TNF-α and myocardial matrix metalloproteinases in heart failure: relationship to LV remodeling

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A MILESTONE IN THE DEVELOPMENT of left ventricular (LV) failure is myocardial remodeling. Myocardial remodeling can be defined as structural changes in the cellular and extracellular matrix resulting in compositional changes within the LV wall. An acceleration of the LV remodeling process is associated with greater morbidity and mortality in patients with LV failure (3, 16). While a number of biological systems influence myocardial matrix structure and function, a family of pro teaseas called matrix metalloproteinases (MMPs) likely contributes to the remodeling process (4, 8, 12–15, 18, 26, 29–31). MMP activity is regulated at several levels, including transcription and posttranslational modification (8, 21). MMP activity can be inhibited by an endogenous class of proteins called the tissue inhibitors of MMPs (TIMPs). A number of biologically active molecules can induce MMP expression, including the pleiotropic cytokine tumor necrosis factor (TNF)-α, which has been demonstrated to increase MMPs in vitro (10, 25). Increased TNF-α levels have been previously associated with LV myocardial remodeling (1, 17). TNF-α is synthesized as a membrane-bound protein and subsequently processed and cleaved to a soluble form that circulates as a stable homotrimer (5). TNF-α binding to cell surface receptors results in the activation of intracellular signaling cascades that can result in the formation of various transcription factors that potentially may modulate protein expression (36).

Chronic pacing in animals has been demonstrated to reliably produce LV dilation and myocardial remodeling (21, 22, 29, 30). Moreover, previous studies have also demonstrated increased myocardial MMP levels that were temporally related to the onset of the LV remodeling process (29, 30). Methods to pharmacologically modulate the actions of TNF-α have been recently described, and a TNF-α blocking protein has been recently deployed in heart failure (6, 33). The goal of this study was to test the hypothesis that concomitant administration of a TNF-α blocking protein with chronic pacing would modify myocardial MMP expression and subsequently LV remodeling in a model of progressive LV remodeling and heart failure.

METHODS

Experimental Design

Twenty-nine mongrel dogs (18–36 kg) were prepared for rapid ventricular pacing as previously described (20–22). Briefly, under isoflurane anesthesia, a unipolar pacemaker lead was placed in the right ventricular apex under fluoroscopic visualization through the external jugular vein, and a...
programmable pulse generator (Prevail 8084, Medtronic; Mississauga, Ontario, Canada) was inserted into a subcutaneous cervical pocket. The animals were allowed to recover for at least 7 days and then randomly assigned to the following groups: 1) chronic ventricular pacing at 250 beats/min for 28 days with concomitant administration of a TNF-α blocking protein (huTNFR:Fc, a soluble p75 TNF- receptor fusion protein, 0.5 mg/kg subcutaneously twice a week, Immunex; Seattle, WA, n = 7); 2) chronic right ventricular pacing at 250 beats/min for 28 days with concomitant administration of a subcutaneous saline vehicle (n = 12); or 3) sham controls (n = 10). The dosage and frequency of administration of the TNF-α blocking protein was based on previous studies (6, 35) and on pilot pharmacokinetic measurements (n = 3). These pilot pharmacokinetic studies demonstrated a plasma level of the TNF-α blocking protein of >200 ng/ml over a 5-day period. These plasma levels of the TNF-α blocking protein exceeded those necessary to demonstrate biologically active inhibition of TNF-α (6, 19, 34). At the conclusion of the treatment protocols, terminal studies were performed as described in Terminal Study: Myocardial Sampling. This protocol was reviewed and approved by the University of Toronto Animal Care and Use Committee. All animals used in this study were treated and cared for in accordance with the Canadian Council on Animal Care, Ottawa, Canada, and the principles set forth in the National Institutes of Health Guide for the Care and Use of Laboratory Animals (1996).

