-β1, and monocyte chemoattractant protein-1) in neonatal rat cardiomyocytes.

Conclusions—Together with our demonstration of activin A–induced gene expression in neonatal cardiomyocytes of mediators related to myocardial remodeling, the expression pattern of activin A during clinical and experimental HF suggests an involvement of this cytokine in the pathogenesis of HF. (Circulation. 2004;109:1379-1385.)

Key Words: heart failure ■ inflammation ■ leukocytes ■ myocardium

Despite state-of-the-art treatment, heart failure (HF) is a progressive disorder characterized by high morbidity and mortality, suggesting that important pathogenic mechanisms remain active and unmodified by current treatment regimens. A growing body of evidence links inflammation to the pathogenesis of HF; consequently, modulation of inflammatory processes has been suggested as a novel treatment modality in this disorder. However, our knowledge about the abnormalities in the cytokine pathway during HF is still limited. To address this issue, we recently performed a screening experiment using cDNA expression arrays to spot differentially expressed inflammatory mediators in peripheral blood mononuclear cells (PBMCs) from HF patients and healthy control subjects. This study revealed increased expression of several cytokine-related genes, including ligands in the tumor necrosis factor (TNF) superfamily, but also genes related to the activin A pathway.

Activin A, a homodimer of activin β3 subunits, is a member of the transforming growth factor (TGF)-β superfamily. Although originally described as an inducer of follicle-stimulating hormone release, activin A has been recognized as a multifunctional cytokine expressed in a wide range of tissues and cells with roles in regulation of wound repair, cell differentiation, apoptosis, embryogenesis, and inflammation. Moreover, a role for activin A has been proposed in several pathological processes such as carcinogenesis and fibrosis, and this cytokine may also be involved in the pathogenesis of various inflammatory disorders such as inflammatory bowel disease and rheumatoid arthritis. In addition, activin A seems to be
TABLE 1. Clinical Characteristics of the Study Group

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>HF Patients (n=86)</th>
<th>Healthy Control Subjects (n=20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>50±10</td>
<td>46±2</td>
</tr>
<tr>
<td>Gender, M/F, n</td>
<td>74/12</td>
<td>15/5</td>
</tr>
<tr>
<td>Origin, CAD/IDCM, n</td>
<td>46/40</td>
<td></td>
</tr>
<tr>
<td>NYHA class II/III/IV, n</td>
<td>20/38/28</td>
<td></td>
</tr>
<tr>
<td>LVEF, %</td>
<td>28±13</td>
<td></td>
</tr>
<tr>
<td>Pro-ANP, pmol/L^*</td>
<td>3485±2124</td>
<td></td>
</tr>
<tr>
<td>Medication, %</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACE inhibitor</td>
<td>82</td>
<td></td>
</tr>
<tr>
<td>β-Blocker</td>
<td>42</td>
<td></td>
</tr>
<tr>
<td>Diuretics</td>
<td>89</td>
<td></td>
</tr>
<tr>
<td>Aldosterone antagonist</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>Digitoxin</td>
<td>51</td>
<td></td>
</tr>
<tr>
<td>Nitrate</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>HMG-CoA reductase inhibitors</td>
<td>31</td>
<td></td>
</tr>
</tbody>
</table>

CAD indicates coronary artery disease; IDCM, idiopathic dilated cardiomyopathy; LVEF, LV ejection fraction; and ACE, angiotensin-converting enzyme. Data are presented as mean±SD when appropriate.

^*Pro-ANP levels in healthy control subjects are <900 pmol/L.

Serum levels of activin A (Serotec), N-terminal pro-brain natriuretic peptide (Nt-pro-BNP; Biomedica), monocyte chemotactic peptide-1 (MCP-1; R&D Systems), and tumor necrosis factor-α (TNF-α; R&D Systems) were analyzed by enzyme immunoassay; N-terminal pro-atrial natriuretic peptide (Nt-pro-ANP) was analyzed by radioimmunoassay.

