Dysregulated Osteoprotegerin/RANK Ligand/RANK Axis in Clinical and Experimental Heart Failure

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Background—Persistent inflammation appears to play a role in the development of heart failure (HF). Osteoprotegerin (OPG), the receptor activator of nuclear factor-κB (RANK), and RANK ligand (RANKL) are newly discovered members of the tumor necrosis factor superfamily that are critical regulators in bone metabolism but appear also to be involved in immune responses. We hypothesized that the OPG/RANK/RANKL axis could be involved in the pathogenesis of heart failure, and this hypothesis was investigated in both experimental and clinical studies.

Methods and Results—Our main and novel findings were as follows: (1) In a rat model of postinfarction HF, we found persistently increased gene expression of OPG, RANK, and RANKL in the ischemic part of the left ventricle (LV) and, for OPG, in the nonischemic part that involved both noncardiomyocyte and in particular cardiomyocyte tissue. (2) Enhanced myocardial protein levels of OPG, RANK, and RANKL, in particular, were also seen in human HF, and using immunohistochemistry, we localized these mediators to cardiomyocytes within the LV in both experimental and clinical HF. (3) In human HF, we also found increased systemic expression of RANKL (T cells and serum) and OPG (serum), with increasing levels according to functional, hemodynamic, and neurohormonal disease severity. (4) RANKL increased total matrix metalloproteinase activity in human fibroblasts, which indicates a matrix-degrading net effect and suggests a potential mechanism by which enhanced RANKL expression in HF may contribute to LV dysfunction.

Conclusions—These findings suggest a potential role for known mediators of bone homeostasis in the pathogenesis of HF and possibly represents new targets for therapeutic intervention in this disorder. (Circulation. 2005;111:2461-2468.)

Key Words: heart failure ■ immunohistochemistry ■ molecular biology ■ myocytes ■ immunology

Persistent inflammation appears to play a role in the development of heart failure (HF). In particular, tumor necrosis factor (TNF)-α has been implicated as a possible pathogenic factor in this process. Mice with cardiac overexpression of TNF-α develop myocardial inflammation, cardiac hypertrophy, and dilated cardiomyopathy. Furthermore, these effects are mimicked by infusion of TNF-α, which indicates that both circulating and locally produced TNF-α may induce myocardial dysfunction. However, although several studies have focused on the possible pathogenic role of TNF-α, other inflammatory mediators may also be of importance in the pathogenesis of HF. We have recently shown enhanced gene expression of several ligands of the TNF superfamily in peripheral blood mononuclear cells (PBMCs) from HF patients, which suggests that other members of this family also could be involved in the development of myocardial failure.

Osteoprotegerin (OPG) is a member of the TNF receptor superfamily that can function as a soluble decoy receptor by binding the receptor activator of nuclear factor-κB (RANK) ligand (RANKL) and competitively inhibiting the interaction between RANKL and its receptor. These factors have been identified as candidate mediators for paracrine signaling in bone metabolism and extracellular matrix regulation but have also been shown to modulate dendritic cells and activated T cells, as well as to promote B-cell maturation and antibody response, which suggests a role in both innate and adaptive immunity.

Furthermore, mRNA and protein expression of both RANKL and OPG has been detected in murine heart tissue. We hypothesized that the OPG/RANK/RANKL axis also could be involved in the pathogenesis of myocardial failure. In the present study, this hypothesis was investigated in both rat and human HF through both experimental and clinical studies.

