Tumor Necrosis Factor Receptors 1 and 2 Differentially Regulate Survival, Cardiac Dysfunction, and Remodeling in Transgenic Mice With Tumor Necrosis Factor-α–Induced Cardiomyopathy

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Background—Tumor necrosis factor (TNF)-α plays a pathophysiological role in heart failure. Although both TNF receptor 1 (TNFR1) and 2 (TNFR2) are present in the heart, comparatively little is known about the role of TNFR2.

Methods and Results—We bred TNFR1-knockout (KO) or TNFR2KO mice to transgenic (TG) mice with cardiac-specific overexpression of TNF-α and analyzed resultant progeny. Six groups of male and female mice were studied: wild type (WT) with wild receptors (WT/W), TG with wild receptors (TG/W), TG with heterozygous receptor KO (TG/R1+/− or TG/R2+/−), and TG with homozygous receptor KO (TG/R1−/− or TG/R2−/−). Both male and female TG mice displayed cardiac hypertrophy, dilatation, and reduced cardiac function. Male TG mice were more severely affected than genotypically matched females and died of heart failure at a younger age. Survival, cardiac function, and remodeling of TG/R1+− and TG/R1−− mice were improved relative to TG/W mice in both males and females. However, the survival of female TG/R2+− and TG/R2−− mice was worse than that of TG/W mice, with increased left ventricular dimension and left ventricular weight/body weight ratios. The cardiac TNF-α protein level was upregulated in TG/R1−− and TG/R2−− compared with TG/W mice, whereas the level of TNF receptors was not downregulated in TG/W relative to WT/W mice.

Conclusions—Ablation of the TNFR2 gene exacerbates heart failure and reduces survival, whereas ablation of TNFR1 blunts heart failure and improves survival. Signaling via TNFR2 may play a cardioprotective role in the pathogenesis of cytokine-mediated heart failure. (Circulation. 2004;109:1892-1897.)

Key Words: heart failure ■ receptors ■ signal transduction

Tumor necrosis factor (TNF)-α may play an important role in the development of heart failure. In humans, there is a direct correlation between functional capacity and survival and circulating levels of TNF-α. Transgenic (TG) mice with cardiac-restricted overexpression of TNF-α develop cardiac dilatation, interstitial infiltrates, abnormal calcium homeostasis, increased apoptosis, extracellular matrix remodeling, ventricular arrhythmias, and early death. Although the administration of either a soluble TNF receptor or an anti-TNF-α antibody abrogates the development of heart failure in experimental animals, the use of anti-cytokines has not proven efficacious in patients with chronic heart failure. Thus, the role of and mechanism by which TNF-α may contribute to heart failure pathophysiology remain incompletely understood.

TNF-α initiates its biological effects by binding to 2 distinct cell surface receptors with approximate molecular masses of 55 kDa (TNFR1) and 75 kDa (TNFR2). Both receptors are expressed in most cell types, including cardiac myocytes. The cytoplasmic domains of TNFR1 and TNFR2 are different, and each receptor activates both distinct and overlapping intracellular signal pathways. Although most biological activities of TNF-α are signaled through TNFR1, the role of TNFR2 remains unclear. To investigate the pathophysiological importance of these receptors in a model of heart failure, we assessed whether disruption of the TNFR1 or TNFR2 gene altered the severity of heart failure in mice with cardiac-specific overexpression of TNF-α.

Methods

Generation of Mice

To disrupt TNFR1 or TNFR2 in previously characterized TNF-α TG mice (TNF1.6), we crossed TNF1.6 with TNFR1-knockout (KO)
(strain name B6.129-Tnfrsf1a<sup>tm1Mak</sup>) or TNFR2KO mice (strain name B6.129-Tnfrsf1a<sup>tm1Mak</sup>) (Jackson Laboratory, Bar Harbor, Maine). Because TNF1.6 (FVB background) and KO (C57BL/6) mice arise from different genetic backgrounds, we generated all mice as mixed genetic background (1:1 ratio of C57BL/6 and FVB) to minimize genetic heterogeneity of these mice, as previously described. Briefly, TNF1.6 female mice were crossed withTNFR1KO or TNFR2KO male mice, and resulting heterozygous mice were interbred to yield offspring with 6 different combinations of transgene (present or absent) and TNF receptor number (wild type [WT], heterozygous KO, or homozygous KO). Six groups of male and female mice were studied: wild type with wild receptors (WT/W), TNF1.6 with wild receptors (TG/W), TNF1.6 with heterozygous receptor KO (TG/R1<sup>+/−</sup> or TG/R2<sup>+/−</sup>), and TNF1.6 with homozygous receptor KO (TG/R1<sup>−/−</sup> or TG/R2<sup>−/−</sup>). The institutional animal care and use committee of Thomas Jefferson University approved the animal use protocols.