LV Geometry and Function

Indexes of LV size and function were obtained using echocardiographic measurements as previously described (32). The pacemakers were deactivated (chronic pacing groups only), and two-dimensional and M-mode echocardiographic studies (HP SONOS 1000, 3.5-MHz transducer; Bothell, WA) were used to image the LV from a right parasternal approach. The two-dimensional parasternal long-axis view of the LV was first recorded to precisely define the LV long axis and papillary muscles. A perpendicular view with respect to the LV long axis was then obtained to obtain the two-dimensional parasternal short-axis view. With the use of the short- and long-axis dimension, LV end-diastolic volume, LV wall thickness, and ejection fraction were computed (37).

Terminal Study: Myocardial Sampling

Under a surgical plane of thiopental anesthesia, the heart was arrested with KCl and quickly extirpated and placed in cold saline. The great vessels, atria, and right ventricle were carefully trimmed away. The lateral LV free wall was excised, cut into 500-mg samples, and snap-frozen in liquid nitrogen for subsequent analysis of MMP zymographic activity and MMP levels.

LV Zymographic MMP Assays

LV myocardial samples (500 mg) were prepared for MMP extraction as previously described (4, 24, 31). The myocardial extracts (20 μg total protein) were loaded onto electrophoretic gels (SDS-PAGE) containing a gelatin substrate (0.1%, NOVEX; San Diego, CA). After SDS-PAGE, the gels were incubated overnight in a substrate buffer (NOVEX). After incubation, the gels were stained with 0.1% amido black and digitized (AGFA Arcus II scanner). Purified human MMP-2 and MMP-9 were used as positive controls (Catalog No. CC073, Chemicon International; Temecula, CA). The prolytic regions for each sample were determined by quantitative image analysis (Gel-Pro Analyzer, Media Cybernetics; Silver Spring, MD). A 25-pixel-wide profile was constructed along the long axis of each lane, and the peaks corresponding to proteolytic zones were summated by two-dimensional integrated optical density (IOD).

LV Myocardial MMP Abundance

The relative abundance of MMPs was examined in LV myocardial extracts using standard immunoblotting procedures (4, 31, 33). Briefly, LV myocardial extracts (8.0 μg total protein) were subjected to electrophoresis on a 8% SDS-polyacrylamide gel, and the separated proteins were transferred to a nitrocellulose membrane (Trans-blot transfer medium, 0.45 μm, Bio-Rad; Hercules, CA). After the blocking and washing, the membranes were incubated overnight at 4°C in MMP-2, MMP-9, or MMP-13 antibody solutions (2 μg/ml, mouse anti-MMP2, Oncogene Research Products; Cambridge, MA; rabbit anti-MMP-9 and mouse anti-MMP-13, Chemicon). After stringent washing, the membranes were subjected to chemiluminescent activation (Renaissance Western Blotting Chemiluminescence Kit, NEQ Life Sciences; Boston, MA). The luminescent signal was detected by exposure to X-ray film (Eastman Kodak; Rochester, NY) for a time period of 5 min. Positive controls consisting of purified human MMP-2 and MMP-9 and recombinant MMP-13 (Catalog Nos. CC073 and CC068, Chemicon) were included as appropriate.

LV Myocardial TIMP Levels

Quantitative ELISA analysis for TIMP-1 was performed using previously described methods (4, 24, 28). TIMP-1 standards and LV myocardial samples were incubated in microtiter wells coated with a TIMP-1 antibody (RPN 2611, Amersham Pharmacia Biotech; Piscataway, NJ). After a wash step and incubation with a secondary antibody, a colorimetric reaction was initiated by the addition of 3,3',5,5'-tetramethylbenzidine-hydrogen peroxide in dimethylformamide, and the absorbance was read at 450 nm (Multiskan EX, Labsystems; Helsinki, Finland). The results were expressed as nanograms of TIMP-1 per gram of LV myocardial protein. One of the TIMPs, TIMP-4, has been reported previously to be highly expressed in myocardium (11). Accordingly, immunoblotting was performed for TIMP-4 using methods described previously (31). Briefly, LV myocardial extracts were prepared, transferred to nitrocellulose membranes as described in LV Myocardial MMP Abundance, and incubated overnight at 4°C in a TIMP-4 antibody solution (0.2 μg/ml, rabbit anti-TIMP-4, Chemicon). The membranes were then washed and developed as described in LV Myocardial MMP Abundance. A positive control (human TIMP-4, Catalog No. CC1066, Chemicon) was included in all immunoblotting procedures.