Methods

Rat Model of Experimental HF
The course of myocardial OPG, RANK, and RANKL mRNA expression was investigated at various time points after ligation of...
TABLE 1. Clinical and Hemodynamic Characteristics of the HF Population (n=102)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>52 ± 9</td>
</tr>
<tr>
<td>% Male</td>
<td>89%</td>
</tr>
<tr>
<td>NYHA functional class II/III/IV, n</td>
<td>25/47/30</td>
</tr>
<tr>
<td>Cause of HF, n (%)</td>
<td></td>
</tr>
<tr>
<td>Coronary artery disease</td>
<td>54 (52%)</td>
</tr>
<tr>
<td>Idiopathic dilated cardiomyopathy</td>
<td>43 (42%)</td>
</tr>
<tr>
<td>Duration of heart failure, y</td>
<td>4 ± 3</td>
</tr>
<tr>
<td>LV ejection fraction, %</td>
<td>32 ± 16</td>
</tr>
<tr>
<td>Pulmonary capillary wedge pressure, mm Hg</td>
<td>18 ± 9</td>
</tr>
<tr>
<td>Cardiac index, L - min⁻¹ - m⁻²</td>
<td>2.1 ± 0.6</td>
</tr>
<tr>
<td>Medication, n</td>
<td></td>
</tr>
<tr>
<td>ACE inhibitors</td>
<td>79%</td>
</tr>
<tr>
<td>β-Blocker</td>
<td>40%</td>
</tr>
<tr>
<td>Diuretics</td>
<td>87%</td>
</tr>
<tr>
<td>Nitrate</td>
<td>24%</td>
</tr>
<tr>
<td>Aldosterone antagonist</td>
<td>22%</td>
</tr>
<tr>
<td>Statins</td>
<td>29%</td>
</tr>
<tr>
<td>Digitalis</td>
<td>54%</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD or number of patients or subjects. Hemodynamic parameters were available in 84 patients.

the left coronary artery during the development of HF in male Wistar rats (Møllegaard Breeding Center, Denmark). The procedure generally resulted in transmural infarction of the left ventricular (LV) free wall, comprising 40% to 50% of the ventricular circumference (as assessed by perimetry of LV tissue sections). Except for ligation of the coronary artery, sham-operated rats underwent the same procedure. Assessment of hemodynamic function and tissue sampling procedures were performed as described previously. In a separate set of experiments, the LV was divided into cardiomyocytes and noncardiomyocytes. Total RNA was isolated from ischemic and nonischemic areas in LV by acid-phenol extraction in the presence of chaotropic salts (TRlzol, Invitrogen) and subsequent isopropanol-ethanol precipitation. The animal experiments and housing were in accordance with institutional guidelines and national legislation concerning the use of animals for scientific purposes.

HF Patients

One hundred two patients with stable HF for >4 months in New York Heart Association (NYHA) functional class II, III, or IV were consecutively included from Rikshospitalet University Hospital (Table 1). Most of the patients were evaluated by standard right-sided (n=84) and left-sided (n=70) cardiac catheterization. Patients with acute coronary syndromes during the past 6 months and patients with significant concomitant disease, such as infection, malignancy, or collagen vascular disease, were not included. The underlying cause of HF was classified as ischemic (n=54) or idiopathic dilated cardiomyopathy (n=48) on the basis of disease history and coronary angiography. Control subjects were 20 sex- and age-matched healthy blood donors. Informed consent for participating in the study was obtained from all individuals. The study was approved by the local ethics committee and conducted according to the Declaration of Helsinki.

Biochemical Measurements

Serum levels of OPG were quantified by ELISA with commercially available matched antibodies (R&D Systems). Serum levels of N-terminal pro-brain natriuretic peptide (N-BNP) and RANKL were quantified by ELISA (Biomedica GmBH). Serum samples were collected and stored as described previously.

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 GmBH) and CD3 T cells (negative selection by monodisperse immunomagnetic beads; Dynal, Nycomed) was performed as described elsewhere. The selected T cells consisted of >90% CD3 cells and the isolated monocytes of >95% CD14 cells (flow cytometry). Cell pellets were stored in liquid nitrogen until used. Total RNA was isolated from frozen T cells and monocytes with the RNaseq Minikit (Qiagen).