Isolation of Cells
PBMCs were obtained from heparinized blood by Isopaque Ficoll (Lymphoprep, Nycomed) gradient centrifugation. Further separation of monocytes (CD14-labeled magnetic beads; MACS, Miltenyi Biotec) and CD3^+ T cells (negative selection by monodisperse immunomagnetic beads) was performed as described elsewhere. After isolation, the cells were immediately stored in liquid nitrogen. The selected T cells consisted of >90% CD3^+ cells; the isolated monocytes consisted of >95% CD14^+ cells (flow cytometry).

Real-Time Quantitative RT-PCR
Total RNA was isolated from frozen PBMCs, T cells, and monocytes with RNeasy Minikit (Qiagen), subjected to DNase I treatment (RQ1 Dnase, Promega), and stored at −80°C until analysis. Primers were designed with the Primer Express software, version 2.0 (Applied Biosystems) (Table 2). mRNA was quantified with the ABI Prism 7000 (Applied Biosystems). SyBr Green assays (Table 2) were performed with the qPCR Master Mix for SYBR Green I (Eurogentec) and 300 nmol/L sense and antisense primers. Specificity of the SyBr Green assays was confirmed by melting point analysis and gel electrophoresis. Gene expression of the housekeeping gene GAPDH (Applied Biosystems) was used for normalization.

Rat Model of Experimental HF
Male Wistar rats (~290 g) were subjected to left coronary artery ligation or sham operation during halothane anesthesia (1% halothane in a mixture of one third O_2 and two thirds N_2O) as previously described. The rats were euthanized 2, 7, and 28 days after the surgical procedure. LV end-diastolic pressure was measured under halothane anesthesia with a 2F micromanometer-tipped catheter (model SPR-407, Millar Instruments) before the rats were euthanized. Rats with transmural infarction of the LV free wall comprising >40% of LV circumference and with LV end-diastolic pressure >10 mm Hg were considered to have HF, and only these rats were included in the study. Hearts were sectioned by separating the LV (LV free wall and interventricular septum) from the rest of the heart. The LV was further divided into ischemic (infarcted zone and 1 to 2 mm of the border zone) and nonischemic areas, with care taken to avoid contamination of viable myocardial tissue with necrotic tissue. The animal experiments, procedures, and housing were in accordance with institutional guidelines and national legislation conforming to The European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes of March 18, 1986.

Immunohistochemistry
For immunohistochemical analysis, rat hearts (7 days after myocardial infarction [MI]; see above) were fixed in Bouin’s solution and embedded in paraffin. Immunohistochemical analysis was performed by use of an affinity-purified polyclonal anti-activin A antibody (Sigma-Aldrich). Unmasking of antigens was improved by heating the sections in citrate buffer, pH 6.0, in a microwave oven. Diaminobenzidine was used as the chromogen in a commercial Diaminobenzidine was used as the chromogen in a commercial Diaminobenzidine was used as the chromogen in a commercial Diaminobenzidine was used as the chromogen in a commercial Diaminobenzidine was used as the chromogen in a commercial Diaminobenzidine was used as the chromogen in a commercial Diaminobenzidine was used as the chromogen in a commercial Diaminobenzidine was used as the chromogen in a commercial Diaminobenzidine was used as the chromogen in a commercial Diaminobenzidine was used as the chromogen in a commercial Diaminobenzidine was used as the chromogen in a commercial Diaminobenzidine was used as the chromogen in a commercial Diaminobenzidine was used as the chromogen in a commercial Diaminobenzidine was used as the chromogen in a commercial Diaminobenzidine was used as the chromogen in a commercial antibody served as a negative control.

Stimulation of Neonatal Rat Cardiomyocytes
Cardiomyocytes were prepared as previously described with minor modifications. Hearts from 1- to 3-day-old Wistar rats were cut into small pieces and digested with collagenase II (Worthington) and protease (Sigma). The cell suspension was centrifuged through a discontinuous Percoll gradient (Amersham Pharmacia Biotech), and

Methods
Patients
The study population was recruited during 1999 to 2000 and consisted of white patients with stable HF for >4 months classified as New York Heart Association (NYHA) functional classes II through IV with no changes in medication during the last 3 months (Table 1). None of the patients had significant concomitant disease such as infections, malignancies, autoimmune disorders, diabetes, or pulmonary disease. The cause of HF was classified as coronary artery disease or idiopathic dilated cardiomyopathy on the basis of disease history and coronary angiographic examination. Left ventricular (LV) ejection fraction and ventricular dimensions were visually estimated by 1 independent experienced cardiologist from 2-dimensional and M-mode echocardiographic images. For comparison, blood samples were also collected from sex- and age-matched healthy blood donors. Signed informed consent was obtained from each individual. The investigation conformed to the principles outlined in the Declaration of Helsinki.

