Targeted Overexpression of Transmembrane Tumor Necrosis Factor Provokes a Concentric Cardiac Hypertrophic Phenotype

Ziad I. Dibbs, MD*; Abhinav Diwan, MBBS*; Shintaro Nemoto, MD, PhD; Gilberto DeFreitas, BS; Maha Abdellatif, MD, PhD; Blase A. Carabello, MD; Francis G. Spinale, MD, PhD; Giora Feuerstein, PhD; Natarajan Sivasubramanian, PhD; Douglas L. Mann, MD

Background—Tumor necrosis factor (TNF) is initially synthesized as a 26-kDa transmembrane protein that is enzymatically cleaved by TNF-α converting enzyme (TACE) to generate a 17-kDa form of “secreted” TNF. Whereas the effects of secreted TNF in the heart have been characterized extensively, the effects of transmembrane TNF in the heart are unknown.

Methods and Results—We generated lines of transgenic mice with cardiac-restricted overexpression of a noncleavable, transmembrane form of TNF. We next treated a previously generated transgenic line of mice with cardiac-restricted expression of cleavable TNF (referred to as MHCstTNF mice) with a TACE inhibitor (DPC-IDR1) to determine whether TACE inhibition would prevent the transition from concentric hypertrophy to left ventricular (LV) dilation that occurs in this line of transgenic mice. Two of the founder lines did not have a demonstrable phenotype (M-41 and M-45), whereas a third line developed a concentric hypertrophic cardiac phenotype (M-48). Characterization of the M-48 line at 6 weeks of age showed that this line developed concentric hypertrophy, with an increase in myocardial cross-sectional area and reexpression of the fetal gene program. Four weeks of TACE inhibition abrogated the LV dilation in the MHCstTNF mice and resulted in an increase in LV wall thickness and increased myocyte cross-sectional area, thus mimicking the effects observed in the mice with noncleavable, transmembrane TNF.

Conclusions—These studies show that transmembrane TNF is biologically active and provokes a concentric hypertrophic cardiac phenotype, thus suggesting that posttranslational processing (ie, secretion) of TNF is responsible for the dilated cardiomyopathic phenotype in mice with targeted, cardiac-restricted overexpression of TNF. (Circulation. 2003;108:1002-1008.)

Key Words: hypertrophy ■ growth substances ■ genes ■ tumor necrosis factor

Tumor necrosis factor (TNF) is a pleiotropic cytokine that exerts both beneficial and deleterious effects in the heart, depending on the duration, degree, and context of cytokine signaling (reviewed in Mann1). Recently, we and others have developed lines of mice with cardiac-restricted overexpression of wild-type, secreted TNF.2–4 These mice initially develop a concentric hypertrophic cardiac phenotype that transitions to a dilated cardiac phenotype over weeks to months, thus recapitulating the classic “transition to failure” that has been reported in numerous experimental models of cardiac decompensation. Although several of the potential downstream targets that are responsible for this transition have been identified in the TNF-transgenic lines of mice, the basic mechanisms that govern the transition from a concentric hypertrophic phenotype to a dilated cardiac phenotype remain poorly characterized.

Relevant to the aforementioned discussion is the observation that TNF is initially synthesized as a 26-kDa, type II transmembrane protein that is subsequently cleaved (ie, secreted) by a membrane-bound enzyme termed TNF-α-converting enzyme (TACE).5 The resultant 17-kDa TNF monomer that is “shed” from the cell surface membrane subsequently assembles as a biologically active homotrimer that is capable of initiating cellular signaling.6 Although the
presence of transmembrane TNF has been identified in the heart after acute myocardial infarction, and although TACE mRNA and protein have been demonstrated in the failing human heart, the functional significance of transmembrane TNF in the heart remains unknown.6,7 Noting that previous reports of nonmyocyte cell types have suggested that transmembrane and secreted TNF have different biological actions,8,9 we asked whether transmembrane and secreted forms of TNF might confer different biological responses in the heart and thereby explain some of the disparate beneficial and deleterious effects that have been observed for TNF. As a first step toward addressing this question, we have generated and characterized lines of transgenic mice with cardiac-restricted overexpression of a noncleavable, transmembrane form of TNF.