Human Myocardial Tissue Samples

Tissue aliquots from the failing myocardium were removed from still-beating hearts immediately on explantation from patients with end-stage HF (NYHA class III or IV, LV ejection fraction <34%) undergoing cardiac transplantation (16 samples for RNA extraction, 10 for Western immunoblot), snap-frozen in liquid nitrogen, and stored at −80°C until use. Control (nonfailing) human LV tissue was obtained from sex- and age-matched subjects whose hearts were rejected as cardiac donors for surgical reasons (3 samples for RNA extraction, 6 for Western immunoblot). The cause of death of donors was cerebrovascular accident or trauma, and none had a history of heart disease. Myocardium from these subjects was kept on ice water for 1 to 4 hours before tissue sampling was conducted as described above. Total RNA was isolated from myocardial tissue by the acid guanidinium thiocyanate phenol chloroform method. Samples for Western immunoblot were homogenized separately, and proteins were extracted with ice-cold lysis buffer that contained a protease inhibitor cocktail at a ratio of 500 μL per 50 mg of frozen weight tissue. Extracts were incubated on ice for 15 minutes and centrifuged at 12 000g for 15 minutes at 4°C. The supernatants were retained and protein concentrations of the sample measured by the BCA method (Pierce Chemical).

Isolation of Cardiomyocytes for Immunohistochemistry

Ventricular cardiomyocytes were isolated from adult rat hearts by enzymatic digestion with trypsin (60 U/mL) and collagenase (90 U/mL) as described by others and mounted on glass slides for immunohistochemistry.

Real-Time Quantitative Reverse Transcription–Polymerase Chain Reaction

All total RNA samples were subjected to DNase I treatment (RQI DNase; Promega) and stored at −80°C until analysis. Sequence-specific polymerase chain reaction (PCR) primers were designed with Primer Express software version 1.5 (Applied Biosystems; Table 2). Quantification of mRNA was performed with ABI Prism 7000 (Applied Biosystems). Gene expression of the housekeeping gene GAPDH (Applied Biosystems) and β-actin (Table 2) was used for normalization.

Immunohistochemistry

Immunohistochemical analysis was performed on LV myocardial tissue from cardiac explants, rat hearts, and isolated rat ventricular cardiomyocytes as previously described with purified polyclonal antibodies against human OPG, RANK, and RANKL (Santa Cruz Biotechnology). 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 metal-enhanced system (Pierce Chemical). The sections were counterstained with hematoxylin. Omission of the primary antibody served as a negative control. The specificity of the human OPG and RANKL antibodies was also confirmed by the use of specific blocking peptides (Santa Cruz Biotechnology). As for human RANK, the antibody gave a high intensity of positive staining, but unfortunately, no specific blocking peptides were available, and we
TABLE 2. Characteristics of Real-Time PCR Assays in the Study

<table>
<thead>
<tr>
<th>Target</th>
<th>Sequence (5′→3′)</th>
<th>GenBank Accession Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>OPG (+) -AATCAAATCTAAAATTGAGAATATGGTGT</td>
<td>U94332</td>
</tr>
<tr>
<td></td>
<td>(-) -GCTGGAAGCAGAGGAAGAAGGAA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>RANK (+) -CCCTGGTCAGCTCTCAACAG</td>
<td>NM_003839</td>
</tr>
<tr>
<td></td>
<td>(-) -GCTCAAAAGGAATTCAACATATG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>RANKL (+) -GCCTGGTCAGCTCTCAACAG</td>
<td>NM_003701</td>
</tr>
<tr>
<td></td>
<td>(-) -AACATGAGGACCTCACCAT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>β-actin (+) -AGGCAACAGGGGCGTGAT</td>
<td>NM_001101</td>
</tr>
<tr>
<td></td>
<td>(-) -TCGCTCAAGGTGCTGACGAT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MMP-2 (+) -ATGACAGCTGACCACTGAG</td>
<td>NM_004530</td>
</tr>
<tr>
<td></td>
<td>(-) -TCAGGGCGAGAAGAACATCTT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MMP-9 (+) -GCTAAGCTGAGCTGGTGTA</td>
<td>NM_004994</td>
</tr>
<tr>
<td></td>
<td>(-) -TCTAGGTCAGCTCCGCGACA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TIMP1 (+) -GCTATCAAGGAGAATGCTT</td>
<td>NM_003254</td>
</tr>
<tr>
<td></td>
<td>(-) -TTTACATACGTTGCTCACTCTCA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TIMP2 (+) -AGGTGGACCTTGAAGAAGCCATTT</td>
<td>NM_003255</td>
</tr>
<tr>
<td></td>
<td>(-) -TCTGACGGCCTTGAAGAATCTT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GAPDH (+) -AAATTCCTGAGGACCTGCA</td>
<td>NM_001101</td>
</tr>
<tr>
<td></td>
<td>(-) -AGGATCTGCTCCTGCAA</td>
<td></td>
</tr>
</tbody>
</table>