Myocardial Fibroblast Incubation With TNF-α Blocking Antibody

The in vivo and ex vivo measurements outlined in the preceding three sections do not directly address whether the TNF-α-blocking antibody has direct effects on steady-state MMP levels. To begin to address this issue and the minimize confounding influences that could be encountered in vivo, primary cultures of myocardial fibroblasts were incubated in the presence and absence of TNF-α blocking antibody. Primary cultures of myocardial porcine fibroblasts were established using well-described techniques (2, 27). Briefly, LV...
midmyocardial samples (1 × 2 mm) were harvested from anesthetized pigs (25 kg, n = 3, Yorkshire; Hambone Farms, SC) using sterile techniques and plated onto flasks (75 cm², Falcon, Becton-Dickinson; Franklin Lakes, NJ) containing standard cell culture media (DMEM, GIBCO-BRL/Invitrogen; Grand Island, NY) supplemented with 10% fetal bovine serum. After a 2-wk incubation period, myocardial fibroblasts had expanded and were adherent in the bottom of the flask. These primary cultures were used during cell passages 2–4 and were subsequently grown to an 80% confluent monolayer in 25-cm² flasks (Falcon). These cultures were confirmed to be fibroblasts due to the spindle-shaped morphology and a complete absence of staining for smooth muscle actin (2, 4). For these experiments, identically matched fibroblast cultures were incubated in serum-free media in the absence or presence of TNF-α blocking protein (2,000 ng/ml, huTNFR:Fc, Immunex) for 24 h. The conditioned cell media were then collected and immediately frozen for MMP zymographic analysis. This concentration of TNF-α blocking protein was chosen for these studies because it was equivalent to or exceeded previously reported peak plasma levels (6, 34). For the zymographic assays, the conditioned media were prepared in electrophoresis sample buffer (1 μg total protein) and run in duplicate for each experiment.

**Data Analysis**

To provide for a means of comparison and standardization of each zymogram, the 10 control myocardial extracts were loaded onto every zymogram in a blinded fashion along with randomly selected extracts from the two pacing groups. The composite control zymogram value in IOD units was compared with each individual experimental myocardial extract. For the immunoblotting analyses, each gel was loaded with myocardial extracts in a blinded and randomized fashion, and the values for the controls were pooled and set at 100%. IOD values for experimental myocardial extracts were expressed as the percent change from 100%. One-way ANOVA was performed on indexes of the absolute values from the MMP zymographic assays and TIMP-1 levels as well as on the indexes of LV geometry and function. If the ANOVA models revealed significant differences between experimental groups, pairwise tests of individual group means were conducted using Bonferroni-adjusted t-tests. The normalized zymographic and immunoblotting values were analyzed with using a one-sided t-test in which the mean for the null hypothesis was set at 100%. For the MMP zymographic measurements obtained from the fibroblast cell culture studies, a standard t-test was used. All statistical procedures were performed using the SYSTAT (SPSS; Chicago, IL) statistical software program. Results are presented as means ± SE. Values of P < 0.05 were considered to be statistically significant.

**RESULTS**

**Chronic Pacing and TNF-α Blocking Protein**

**Effect on LV geometry.** Chronic pacing resulted in LV dilation and pump dysfunction (Table 1). These changes in LV size and function with chronic pacing were accompanied by reduced LV wall thickness and were consistent with previous reports (21, 30, 32). In the pacing with TNF-α blocking protein group, the degree of LV dilation and wall thinning was attenuated from the chronic pacing-only values. However, the LV ejection fraction remained equivalently reduced in both chronic rapid pacing groups.