**Experimental Design**

Echocardiography was performed at 4 and 13 weeks of age to compare cardiac structure and function. After measurement of body and heart weight, hearts were fixed in 4% neutral buffered paraformaldehyde for hematoxylin-eosin staining and assessment of the degree of cell infiltration, or hearts were snap-frozen in liquid nitrogen for protein analysis.

**Echocardiographic Measurements**

M-mode echocardiographic analyses were performed with the use of a 14-MHz transducer (Sequoia C256, Acuson) as previously described. Measurements included left ventricular diastolic dimension (LVDd), anterior wall thickness (AWT), and fractional shortening (FS).

**Enzyme-Linked Immunosorbent Assay**

The protein levels were assessed with the use of enzyme-linked immunosorbent assay kits for mouse TNF-α (Quantikine, R&D Systems) according to manufacturer’s instructions, as previously described. Results were expressed as picograms of target proteins per gram of total protein.

**Quantification of Myocardial Infiltrates**

To quantify myocardial infiltrates, nuclear density (nuclei per square millimeter) was determined. In each animal, 5 independent high-powered fields (0.250×0.300 mm; 0.075 mm<sup>2</sup> area) were analyzed with the use of Image-Pro Plus Software and averaged by investigators blinded to the groups.

**Statistical Analysis**

Results are presented as mean±SD. Analysis was performed with SPSS for Windows (version 11.5). Kaplan-Meier survival curves were compared between groups with the use of log-rank tests. Data were analyzed by a 1-way ANOVA with Bonferroni multiple comparison test. Comparisons between mice analyzed at 4 and 13 weeks and between males and females were performed by unpaired t tests. A value of <0.05 was considered significant.

**Results**

**Survival Analysis**

Mice with homozgyous ablation of the TNFR1- and cardiaspecific overexpression of TNF-α (TG/R1<sup>−/−</sup>) had a markedly improved survival compared with TG mice having the WT TNFR1 genotype (TG/W). Indeed, these mice had a survival pattern that was identical to that of WT mice (WT/W). The benefit of ablating the TNFR1 was obvious in both male and female mice (Figure 1). Ablation of 1 TNFR1 allele in TG mice (TG/R1<sup>+/−</sup>) also improved survival in both males and females compared with TG/W mice, albeit to a lesser extent. By contrast, ablation of either 1 or both alleles for the TNFR2 gene in male TG mice had no effect on survival (TG/R2<sup>−/−</sup> or TG/R2<sup>−/−</sup> versus TG/W). However, ablation of the TNFR2 gene in female TG mice (TG/R2<sup>−/−</sup>) resulted in a substantially higher mortality rate compared with female TG having the WT TNFR2 genotype (TG/W). Interestingly, no significant differences in survival were observed between WT mice having ablation of a single or both TNFR2 gene alleles in either male or female mice. All male mice except WT/W and TG/R1<sup>−/−</sup> died of apparent congestive heart failure (tachypnea and dyspnea; postmortem cardiac hypertrophy and dilatation) at a significantly younger age than the genotypically matched female. These observations of gender differences in survival rate were consistent with our previous data; however, the survival rate of TG/W (50% each strain FVB, C57BL/6) was worse than that observed in TNF1.6 (100% FVB strain) in both male and female mice. In the FVB strain, the 21-week survival rate was approximately 52% for male TNF1.6 and 96% for female TNF1.6. By contrast, in the 1:1 mix of FVB and C57BL/6, all TG/W male died by 9 weeks, and only approximately 65% of TG/W females survived to 21 weeks.