(+) indicates forward primers; (−), reverse primers; and TIMP, tissue inhibitor of metalloproteinases.

were therefore unable to confirm its specificity by blocking experiments.

Western Immunoblotting

Western blotting was performed as described previously,13 with equal amounts of protein being separated from each sample by SDS-PAGE (10%) before being transferred to PVDF membranes. Filters were incubated with the same antibodies against OPG, RANK, and RANKL as used for immunohistochemistry. Proteins were detected by enhanced chemiluminescence with horseradish peroxidase–labeled anti-goat IgG (Santa Cruz Biotechnology) or anti-rabbit IgG (Cell Signaling). All proteins were normalized against GAPDH (Chemicon International). The enhanced-chemiluminescence–exposed films were scanned (Kodak 440 CF imaging station) and quantified with Total Laboratory 1.10 software (Phoretix).

Stimulation of Neonatal Rat Cardiomyocytes

Cardiomyocytes were isolated from 1- to 3-day-old Wistar rats as described previously.18 The purity of cardiomyocyte cultures was estimated with Hoechst dye (Molecular Probes) and Oregon green 488 (Molecular Probes) for staining of nuclei and F actin, respectively. The purity of the cultures was >92% cardiomyocytes. Cells were treated with recombinant RANKL (300 ng/mL; R&D Systems) or vehicle for 48 hours. Total RNA was isolated from harvested cells on a MagnaPure LC robot (Roche) with an RNA isolation kit II that included DNase treatment.

Stimulation of Human Skin Fibroblasts

Fibroblasts were obtained from skin biopsies of 2 healthy individuals. Human skin fibroblast (HSF) cells were cultured as described previously.19 For subcultures, the media were removed, and cells were detached from culture flasks with 0.25% trypsin. Culture medium with 20% fetal calf serum was added to stop trypsinization. Cells were then seeded at 104/dish (35 mm in diameter), and after 3 days, the culture media were removed and replaced by recombinant RANKL (1500 ng/mL; R&D Systems) or vehicle dissolved in OPTI-MEM (Invitrogen Life Technologies) before being cultured for an additional 48 hours. All studies with HSF were performed between the 10th and 19th passages in culture. Total RNA was isolated from harvested cells on a MagnaPure LC robot (Roche Molecular Systems) with an RNA isolation kit II that included DNase treatment. Cell-free supernatants were harvested after 48 hours and stored at −80°C until analysis.

Matrix Metalloproteinase Activity Assays

Total matrix metalloproteinase (MMP) activity in cell-free HSF supernatants (cultured for 48 hours) was measured by a fluorogenic peptide substrate (R&D Systems) used to assess broad-range MMP activity (MMP-1, -2, -7, -8, -9, -12, and -13 can cleave the peptide) by the protocol recommended by the manufacturer. Briefly, the MMP substrate was diluted in TCN buffer (50 mmol/L Tris HCl, by the protocol recommended by the manufacturer. Briefly, the MMP substrate was diluted in TCN buffer (50 mmol/L Tris HCl, 150 mmol/L NaCl, 10 mmol/L CaCl2; pH 7.5) and added to the supernatants (preactivated by aminophenylmercuric acetate [Sigma] for 1 hour) before incubation at 37°C. After 30 minutes, total MMP activity was determined on a fluorometer (FLX 800 Microplate Fluorescence Reader, Bio-Tek Instruments). Gelatinase activity was determined in HSF supernatant after incubation for 48 hours as described previously.20 Briefly, 20 μg of protein was incubated with a sample buffer that contained 2% SDS. Both human MMP-2 and MMP-9 standards (Chemicon) were run in separate lanes to ensure identification of MMP activity. Gels were scanned by a Kodak 440 CF imaging station (Vector), and Total Laboratory software (Phoretix) was used for quantification.