**Effects on myocardial MMP and TIMP levels.** Myocardial zymographic activity increased twofold in the chronic pacing-only group (Table 1). With control values normalized at 100%, an increase in the 92-kDa zymographic activity was observed in the chronic pacing-only group, which was attenuated to within control levels with concomitant administration of the TNF-α blocking protein (Fig. 1). A small but statistically significant increase in the 72-kDa zymographic activity was observed in the chronic pacing-only group, but values for the TNF-α blocking protein group were not different from control (Fig. 1). However, because the lower sample size in the TNF-α blocking protein group (n = 7), the statistical power for this comparison was low (0.40). Relative myocardial MMP-9 levels were increased in the chronic pacing group and were reduced to within control values in the TNF-α blocking protein group (Fig. 2). There were no observable differences in MMP-2 levels for either pacing group compared with controls (Fig. 2). MMP-13 levels were increased in the chronic pacing-only group and reduced to within-control values in the TNF-α blocking protein group (Fig. 3). TIMP-1 levels were 2.19 ± 0.29 ng TIMP-1/g protein for the controls and remained unchanged in the chronic pacing or the chronic pacing with concomitant TNF-α blocking protein group (2.08 ± 0.33 and 1.88 ± 0.36 ng TIMP-1/g protein). A robust immunoreactive signal corresponding to TIMP-4 was readily detectable in all myocardial samples (Fig. 4). TIMP-4 levels were slightly reduced from

<table>
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<th>Control</th>
<th>Chronic Pacing With Saline</th>
<th>Chronic Pacing With TNF-α Blocking Protein</th>
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<tr>
<td>LV geometry and function</td>
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<tr>
<td>End-diastolic volume, ml</td>
<td>83 ± 12</td>
<td>118 ± 10*</td>
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<td>End-diastolic wall thickness, cm</td>
<td>0.86 ± 0.06</td>
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<td>MMP zymography</td>
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<tr>
<td>92-kDa activity, pixels</td>
<td>36,848 ± 5,939</td>
<td>87,247 ± 12,912*</td>
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<tr>
<td>72-kDa activity, pixels</td>
<td>120,784 ± 7,431</td>
<td>159,637 ± 16,232*</td>
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Values are means ± SE. LV, left ventricular; MMP, matrix metalloproteinase; TNF-α, tumor necrosis factor alpha. *P < 0.05 vs. control.
control values in both pacing groups, but this did not reach statistical significance.

To determine whether the TNF-α blocking antibody influenced basal MMP levels, zymographic measurements were performed on conditioned media taken from porcine myocardial fi broblast cultures. Robust gelatinolytic activity was detected corresponding to the 72-kDa region in conditioned media in the presence and absence of the TNF-α blocking protein. After a 24-h incubation period under serum-free conditions, 72-kDa zymographic activity was similar in the conditioned media with and without the TNF-α blocking antibody (60.1 ± 4.1 × 10² vs. 50.3 ± 2.0 × 10⁴ pixels, P = 0.164). Thus the TNF-α blocking protein did not appear to significantly influence the steady-state gelatinolytic activity in a cultured myocardial fi broblast system.

**DISCUSSION**

Cytokine activation has been implicated to contribute to the progression of myocardial remodeling and LV pump dysfunction in both clinical and experimental forms of heart failure (1, 17, 33). Notably, increased activity of the cytokine TNF-α has been causally linked...
to myocardial remodeling (1, 17). While the biological basis for tissue remodeling is complex, increased expression and activation of MMPs likely contribute to the myocardial remodeling process (4, 8, 12, 14, 15, 26, 29–31). Previous in vitro studies (10, 25) have suggested that TNF-α receptor activation can induce MMPs. The present study examined the effects of TNF-α inhibition with chronic pacing and measured indexes of myocardial remodeling and MMP levels. The significant findings of the study were twofold. First, concomitant TNF-α blocking protein administration attenuated the degree of LV dilation invariably observed with chronic pacing. Second, certain myocardial MMPs were increased with chronic pacing and normalized with concomitant TNF-α blocking protein administration. These unique findings provided evidence to suggest that the cytokine TNF-α contributes to the myocardial remodeling process in evolving heart failure through the induction of MMPs.