Methods

Generation of mTNF Transgene Construct

Polymerase chain reaction–based, site-directed mutagenesis was used to generate a transgene lacking the cleavage site (TACE site) that is responsible for TNF shedding. On the basis of the known protein sequence for the TACE site in human TNF,10 protein sequence alignment identified a cleavage site for wild-type, murine TNF located near the amino terminus between amino acids 78 and 91 (shown in red in Figure 1A). An antisense deletion oligonucleotide primer (50-mer 5′-CTCCACTTGGTGGTTTGC-3′) and the aforementioned antisense primer to generate a cDNA were performed with a sense primer (ATGACAGACAGATCCCTCC-CTGGGCCATAGAAC-3′) and the simian virus 40 polyadenylation signal was ligated to the 3′ untranslated region representing secreted TNF protein is shown in black. B, 24-kDa, membrane-bound, mutated TNF protein generated by deleting TACE site (see text for details).

Figure 1. Schematic of wild-type, cleavable TNF and mutated, noncleavable TNF constructs. A, 26-kDa, cleavable, transmembrane form of wild-type murine TNF protein with intact TACE site spanning amino acids 78 to 91 is shown by hatched bar; membrane-spanning domain is shown in gray, and 17-kDa region representing secreted TNF protein is shown in black. B, 24-kDa, membrane-bound, mutated TNF protein generated by deleting TACE site (see text for details).
>90% reduction in circulating TNF induced by lipopolysaccharide challenge in mice). MHCS-TNF mice were randomly allocated to receive diluent (water) or DPC-IDR1 at a rate of 0.25 μg/h delivered by intraperitoneal micropump (Alzet-1002, Durect Corp) and were then treated from 4 to 8 weeks of age. Serum for TNF measurements was obtained by tail bleeding on day 7 after pump implantation, and TNF levels were measured by ELISA (Biosource). The DPC-IDR1- and diluent-treated mice were studied with 2D-directed M-mode echocardiography at 4 weeks (baseline) and after 4 weeks of treatment (ie, at 8 weeks of age). Hearts were also perfusion-fixed at 8 weeks of age for routine histology and analysis of myocyte cross-sectional area. \(^\text{11}\)

Statistical Analysis
Data are expressed as mean±SEM. One-way ANOVA was used to compare myocardial and serum TNF protein levels between transgenic mice from various founder lines and FVB wild-type mice. Post hoc testing (Tukey’s) was used to assess mean differences between groups. A Student’s \(t\) test was used to detect differences in all transgenes relative to wild type; HW, heart weight; and LM, littermate control. \(^{P}<0.05\) by 1-way ANOVA.

Results
Characterization of the mTNF Construct
Preliminary controls studies with the plaque assay confirmed that expression of the mTNF construct resulted in L929 cell killing, indicating that the mTNF construct was biologically active (see online Data Supplement). Adenovirus-mediated transfection of the mTNF construct in adult feline cardiac myocytes confirmed that the mTNF construct was expressed in cardiac myocytes but was not secreted into the culture medium (see online Data Supplement), indicating that deletion of the TACE site prevented TNF cleavage.

Characterization of Mice With Cardiac-Restricted Overexpression of Membrane-Bound TNF
We obtained 4 heterozygous founder lines with cardiac-restricted overexpression of the noncleavable transmembrane TNF transgene: M-41 (2 copies), M-45 (2 copies), M-48 (6 copies), and M-46 (12 copies). The Table summarizes the morphometric data obtained at necropsy in the various founder lines compared with their respective littermate controls. Both the M-41 and M-45 founder lines did not have a demonstrable cardiac phenotype when compared with their respective littermate controls. In contrast, the M-48 line had a significant increase \((P<0.001)\) in both absolute heart weight and heart weight-to-body weight ratio when compared with littermate control mice. Attempts to characterize the founder line with higher transgene dosage were unsuccessful, insofar as the M-46 line did not breed well.