Statistical Analysis

When >2 groups were compared, the Kruskal-Wallis test was used. If a significant difference was found, the Mann-Whitney U test was used to determine the differences between each pair of groups. Coefficients of correlation were calculated by the Spearman rank test. Data are given as medians and 25th to 75th percentiles unless otherwise stated. Throughout, we report 2-tailed probability values that were considered significant when <0.05.

Results

Expression of OPG/RANK/RANKL in Experimental HF

To characterize the OPG/RANK/RANKL axis in HF, we first examined myocardial gene expression by real-time quantitative reverse transcription-PCR (RT-PCR) in an experimental rat model of postinfarction HF. As shown in Figure 1, rats with myocardial failure had markedly elevated levels of OPG mRNA in both the ischemic and nonischemic regions of LV compared with sham, with persistently raised levels through-
out the observation period. RANKL gene expression was in general low, but significantly elevated mRNA levels were observed in the ischemic region, with increasing levels during the study period that reached noticeably high amounts after 28 days (≈200-fold increase). Finally, increased RANK gene expression was observed in both ischemic and nonischemic tissue at 2 days compared with sham but remained elevated only in the ischemic LV during the entire study. Thus, it appears that postinfarction HF in rats is characterized by persistently elevated expression of OPG, RANKL, and RANK in the ischemic region, and a similar pattern was seen in nonischemic parts of the LV for OPG.

Localization of OPG/RANK/RANKL Within the LV in Experimental HF

Strong RANK immunoreactivity was seen in cardiomyocytes from both nonfailing and failing rat hearts, with the most prominent expression in the failing myocardium (Figure 2A). In HF rats, RANK immunoreactivity was also seen in infiltrating cells in the border region between ischemic and nonischemic rat myocardial tissue (Figure 2A). RANKL and OPG immunoreactivity was also strong in cardiomyocytes, with no differences between nonfailing and failing rat hearts (Figure 2A). Furthermore, specific staining of isolated ventricular rat cardiomyocytes verified that these cells expressed all candidate proteins (Figure 2A). We also analyzed the gene expression of these mediators after separating LV tissue into cardiomyocytes and noncardiomyocytes. As shown in Figure 2B, HF rats had enhanced gene expression of OPG and RANKL in both cardiomyocyte and noncardiomyocyte tissue, with particularly strong expression in cardiomyocytes. In contrast, only cardiomyocytes showed increased RANK expression in these animals (Figure 2B).

OPG/RANK/RANKL System in Human HF

Our findings suggest that the OPG/RANK/RANKL axis is activated within the myocardium in an experimental rat model of postinfarction HF. We then examined the possible relevance of these findings in human HF. First, when analyzing serum levels of these mediators, we found that HF patients (n=102) had significantly raised OPG compared with healthy controls (n=20), with increasing levels according to clinical severity (NYHA functional class), myocardial dysfunction (cardiac index), and neurohormonal activation (N-BNP; Figure 3A). HF patients also had raised RANKL levels, but this increase appeared to be restricted to those with the most severe heart failure (ie, NYHA group IV; Figure 3A) and was significantly correlated with decreased cardiac index (r=−0.32, P<0.05). Second, when analyzing the expression of these mediators by real-time quantitative RT-PCR in T

![Figure 1. Gene expression, quantified by real-time quantitative RT-PCR relative to GAPDH, of OPG, RANKL, and RANK in sham-operated rats (n=4) and in nonischemic and ischemic regions of LV in rats with HF (n=5), 2, 7, and 28 days (d) after induction of myocardial infarction. Values are expressed as mean±SEM *P<0.05 vs sham animals; †P<0.01 vs nonischemic region.](http://circ.ahajournals.org/)