It has been clearly demonstrated that the development of LV failure and myocardial remodeling is accompanied by increased levels of certain MMP species (28, 29, 31). For example, myocardial MMP-9 levels have been shown to be increased in both human and animal models of LV remodeling (24, 30, 32). Moreover, recent studies (8) in transgenic mice suggest that this MMP species plays a role in postinfarction myocardial remodeling. In the present study, increased myocardial zymographic activity and MMP-9 levels occurred with chronic pacing, which may have, in turn, contributed to the remodeling process. Previously thought to be restricted to a rodent collagenase, clinical studies (18, 23) have demonstrated the emergence of the interstitial collagenase MMP-13 in pathological remodeling states such as human breast cell carcinoma and osteoarthritis. More importantly, in a recent study (28), elevated MMP-13 levels were observed in end-stage human heart failure. In the present study, increased myocardial MMP-13 levels were observed to occur with chronic pacing, which were associated with the myocardial remodeling process.

To our knowledge, this is the first study to examine the effects of a TNF-α blocking protein on MMP levels in an evolving heart failure model. However, the relationship between myocardial TNF-α levels and MMP induction has been recently established in a transgenic mouse model. Specifically, in transgenic mice, myocardial overexpression of TNF-α caused LV dilation (19). In the present study, concomitant TNF-α blocking protein administration during chronic pacing normalized myocardial MMP-9 and MMP-13 levels. Interestingly, myocardial MMP-2 levels appeared unaffected by either chronic pacing or the TNF-α blocking strategy. A potential mechanism for the differential myocardial MMP levels may lie in the fact that TNF-α receptor activation results in the formation of specific DNA transcription factors such as those binding to activating protein-1 (AP-1) (36). AP-1 response elements are not uniformly present in MMP gene promoter sequences (9). Notably, AP-1 response elements are present on the genes for MMP-9 and MMP-13 but absent from the MMP-2 gene promoter (9). MMP-9 and MMP-13 expression, therefore, may potentially be induced by TNF-α stimulation and inhibited by TNF-α blockade, whereas MMP-2 induction may not occur via the same signaling pathway. The present study suggests that MMP-2 may not be induced by cytokine activation but rather is constitutively expressed in the myocardium. TNF-α may therefore influence myocardial remodeling by the selective induction of a specific portfolio of MMPs, such as MMP-9 and MMP-13, which, in turn, facilitate myocardial remodeling. Fi-

Fig. 3. Relative myocardial MMP-13 levels were increased in the chronic pacing-only group. MMP-13 myocardial levels were similar to controls in the pacing and TNF-α blocking protein group. *P < 0.05 vs. controls.

Fig. 4. A robust signal for tissue inhibitor of MMP (TIMP)-4 was detected in myocardial extracts from controls and in both rapid pacing groups. While TIMP-4 levels were reduced in the chronic pacing-only and TNF-α blocking protein groups relative to controls, these values did not reach statistical significance (P = 0.45 and P = 0.17, respectively).
nally, it should be noted that there are other signaling mechanisms for increased MMP expression that may be operative in heart failure. These include angiotensin II, catecholamine, and endothelin-1, which can potentially induce MMP expression (13).

Previous studies (12, 28–31) have demonstrated that relative MMP-2 levels were increased in end-stage human heart failure and in pacing-induced heart failure in pigs. These past observations were primarily predicated upon the quantification of 72- and 68-kDa gelatinolytic bands that likely correspond to MMP-2 (13, 23). Similar to these past reports, 72-kDa gelatinolytic activity was increased in the chronic rapid pacing group. However, in the present study, relative myocardial MMP-2 levels appeared unchanged with chronic rapid pacing. The apparent disparity between the 72-kDazymographic activity and the MMP-2 immunoblotting results are likely due to several reasons. First, relative myocardial MMP levels are likely to be determined by the summation of a number of biological and physical stimuli that are operative in the progression of the heart failure process. The severity of the heart failure process is likely to be different between the previously performed human and pig studies and that of the present study, which, in turn, may account for differences in the degree of myocardial MMP induction. Second, there are limitations to the MMP zymographic activity measurements that prevent direct inferences to relative levels of certain MMP species. Specifically, MMPs undergo a cascade of proteolytic steps, yielding an active form of MMP at lower molecular weights (23). A number of active MMP species exist within the 50- to 70-kDa range and can potentially contribute to net zymographic activity. Third, previous results have suggested that the activation of myocardial MMP-2 may be enhanced after the development of pacing-induced heart failure (29). Thus, in the present study, the increased 68- to 72-kDa zymographic activity observed in the chronic pacing group may reflect differences in activational state of MMP-2. However, it must also be recognized that MMP zymographic measurements are performed on detergent-treated myocardial samples after electrophoresis and do not reflect myocardial MMP activity or activational states in vivo.