Myocardial TNF Protein Levels
Figure 3A, which depicts myocardial TNF protein levels in the M-41 (2 copies) and M-48 (6 copies) founder lines at 6 weeks of age, shows that the level of myocardial TNF protein was obtained by tail bleeding on day 7 after pump implantation, and TNF levels were measured by ELISA (Biosource). The DPC-IDR1- and diluent-treated mice were studied with 2D-directed M-mode echocardiography at 4 weeks (baseline) and after 4 weeks of treatment (ie, at 8 weeks of age). Hearts were also perfusion-fixed at 8 weeks of age for routine histology and analysis of myocyte cross-sectional area. \(^\text{11}\)

Characterization of Founder Lines of Transgenic Mice With Targeted Overexpression of Noncleavable TNF

<table>
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<th>Founder</th>
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<th>HW/BW, mg/g</th>
<th>Cardiac Phenotype</th>
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<tr>
<td>M-48</td>
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<td>Hypertrophy</td>
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Data are expressed as mean±SEM. Necropsy data at 6 weeks are presented for the M-41, M-45, and M-48 lines of mice and their respective littermate controls \((n=6/group)\). BW indicates body weight; copy, copy number of transgenes relative to wild type; HW, heart weight; and LM, littermate control. \(^*P<0.001\) vs littermate controls.

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were significantly ($P \leq 0.02$) greater in the M-48 line when compared with the M-41 line, which were in turn significantly ($P \leq 0.02$) greater than the myocardial TNF levels in littermate control wild-type mice. Moreover, the protein levels in the MHCmTNF mice were comparable to those previously reported in lines of transgenic mice with targeted overexpression of a secretable TNF transgene. Circulating levels of TNF in the M-41 and M-48 founder lines were negligible (Figure 3B) and were not significantly ($P = 0.08$) different when compared with those of the wild-type mice.

Kaplan-Meier Analysis

Figure 3C depicts the Kaplan-Meier survival analysis for the M-41 and M-48 founder lines. As shown, survival in the 2 founders with 2 ($n=74$) and 6 ($n=213$) transgene copies was not significantly different when compared with wild-type littermate control mice during a follow-up period of up to 52 weeks ($P = 0.18$).

Characterization of M-48 Founder Line

Given that the M-48 founder developed a cardiac phenotype (see the Table), we focused on characterizing this line of mice in more detail. Northern blot analysis of total RNA from various tissues, including spleen, liver, lung, and heart, showed that the mTNF transgene was selectively expressed in the hearts of transgenic mice and was not detectable in other tissues (data not shown).

Figure 4. Echocardiographic characterization of littermate controls (open bars) and M-48 mice (black bars) at 6 weeks of age. A, LV end-diastolic diameter (LV EDD); B, LV wall thickness; C, LV mass-to-body weight ratio; D, LV fractional shortening ($n=7$ for littermate controls and $n=6$ for M-48 mice). *$P<0.05$ vs littermate controls.
LV Morphology

The major finding shown in Figure 3A and 3B, which depicts representative myocardial sections (midpapillary muscle level) from littermate controls and the M-48 line of mice at 6 weeks of age, is that the M-48 mice developed a concentric hypertrophy phenotype. Light microscopy (200×) of hematoxylin-and-eosin–stained myocardial sections and of cosin-stained hearts from the M-48 (Figure 5D) and littermate control (Figure 5C) mice showed that the myofibrillar architecture was qualitatively similar. As shown in Figure 5F, formal morphometric analysis of cell area in the M-48 and littermate control mice showed that myocyte cross-sectional area was significantly (P<0.05) increased in the M-48 mice (271±11 μm²) compared with littermate controls (213±6.2 μm²).

To further characterize the M-48 line of mice, we performed 2D-directed M-mode echocardiography. As shown in Figure 4, the LV end-diastolic dimension (Figure 4A) was not different (P=0.729) in the M-48 line of mice when compared with littermate controls; however, LV wall thickness (Figure 4B) was significantly (P<0.001) increased in the M-48 line of mice compared with control littermates, and the ratio of LV radius to LV wall thickness was significantly decreased (P<0.001) in the M-48 line of mice (2.01±0.71) compared with littermate controls (2.94±0.09), consistent with the concentric hypertrophic phenotype observed by light microscopy. The LV mass–to–body weight ratio (Figure 4C) was also significantly (P<0.001) increased in the M-48 mice compared with littermate controls. The percentage of fractional shortening (endocardial) was significantly increased (P<0.05) in the M-48 mice compared with littermates (Figure 4D). Thus, targeted overexpression of membrane-bound TNF provokes a concentric LV hypertrophic phenotype. The concentric hypertrophy was not secondary to an increase in afterload, insofar as the systolic blood pressure and heart rate in M-48 mice were significantly lower (P≤0.04) than in the respective littermate controls (70±1/42±4 mm Hg and 205±15 bpm [M-48; n=7] versus 78±3/51±4 mm Hg and 274±25 bpm [littermates; n=6]). Furthermore, in parallel studies (A. Diwan and D.L. Mann, submitted manuscript, July 2003), we have observed that the concentric hypertrophic phenotype in the M-48 line of mice is persistent through 24 weeks of age without evidence of LV dilation.