![Figure 2. A, Representative photomicrographs of rat myocardial tissue sections from LV of sham-operated rat and from border area between infarcted and noninfarcted myocardium of rat with HF. Immunostaining (brown) demonstrated fairly strong RANKL, RANK, and OPG immunoreactivities in cardiomyocytes of both nonfailing and failing myocardial tissue. RANKL immunoreactivity was also seen in numerous infiltrating cells in border region between ischemic and nonischemic rat myocardial tissue, whereas OPG immunoreactivity was only found in a few infiltrating cells. Original magnification ×400. Inset, Isolated ventricular rat cardiomyocytes (original magnification ×1000) demonstrating positive staining for all candidate proteins. B, Gene expression, quantified by real-time quantitative RT-PCR and relative to GAPDH, of OPG, RANKL, and RANK in cardiomyocyte and noncardiomyocyte tissue from LV of sham rats and rats with postinfarction HF. Values are expressed as mean±SEM. *P<0.05 vs sham.](http://circ.ahajournals.org/)
cells and monocytes from 13 HF patients and 9 healthy controls, we found that T cells from HF patients had markedly enhanced gene expression of RANKL (Figure 3B). In contrast, there were no differences in RANK expression in T cells or monocytes between congestive HF patients and controls (Figure 3B). Gene expression of RANKL in monocytes and OPG in T cells and monocytes was too low to yield quantifiable results. Third, when analyzing the expression of these candidate proteins by immunohistochemistry in the failing and nonfailing human myocardium, we found strong RANKL and particularly RANK immunoreactivity in cardiomyocytes, vascular smooth muscle cells (VSMCs), and endothelial cells in both groups, with particularly high expression in the failing myocardium (Figure 4A). OPG immunoreactivity was also strong in cardiomyocytes, particularly in the failing LV (Figure 4A). Moreover, whereas OPG immunostaining was weak in VSMCs and not detectable in endothelial cells of nonfailing hearts, intense OPG immunoreactivity was seen in both VSMCs and endothelial cells of failing human LV (Figure 4A). Furthermore, semiquantitative immunoblots confirmed that myocardial protein levels of OPG/RANK/RANKL were markedly higher in HF patients than in control samples. The most pronounced results were observed for RANKL, with a 10-fold increase and no overlap between the failing and nonfailing myocardium (Figure 4B). Because local biological activity of RANKL may depend on how much of the protein is blocked by OPG, we determined the ratio between OPG and RANKL and found a markedly lower ratio in the failing myocardium, with no overlap between the 2 groups and with a very small SD in HF (Figure 4B), which possibly indicates a local imbalance between these mediators. For all analyses in human HF, there were no significant differences between ischemic and idiopathic dilated cardiomyopathy (as illustrated for serum samples in Figure 3A).

**Effect of RANKL in Neonatal Rat Cardiomyocytes and HSF**

RANKL has been shown to induce MMP expression in osteoclasts, and unrestricted MMP activation appears to be of major importance for LV remodeling. Cardiomyocytes, and particularly cardiac fibroblasts, are of major importance for the regulation of collagen degradation within the failing myocardium by their modulation of MMP activity. To map any possible pathogenic consequences of the enhanced RANKL expression during HF, we therefore examined the effect of RANKL on MMP expression and activity in neonatal rat cardiomyocytes and HSFs. Although RANKL induced a significant increase in MMP-2 and MMP-9 gene expression in cardiomyocytes, there was no change in their inhibitors, ie, tissue inhibitors of MMP (TIMP)-2 and TIMP-1, which suggests matrix-degrading net effects (Figure 5A); however, we could not detect sufficient MMP activity for quantification in culture supernatants. We found that RANKL markedly increased the gene expression of both MMP-2 and MMP-9 in HSFs, with particularly enhancing effects on MMP-9 (≈20-fold increase), along with a modest decrease in TIMP-1 and TIMP-2 expression (Figure 5B). More importantly, these changes in gene expression were accompanied by a significant increase in gelolytic activity (MMP-2 and particularly MMP-9) and total MMP activity, demonstrating a matrix-degrading net effect (Figures 5C through 5E).