One of the better-characterized TIMPs is TIMP-1. An important endogenous system for regulating MMP activity, TIMPs inhibit MMP activity by forming 1:1 stoichiometric complexes with MMPs and blocking access to extracellular matrix substrates (23). In the present study, increased MMP-9 and MMP-13 levels observed in the chronic pacing-alone group were not associated with a concomitant rise in TIMP-1. This alteration of TIMP-1/MMP stoichiometry that occurred with chronic rapid pacing may have favored increased MMP activity within the myocardium. Concomitant administration of the TNF-α blocking protein with chronic pacing did not affect TIMP-1levels. It has been demonstrated previously that TIMP-4 is predominantly expressed within the myocardium (11), and a robust immunoreactive signal for this TIMP species was obtained in the present study. Relative myocardial TIMP-4 levels appeared unchanged from control values by rapid pacing with and without TNF-α blocking protein treatment. Thus the increase in myocardial MMP levels observed with chronic rapid pacing were not accompanied by a parallel increase in TIMP-4 levels. In the TNF-α blocking protein group, TIMP-1 and TIMP-4 levels were similar to control values and MMP-9 and MMP-13 levels were also similar to controls. Thus the TNF-α blocking protein likely restored the balance between myocardial TIMPs and MMPs, which in turn would reduce myocardial MMP activational states. However, the present study did not directly measure in vivo stoichiometric relationships of MMPs and TIMPs.

Consistent with previous reports (21, 22, 29, 30), chronic pacing resulted in LV chamber dilation and wall thinning. Previous studies (29, 30) using this pacing model have also demonstrated increased myocardial MMP activation and myocardial remodeling. In the present study, administration of the TNF-α blocking protein, concomitant with chronic pacing, attenuated the degree of LV dilation and wall thinning, which, in turn, was associated with decreased MMP zymographic and relative MMP-9 and MMP-13 levels. Despite the fact that LV chamber dilation and the degree of wall thinning were attenuated using the TNF-α blocking strategy, the ejection fraction was not improved. However, this finding was not totally unexpected for several reasons. First, the mechanisms that contribute to the depressed LV ejection fraction with chronic rapid pacing are multifactorial and include alterations in intrinsic myocyte contractile performance and activation of a number of neurohormonal pathways (21, 22, 29, 30). Second, direct pharmacological MMP inhibition in this chronic rapid pacing model reduced the degree of LV dilation but did not influence intrinsic contractile function (30). Third, the relative changes in LV volumes with developing heart failure appear to be independent of ejection performance as it relates to clinical outcomes (3, 16). In the present study, LV ejection fraction was unchanged in the TNF-α blocking group but end-diastolic volume was reduced, implying that intrinsic myocardial contractility may have been altered. On the basis of the results from the present study, which demonstrated that interruption of TNF-α signaling influenced LV myocardial remodeling, future studies that more carefully examine these effects with respect to LV ejection performance and contractility would be warranted.

An important limitation of the present study was that the degree of TNF-α activity within the myocardial compartment was not directly measured. Direct measurement of TNF-α activity within the myocardium can be problematic because this cytokine can act in both an autocrine and paracrine manner. However, previous experimental and clinical studies (7) have identified TNF-α receptor activation in the development of severe heart failure. Furthermore, increased circulating and LV tissue TNF-α levels have recently been documented in this pacing model (20). This study employed a pharmacological approach with respect to
elucidating TNF-α myocardial activity through the use of a TNF-α blocking strategy. However, it must be recognized that this study did not definitively establish that TNF-α receptor activation resulted in MMP species induction. Moreover, this study only examined a limited number of MMPs that have been identified within the myocardium (18, 24, 28, 29, 31). Nevertheless, the present study provided in vivo evidence to suggest a mechanistic link between TNF-α induction of MMPs and the remodeling process in a model of heart failure.

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