Serum Levels of Activin A and Other Circulating Factors
For collection of serum, blood was drawn into pyrogen-free tubes (Becton Dickinson) without additives. The tubes were immediately immersed in ice water, allowed to clot for 2 hours, and centrifuged at 1000g for 10 minutes; serum was stored at −80°C until analyzed.
cardiomyocytes were removed from the gradient. The cardiomyocytes were allowed to attach overnight on gelatin-coated culture dishes at a density of 6.2 × 10^4 cells/cm^2. Thereafter, the cardiomyocytes were incubated in maintenance medium (plating medium without horse and fetal calf serum) and stimulated with 100 ng/mL recombinant human activin A (R&D Systems) or vehicle for the indicated time points. The purity of cardiomyocyte cultures was 92%.

**Western Blotting**

Cardiomyocytes were resuspended in ice-cold lysis buffer (PBS containing protease inhibitor cocktail [Roche] with 1% Triton X-100), and extracts were incubated on ice for 15 minutes and then centrifuged at 12,000g for 15 minutes at 4°C. The supernatants were retained, and protein concentrations in the samples were measured with the BCA method (Pierce). Protein extracts (50 μg) were separated on 7.5% SDS-PAGE gels (Bio-Rad) and electrophoretically transferred onto nitrocellulose. After blocking overnight, the membranes were incubated with rabbit anti-human phospho-Smad2 (Cell Signaling Technology Inc), stripped, and reprobed with rabbit anti-human Smad2/3 (Upstate) to ensure equal loading. Proteins were detected by enhanced chemiluminescence with horseradish peroxidase–labeled anti-rabbit IgG (Cell Signaling Technology Inc) and visualized after exposure to Hyperfilm ECL (Amersham).

**Statistical Analysis**

For comparison of 2 groups, the Mann-Whitney U test (2 tailed) was used. Coefficients of correlation (r) were calculated by the Spearman rank test. Data are given as mean ± SEM unless otherwise stated. Probability values are 2 sided and considered significant at P<0.05.

**Results**

**Serum Levels of Activin A in Patients With HF and Healthy Control Subjects**

Patients with HF had significantly elevated serum levels of activin A compared with healthy control subjects, with particularly high concentrations in those with NYHA classes III and IV (Figure 1A). A similar pattern with raised activin A levels was seen in both ischemic (n=46) and idiopathic (n=40) cardiomyopathy, with no difference between these etiologic groups of HF (data not shown). The control group tended to be younger and have a higher proportion of women. However, we found no correlation between activin A levels and either age or gender in the HF group, suggesting that the raised activin A level in HF does not merely reflect differences in age and gender.

In addition to its relation to clinical severity, activin A levels in HF patients were also significantly correlated with decreased cardiac index, increased LV end-diastolic pressure, and increased circulating levels of Nt-pro-ANP, Nt-pro-BNP,
Gene Expression of Activin β₅ in Leukocyte Subsets

We next analyzed the gene expression of the activin A subunit activin β₅ in PBMCs, T cells, and monocytes. As shown in Figure 1B, activin β₅ gene expression was markedly upregulated (∼8.8-fold increase) in PBMCs from HF patients (n=12) compared with levels in healthy control subjects (n=8). Moreover, although activin β₅ mRNA expression in monocytes was too low to yield quantifiable results in both HF patients and control subjects, this cytokine was upregulated in T cells from HF patients compared with nearly undetectable levels in healthy control subjects (∼3.9-fold increase; Figure 1B).