**Effect of Cardiovascular and Immunomodulating Therapy on Serum OPG Levels in Human HF**

Serum levels of OPG are regarded as a stable and reliable marker of activity in the OPG/RANK/RANKL system. We therefore examined the ability of cardiovascular (ie, β-blockers) and immunomodulating (ie, intravenous Ig) therapy to modulate serum OPG levels in human HF. Serum samples were obtained from 40 randomly selected HF pa-
tients from the Metoprolol Controlled-Release Randomised Intervention Trial in Heart Failure (MERIT-HF) trial and from all 40 HF patients in our previously published intravenous Ig trial. Although there was no difference between metoprolol (3.0 ± 0.3 versus 2.9 ± 0.3 ng/mL) and placebo (3.3 ± 0.3 versus 3.1 ± 0.3 ng/mL) in the MERIT-HF trial (data represent baseline values and values after 10 months of therapy, respectively), intravenous Ig induced a significant decrease in OPG levels (2.5 ± 0.2 versus 2.2 ± 0.2 ng/mL) compared with no change in the placebo group (2.4 ± 0.2 versus 2.4 ± 0.2 ng/mL), which resulted in a significant difference in changes (P < 0.05, intravenous Ig versus placebo after 6 months of therapy).

Discussion

The OPG/RANKL/RANK axis has been identified as a candidate mediator for paracrine signaling in bone metabolism. In the present study, we report enhanced systemic and myocardial expression of these mediators in both experimental and clinical HF. In addition, increased serum levels of OPG and RANKL were significantly correlated with functional, hemodynamic, and neurohormonal parameters for disease severity. Furthermore, in a rat model of postinfarction HF, we found increased gene expression of OPG, RANK, and RANKL in the ischemic part of the LV and, for OPG, in the nonischemic part of the LV, with persistently high levels during the observation period that involved both noncardiomyocyte and in particular cardiomyocyte tissue. Finally, RANKL increased gelanolytic (MMP-2 and MMP-9) and total MMP activity in human fibroblasts, which suggests a matrix-degrading net effect. These findings suggest a potential pathogenic role for RANKL/RANK interaction in the pathogenesis of HF.

Figure 4. A, Representative photomicrographs of human myocardial tissue sections from nonfailing donor heart (CTR) and failing explanted heart (HF) mainly showing cardiomyocytes, vascular tissue, and extracellular matrix. Enhancements show vascular tissue. Immunostaining (brown) of LV sections demonstrated strong RANKL, RANK, and OPG immunoactivities in cardiomyocytes of nonfailing and failing myocardial tissue, with staining most prominent in failing myocardium. RANKL and RANK immunoreactivity was also seen in VSMCs and endothelial cells (enhanced) from donor and explanted human hearts. OPG immunoreactivity was also found in VSMCs and endothelial cells (enhanced) but only in explanted failing hearts. Magnification ×400. B, Representative Western immunoblots of candidate proteins performed on myocardial tissue extracts from failing (P) and nonfailing (C) human myocardial tissue and graphs showing semiquantitative levels of these proteins (CHF indicates patients, n = 10; CTR indicates controls, n = 6). All proteins were normalized against GAPDH.