Fetal Gene Expression

We next measured mRNA levels for ANF, SERCA2a, and α- and β-MHC to determine whether there was expression of the fetal gene program characteristic of hypertrophic growth. The salient finding shown by the Northern blot analyses depicted in Figure 5A and the group data in Figure 5B is that there was a significant (P<0.05) increase in expression of ANF and α-MHC and a significant decrease (P<0.05) in expression of β-MHC and SERCA2a in M-48 mice when compared with littermate controls.

TACE Inhibition

As noted earlier, the M-48 mouse line develops a concentric hypertrophic phenotype. Although myocardial TNF protein levels in the M-48 mice that developed concentric hypertrophy are similar to4,14 or greater than3 the protein levels that have been reported in lines of transgenic mice that overexpress wild-type TNF and subsequently develop LV dilation,4,14 we considered the possibility that higher myocardial protein levels might have been necessary to produce LV dilation in the M-48 line. To address this possibility, we used a complementary approach and treated a previously reported line of transgenic mice with cardiac overexpression of wild-type, secretable TNF (referred to as MHCsTNF2) with a TACE inhibitor (DPC-IDR1) to prevent shedding of the wild-type, secretable TNF from the membrane. The MHCsTNF mice with overexpression of secretable TNF had ~15- to 30-fold higher levels of myocardial TNF protein than did the M-48 line reported herein, and they developed LV dilation from 4 to 8 weeks of age.2 As shown in Figure 6A, treatment of MHCsTNF mice with DPC-IDR1 from 4 to 8 weeks of age led to a significant (P=0.005) reduction in the circulating levels of TNF protein compared with diluent-treated MHCsTNF mice. However, the important finding shown by Figure 6 is that treatment with DPC-IDR1 resulted in a significant increase (P=0.035) in LV wall thickness (Figure 6B) and a significant (P=0.006) decrease in LV end-diastolic dimension (Figure 6C) when compared with diluent-treated controls. Moreover, treatment with DPC-IDR1 resulted in a significant (P<0.05) increase in myocyte cross-sectional area (Figure 6D) compared with diluent controls; however, the percent increase was much less pronounced than in the wild-type, secretable TNF mice. These data suggest that TACE inhibition may have an anti-hypertrophic effect in the MHCsTNF mice.
cross-sectional area when compared with diluent-treated controls (Figure 6D). Thus, preventing TNF secretion with a TACE inhibitor prevents LV wall thinning and LV dilation and leads to an increase in myocyte cross-sectional area, thus mimicking the concentric hypertrophic phenotype observed in the M-48 line of mice with cardiac-restricted overexpression of noncleavable TNF.

**Discussion**

The results of this study, in which we have generated and characterized a line (M-48) of transgenic mice with cardiac-restricted overexpression of noncleavable, transmembrane TNF, suggest that membrane-bound TNF provokes a concentric hypertrophic cardiac phenotype. The following lines of evidence support this statement. First, the M-48 line of mice developed cardiac hypertrophy characterized by increased LV wall thickness (Figure 3A and 4B), normal LV cavity dimension (Figure 4A), and increased LV mass (Figures 3B and 4C). Notably, these changes in LV mass were accompanied by an increase in myocyte cell area, activation of the fetal gene program (Figure 5), and activation of the p44/p42 mitogen-activated protein kinase pathway (see online Data Supplement), each of which are emblematic of cardiac hypertrophic growth. Importantly, when we followed up the lines of M-48 mice for as long as 24 weeks, they did not transition to a dilated cardiac hypertrophic phenotype (A. Diwan and D.L. Mann, unpublished data [manuscript in preparation]). Moreover, survival of the M-48 line of mice did not differ significantly from that of littermate controls when followed up for as long as 52 weeks of age (Figure 2C), in contrast to the decreased survival that has been reported in mice with secretable TNF.4 Second, when we treated mice with targeted overexpression of wild-type, cleavable TNF with a hydroxamate compound (DPC-IDR1) that inhibits TNF secretion (Figure 6A), we observed an increase in LV wall thickness (Figure 6B), a decrease in LV dilation (Figure 6C), and an increase in myocyte cross-sectional area (Figure 6D), thus mimicking the cardiac phenotype observed in the M-48 line of mice with cardiac-restricted overexpression of noncleavable TNF.