Expression Pattern of Activin A in Experimental HF

To further characterize the activin A expression in HF, we examined the myocardial expression of this cytokine and its receptors by real-time reverse transcriptase polymerase chain reaction (RT-PCR) in an experimental rat model of postinfarction HF. Rats with myocardial failure had markedly elevated levels of activin β₁ mRNA in the ischemic region of LV, and notably, these levels tended to increase (∼15-fold increase) during the observation period compared with the LV in the sham group after 28 days (Figure 2A). Moreover, the nonischemic regions of the LV of HF rats had enhanced expression of activin β₁ mRNA; again, increased gene expression persisted throughout the observation period (Figure 2A). The ischemic but not the nonischemic regions of the LV in HF rats also had enhanced gene expression of the activin receptors IA, IB, IIA, and IIB (data not shown). Finally, immunohistochemical analysis showed activin A immunoreactivity in only the cardiomyocytes with fairly strong immunostaining in both failing and nonfailing cardiomyocytes (Figure 2B through 2D). However, the strongest activin A immunoreactivity was detected in cardiomyocytes adjacent to the infarcted zone (Figure 2D).

Effects of Activin A on Neonatal Cardiomyocytes

We next examined the effects of activin A on neonatal rat cardiomyocytes. First, we found that these cells expressed mRNA for all described activin A receptors (ie, activin receptors IA, IB, IIA, and IIB; Figure 3A). Second, we observed that stimulation with activin A for 30 and 60 minutes led to increased phosphorylation of Smad2, representing an important part of the activin A signaling system (Figure 3B). Third, to elucidate a possible role for activin A in myocardial remodeling, these cardiomyocytes were stimulated with activin A for 6 and 24 hours, and mRNA levels of mediators known to play an important role in this process (ie, ANP, BNP, TGF-β₁, MCP-1, matrix metalloproteinase-9 [MMP-9], and tissue inhibitor of metalloproteinase-1 [TIMP-1]) were analyzed by real-time quantitative RT-PCR. As depicted in Figure 4A and 4B, cardiomyocytes responded to activin A by significantly increased gene expression of the neurohormones ANP and BNP after 24 hours of stimulation. Furthermore, activin A increased the mRNA levels of TGF-β₁ and
MCP-1 after both 6 and 24 hours of stimulation (Figure 4C and 4D). Finally, although activin A caused increased cardiomyocyte gene expression of MMP-9 after 24 hours (Figure 4E), the mRNA levels of its endogenous TIMP-1 were elevated after both 6 and 24 hours of stimulation (Figure 4F).

Discussion
In the present study, we demonstrate that HF patients have markedly elevated serum levels of activin A that were significantly correlated with functional, hemodynamic, and neurohormonal parameters for disease severity. Furthermore, in a rat model of postinfarction HF, we found...
found that enhanced expression of activin A in PBMCs from HF patients was localized to T cells rather than monocytes. Furthermore, the failing myocardium itself may contribute to the enhanced activin A levels during HF, with cardiomyocytes as the primary cellular source. Together with the enhanced expression of activin receptors within the failing myocardium, these findings may suggest a potential for activin A-mediated responses involving both autocrine and paracrine interactions.

From previously reported effects on wound repair and fibrosis, one might hypothesize a role for activin A in processes related to healing of the MI. Moreover, after MI, we found that the raised gene expression of activin βα and its receptors in the ischemic LV tended to increase throughout the study, and as for activin βα, raised gene expression was also seen in the nonischemic region of the LV. Although the myocardial protein levels were not measured, these expression patterns may suggest a role for activin A in myocardial remodeling and the development of HF. Such a notion is further supported by our in vitro experiments showing an activin A–induced gene expression of ANP, BNP, TGF-β1, MMP-9, TIMP-1, and MCP-1 in neonatal rat cardiomyocytes, and in clinical HF, serum levels of some of these parameters were significantly correlated with activin A. These mediators have all previously been demonstrated to play a role in myocardial remodeling and tissue repair, especially in the development of interstitial fibrosis.13,14 Hence, although activin A may have a beneficial effect on infarction healing, persistently elevated myocardial levels of this cytokine may contribute to remodeling and ultimately HF.

Activin A is induced by angiotensin II in smooth muscle cells and may be induced by inflammatory cytokines such as TNF-α and interleukin-1.17,18 Raised activin A levels in HF may therefore reflect enhancement of both neurohormonal and inflammatory activities in this disorder. However, although the present study may suggest a pathogenic role for activin A in the development of HF, particularly in myocardial remodeling after MI, further studies are needed to identify more precisely the role of this cytokine in the progression of myocardial failure.

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References


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