**Myocardial Infiltrates**

Histological examination showed obvious interstitial infiltration in the myocardium of transgenic mice overexpressing TNF-α (TG/W) (Figure 2A). However, ablation of the TNFR1 gene markedly reduced the level of nuclear infiltration, with the nuclear density in TG/R1<sup>−/−</sup> equivalent to WT/W (Figure 2B) in both male and female mice. The extent of infiltrates in TG/R1<sup>−/−</sup> was significantly lower than that of TG/W in males (<0.05), whereas there was a trend toward
male TG/R1−/− showed a small but significant increase in LVDD relative to WT/W, which was smaller than the increase observed in male TG/R1−/+ mice. Indeed, for both males and females at both ages, the severity of cardiac dysfunction and remodeling induced by the presence of the transgene showed a direct relationship to the TNFR1 gene copy number (ie, cardiac dysfunction and dilation were minimal in TG/R1−/+ mice, worsened in TG/R1−/− mice, and severe in TG/W mice).

TG mice with the normal complement of TNF receptors (TG/W) displayed reduction of AWT, with male TG/W mice (P<0.05 relative to TG/W males at 4 weeks of age) showing a more rapid onset of this remodeling than females (P<0.05 relative to TG/W females at 13 weeks of age). Whereas ablation of the TNFR1 gene normalized AWT in 13-week-old female TG/R1−/− mice, AWT was significantly increased relative to WT mice (WT/W) in 13-week-old male TG/R−/− mice. The measurement of AWT at 13 weeks reflects the lack of cardiac hypertrophy (VW/BW) in female TG/R1−/− and the residual hypertrophy observed in male TG/R1−/−.

Consistent with the results from survival analyses, removal of the TNFR2 gene clearly did not exhibit a beneficial effect on either male or female TG mice. At 4 weeks of age, male TG/W, TG/R2+/−, and TG/R2−/− mice showed no significant difference in FS, LVDD, AWT, or VW/BW ratio. Compared with female TG/W, mice with removal of 1 or 2 TNFR2 genes (TG/R2+/− and TG/R2−/−) showed a statistically significant increase in LVDD and VW/BW at 13 weeks but not at 4 weeks, a nonsignificant trend toward decreased FS at 13 weeks, and no normalization of AWT at 13 weeks (ie, still significantly thinner than WT/W females).

Protein Expression of TNFR1, TNFR2, and TNF-α
Ablation of the appropriate TNF receptor was evidenced by the fact that TNFR1 and TNFR2 protein levels were not appreciable in the TG/R1−/+ or TG/R2+/− mice, respectively (approximately equal to or lower than the limit of detection, <1.25 pg/mg protein). In addition, no significant difference of cardiac TNFR1 protein levels was observed between the groups of mice in which TNFR1 gene was not ablated, regardless of transgene or TNFR2 gene status. Not unexpectedly, TG/R1−/− mice expressed approximately half of the TNFR1 protein detected in either WT/W or TG/W mice. Interestingly, the level of TNFR2 expression was elevated 8- to 9-fold in TG/W relative to WT/W, was decreased by removal of 1 TNFR1 gene copy (TG/R1−/+), and was completely normalized in TNFR1KO mice (TG/R1−/−). TNFR2 levels were only partially elevated by the presence of the transgene in mice with only a single copy of the TNFR2 gene (TG/R2−/+). There was no significant gender difference for expression of cardiac TNFR1 and TNFR2 protein (Table).

The expression of TNF-α protein was significantly elevated in each group of transgene-positive mice relative to WT/W, with levels in TG/R1−/+ and TG/R2−/+ significantly higher than in TG/W mice. Despite a significant elevation of TNF-α in TG/R1−/+ mice, downstream cytokines such as interleukin-1β and monocyte chemoattractant protein-1 were not detected (data not shown). In contrast to the expression of TNF receptors, the protein level of TNF-α in males was
approximately double that of females in each group except WT/W.

Discussion

This study investigated the relative importance of the 2 TNF receptors (TNFR1 and TNFR2) in a model of heart failure (TNF1.6 mice) consequent to cardiac-specific overexpression of TNF-α. Disruption of the TNFR1 gene completely normalized survival, prevented inflammation (myocarditis), limited cardiac remodeling (VW/BW ratio, LVDD, AWT), and preserved cardiac function (%FS) in mice with TNF-α-induced heart failure. By contrast, disruption of the TNFR2 gene did not alter myocarditis in transgene-positive mice but markedly exacerbated heart failure and remodeling and worsened the survival rate in mice with TNF-α-induced heart failure. The observations for TNFR2 ablation were most apparent in females because male mice expressing the TNF1.6 transgene and normal expression of the TNFR1 receptor already displayed very poor survival.