Figure 5. In vitro effects of RANKL in neonatal rat cardiomyocytes and HSFs; see Methods for details. Effects of 48-hour stimulation with RANKL or vehicle (CTR) on mRNA levels of MMPs and their endogenous tissue inhibitors (TIMPs) in neonatal rat cardiomyocytes (A) and HSFs (B). All gene expression data are normalized against β-actin. C, Effect of 48 hours of stimulation with RANKL or vehicle (CTR) on total MMP activity in HSF. D, Representative zymography gel showing specific activity of pro- and active MMP-2 and MMP-9 after 48 hours’ stimulation with RANKL or vehicle (CTR) in HSF. E, Densitometric quantification of zymography gels shown in Figure 5D. Data are given as mean ± SEM. *P < 0.05 vs vehicle.
Increased serum levels of OPG have been reported in coronary artery disease, with increasing levels according to disease severity, but notably, the increase in OPG in human HF was not restricted to those with ischemic cardiomyopathy. Furthermore, although increased circulating levels of RANKL have been found in myelomatosis and some bone disorders, herein we for the first time report enhanced expression of RANKL in serum and in circulating T cells in HF, with no differences in relation to etiology, which possibly reflects the fact that any potential variations in levels of these proteins (ie, OPG and RANKL) with regard to etiology may have been overshadowed by the contribution from chronic HF in itself. In fact, we have recently shown that serum OPG levels are markedly higher in the acute phase (median 3 days after myocardial infarction) than in the chronic phase (>1 month after myocardial infarction) in patients with myocardial infarction who develop HF, which further supports the theory that the influence of acute ischemia may be diluted over time. In the rat model of post–myocardial infarction HF, we found enhanced expression of OPG/RANKL/RANK in the ischemic compared with the nonischemic region of the myocardium, which illustrates that these 2 models (ie, chronic HF in human and post–myocardial infarction HF in rats) represent different aspects of the development of HF. Nonetheless, together these models show that the RANK/RANKL/OPG axis is altered in the setting of myocardial damage or failure.

Enhanced systemic expression of RANKL was accompanied by increased expression of the receptor RANK in cardiomyocytes, as shown in both human and rat HF; with regard to the failing human myocardium, enhanced RANK immunostaining was also seen in VSMCs and endothelial cells. These findings suggest a potential role for RANKL/RANK interaction in the pathogenesis of HF that involves both endocrine, paracrine, and autocrine mechanisms such as interactions between infiltrating activated T cells expressing RANKL and cardiomyocytes expressing RANK. OPG can act as a decoy receptor, binding to RANKL, preventing the interaction between RANK and RANKL. Thus, the increased expression of OPG within the failing myocardium could potentially protect against deleterious effects of RANKL; however, the markedly lower OPG/RANKL ratio found in myocardial extracts from HF patients, primarily due to a markedly higher RANKL protein expression, may possibly indicate a local inadequacy for OPG in counterbalancing the effects of RANKL. Nevertheless, stabilizing effects of OPG on the RANKL molecule cannot be excluded, and the pathophysiological consequences of the increased myocardial OPG and systemic RANKL expression in HF will have to be further elucidated.

Collagens, which surround and support myocytes and muscle fibers, are the major extracellular matrix proteins in the heart. Disruption of this collagen network may lead to myocyte slippage, ventricular dilatation, and progressivecontractile dysfunction. Thus, modulation of the balance between extracellular matrix synthesis and degradation has been suggested to play a major role in ventricular remodeling and dilation in HF. Unrestricted MMP activation appears to be of particular importance for increased matrix degrading in various disorders, and recent evidence also implicates the MMP family as potential mediators in the pathogenesis of HF. Moreover, inhibition of MMP activity in animal models of HF has been found to attenuate the onset of LV dilatation. Cardiac fibroblasts are the major site for the synthesis of collagen in the myocardium, and these cells also play an important role in the regulation of collagen degradation by MMPs. Herein, we show that RANKL markedly enhances MMP activity in human fibroblasts, with a particularly enhancing effect on MMP-9, which suggests a potential mechanism by which increased RANKL expression in HF may contribute to LV dysfunction. Thus, it is not inconceivable that enhanced RANKL expression in infiltrating T cells and myocardial tissue in HF could promote matrix degradation within the failing myocardium by stimulating MMP activity in cardiac fibroblasts, ultimately contributing to LV remodeling.

In conclusion, we show enhanced systemic and myocardial expression of members of the OPG/RANKL/RANK system in both clinical and experimental HF, which potentially contributes to LV remodeling and dilation. This suggests a role for known mediators of bone homeostasis in the pathogenesis of HF and possibly represents new targets for therapeutic intervention in this disorder.

References


Dysregulated Osteoprotegerin/RANK Ligand/RANK Axis in Clinical and Experimental Heart Failure
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