**Biological Role of Transmembrane TNF**

Whereas previous studies have focused on the deleterious role of circulating (cleavable) TNF,15–17 very little is known about the role of the transmembrane form of TNF in the heart. Thus far, only one study has systematically examined the time course of myocardial TNF protein expression after myocardial infarction.6 In that study, elevated TNF protein levels were detected in the myocardium from days 1 to 35 after infarction, whereas circulating levels of TNF were not detectable, suggesting that the membrane-bound form of TNF was the primary form of TNF expressed in this model of cardiac injury. However, the biological role of transmembrane TNF was not addressed in that study. Here, we show for the first time that the transmembrane form of TNF is biologically active in the heart and provokes a concentric hypertrophic cardiac phenotype. Although the actual mechanisms by which transmembrane TNF provokes myocyte hypertrophy are unknown, 2 possibilities warrant discussion. First, TNF triggers increased protein synthesis in cultured adult cardiac myocytes.18 Given that the transmembrane form of TNF can signal in an autotrophic or juxtatrophic manner,19 transmembrane TNF might serve as an autocrine peptide growth factor in the heart. Alternatively, transmembrane TNF might stimulate hypertrophic growth through the recently described phenomenon of “outside-to-inside” signaling, wherein the cytoplasmic tail of TNF is believed to trigger intracellular signaling.20

Second, TNF upregulates the expression of a variety of different peptide growth factors, including transforming growth factor-β, endothelin, and angiotensin II.21 Accordingly, the hypertrophic effects of transmembrane TNF might also be mediated indirectly, through the upregulation of 1 or more different hypertrophic growth factors in the heart.
As noted earlier, in contrast to what has been observed in mice with cardiac-restricted overexpression of wild-type, cleavable TNF,2–4 the M-48 mice with noncleavable, transmembrane TNF developed a concentric hypertrophic phenotype. One possible explanation for this finding is the absence of a dilated cardiac phenotype in the M-48 line was simply the result of low and/or insufficient myocardial TNF protein levels. We consider this possibility unlikely for 2 reasons. First, as noted earlier, the levels of myocardial TNF protein in the M-48 line were comparable to those in transgenic mice that develop a dilated cardiac phenotype.3,14 Second, when we treated transgenic mice expressing high levels of cleavable TNF with a TACE inhibitor (DPC-IDR1), the resulting cardiac phenotype of the DPC-IDR1–treated mice mimicked the phenotype of the M-48 line of transgenic mice (Figure 6). That is, there was an increase in LV wall thickness (Figure 6B), a decrease in LV dilation (Figure 6C), and an increase in myocyte cross-sectional area (Figure 6D) in the DPC-IDR1–treated mice overexpressing cleavable TNF. Accordingly, we consider that a more likely explanation is that transmembrane TNF might not activate matrix metalloproteinases, as has been reported recently in transgenic models that develop LV dilation.2,22 Further studies will be necessary to address this interesting question.

Conclusions

There is a growing body of evidence that suggests that the short-term and self-limited expression of TNF in the heart plays an important homeostatic role in the heart (reviewed in Mann1). Nonetheless, these salutary effects of TNF have been difficult to reconcile with the known untoward effects of sustained TNF expression, including LV dysfunction,23 degradation of extracellular matrix proteins,2,22 and LV remodeling,2,4 any or all of which might directly contribute to the development of cardiac decompensation. In this regard, the results of the present study might help, at least in part, to explain these disparate findings. That is, these studies suggest that it is not necessarily TNF expression per se that is maladaptive but rather posttranslational processing of TNF that provokes maladaptive LV dilation in the heart. Accordingly, a more detailed characterization of the M-48 line of mice in comparison with lines of transgenic mice with targeted overexpression of wild-type, cleavable TNF might provide a useful method for dissecting out the beneficial and deleterious effects of TNF-induced responses in the heart.

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References

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