These studies demonstrate that the balance of TNFR1- or TNFR2-mediated signaling pathways is crucial to the nature and severity of heart failure and cardiac remodeling. The results from ablation of the TNFR1 gene are consistent with our prior studies, in which soluble TNF receptor–IgG fusion protein or anti–TNF-α antibody was able to limit development of cardiac dysfunction and dilation in the TNF1.6 (cardiac-restricted TNF-α overexpression in the FVB strain) model of heart failure, presumably through interaction with and sequestration of soluble, secreted TNF-α. However, both of those approaches did not completely normalize cardiac hypertrophy (VW/BW ratio), and ablation of the TNFR1 gene did not fully restrict the development of cardiac hypertrophy or increased AWT in transgene-positive male mice (TGR1/H11002/H11002). Before release from cells (to generate soluble TNF [sTNF]) by a TNF-converting enzyme, TNF-α exists as a membrane-bound protein (mTNF). The biological effects of sTNF and mTNF are not identical, with mTNF showing a greater activation of TNFR2, whereas sTNF may preferentially show a greater

Myocardial Protein Levels of TNF-α and Receptors

<table>
<thead>
<tr>
<th>TNF-α Receptors</th>
<th>WT/W</th>
<th>TG/W</th>
<th>TG/R1 −/−</th>
<th>TG/R1 +/+</th>
<th>TG/R2 +/+</th>
<th>TG/R2 −/−</th>
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<tr>
<td>TNFR1</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>M</td>
<td>32.58±6.70</td>
<td>39.20±13.16</td>
<td>14.91±4.93</td>
<td>1.95±0.45†</td>
<td>35.16±9.59</td>
<td>21.91±6.38</td>
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<tr>
<td>F</td>
<td>27.14±4.14</td>
<td>31.42±3.38</td>
<td>10.47±1.67†</td>
<td>1.84±0.34†</td>
<td>28.78±11.06</td>
<td>24.88±5.74</td>
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<tr>
<td>TNFR2</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>M</td>
<td>23.58±3.21†</td>
<td>203.27±33.44*</td>
<td>153.75±48.91*</td>
<td>21.71±7.75†</td>
<td>70.08±22.75†</td>
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<td>20.46±5.70†</td>
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<td>111.59±37.18*</td>
<td>19.02±1.84†</td>
<td>45.78±10.61†</td>
<td>3.10±1.45†</td>
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<td>TNF-α</td>
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<td>M</td>
<td>5.96±5.66†</td>
<td>256.49±150.14*</td>
<td>330.42±78.24*†</td>
<td>562.90±93.42†‡</td>
<td>262.84±115.69†‡</td>
<td>571.71±269.68†‡</td>
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<tr>
<td>F</td>
<td>2.78±1.65†</td>
<td>122.21±56.79*</td>
<td>173.90±50.14*</td>
<td>317.13±126.45†‡</td>
<td>149.05±84.09*</td>
<td>297.67±83.34†‡</td>
</tr>
</tbody>
</table>

Values are mean±SD pg/mg; n=5 to 10. M indicates male; F, female.
*P<0.05 vs WT/W; †P<0.05 vs TG/W; ‡P<0.05 vs female.
activation of TNFR1. Interestingly, TG overexpression of mTNF produces a concentric cardiac hypertrophy without progression to cardiac dilation and failure. Thus, one might speculate that preferential stimulation of the TNFR2 receptor through sequestration of sTNF, absence of the TNFR1 protein, or exclusive overexpression of mTNF may drive the cardiac hypertrophy observed in these various studies.

In the present study, TG/R1−/− mice were completely protected from myocardial inflammation, consistent with the previous report showing that TNFR1KO mice did not develop any cardiac inflammatory infiltrates in a murine model of myosin-induced myocarditis. TNFR2 signaling was not involved in myocardial inflammation in that mouse model and appears not to mediate inflammatory infiltrates consequent to TNF-α overexpression in the present model. Taken together, these results suggest that TNFR1 is a key receptor for induction of cardiac inflammatory infiltrates in TNF-α–induced cardiomyopathy. Nonetheless, all aspects of the cardiac pathology consequent to TNF-α overexpression are not related to the extent of myocardial inflammation because ablation of the TNFR2 gene markedly worsened cardiac function and remodeling without obvious increases in the severity of cardiac inflammation.

The mechanisms by which TNFR2 ablation may worsen cardiac function and remodeling are not known. In humans, elevation of soluble TNFR1 and, to a greater level, TNFR2 occurs in heart failure, and these may serve as endogenous antagonists to modulate the effects of elevated TNF-α. Thus, the worsening of heart failure and remodeling in the TG/R2−/− mice could arise from a loss of the circulating sTNFR2 antagonist. However, although we observed a striking increase in TNFR2 protein expression in the myocardium of TG/W mice (both in a 1:1 strain mixture of FVB: C57BL/6J, [this report] or a 100% background of FVB [Y. Higuchi, MD, PhD, et al, unpublished data, 2003]), we did not observe an increase in serum sTNFR2 levels in TNF1.6 mice (100% FVB; Y. Higuchi, MD, PhD, et al, unpublished data, 2003). Thus, it appears unlikely that the “cardioprotective” role of the TNFR2 gene in blunting heart failure and remodeling arose from an increase in serum sTNFR2. Although such studies need further confirmation, it seems more likely that in the present model, alterations in the balance of TNFR1 and TNFR2 signal transduction pathways define the severity of heart failure induced by chronic TNF-α exposure rather than alterations in the expression of potential TNF-α antagonists.

The observed increase of cardiac TNFR2 but not TNFR1 expression in TG/W mice is consistent with a previous report that, unlike TNFR2, the expression level of TNFR1 appears to be stable and relatively unaffected by cytokines and activating molecules. Moreover, TNFR2 is expressed broadly in a number of cells, including macrophages and CD4-positive lymphocytes, which constitute most of the infiltrating cells in TG myocardium. However, this increase in cardiac TNFR2 levels is in contrast to the observation that myocardial TNFR receptor proteins are downregulated in patients with advanced heart failure. This discrepancy may be explained by differences of species, etiology of heart failure, or severity of myocardial infiltrates.

Unexpectedly, the level of TNF-α protein was markedly increased by ablation of either the TNFR1 or TNFR2 gene. Expression of cardiac TNF-α was increased in our previous studies, in which TNF-α binding proteins were believed to increase the half-life of TNF-α while reducing biological activity. However, no such proteins were used in this study. Expression of cardiac TNF-α in TG mice arises both from the α-myosin heavy chain promoter–driven transgene (expressed in cardiac myocytes) and the endogenous TNF-α gene (expressed in infiltrating macrophages). The mechanisms leading to enhanced TNF-α production are unclear but may arise from (1) a relative preservation of transgene expression from the α-myosin heavy chain promoter due to interruption of TNF-α signaling, which is observed to downregulate α-myosin heavy chain gene expression, or (2) an inability of TNF-α to mediate a negative feedback loop because of the absence of either TNFR1 or TNFR2. Additionally, although we observed a gender-related variance in cardiac TNF-α expression, it is not clear how that difference may relate to the gender difference in survival of TG mice.

A large body of evidence suggests that TNF-α may participate in the pathophysiology of heart failure. The results of the present study suggest that TNFR1-dependent pathways may mediate adverse remodeling, whereas TNFR2-dependent mechanisms may elicit cardioprotective effects that counter heart failure progression. One possible mechanism is that these 2 receptors oppositely regulate Akt, a pro-survival protein kinase. Akt is potently inhibited by the TNFR1-induced second messenger ceramide. On the other hand, TNFR2 can specifically activate Akt through interactions between ETK tyrosine kinase and VEGFR2 in endothelial cells. A more complete elucidation of these receptor-mediated pathways in the myocardium may provide novel insights into the role of TNF-α and/or TNF receptors as targets for therapeutic interventions in patients with heart failure.

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Circulation. 2004;109:1892-1897; originally published online March 29, 2004; doi: 10.1161/01.CIR.0000124227.00670.AB
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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