Heart Failure

Excessive Tumor Necrosis Factor Activation After Infarction Contributes to Susceptibility of Myocardial Rupture and Left Ventricular Dysfunction

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Background—We investigated the potential contributions of tumor necrosis factor-α (TNF-α) on the incidence of acute myocardial rupture and subsequent chronic cardiac dysfunction after myocardial infarction (MI) in TNF knockout (TNF−/−) mice compared with C57/BL wild-type (WT) mice.

Methods and Results—Animals were randomized to left anterior descending ligation or sham operation and killed on days 3, 7, 14, and 28. We monitored cardiac rupture rate, cardiac function, inflammatory response, collagen degradation, and net collagen formation. We found the following: (1) within 1 week after MI, 53.3% (n=120) of WT mice died of cardiac rupture, in contrast to 2.5% (n=80) of TNF−/− mice; (2) inflammatory cell infiltration and cytokine expression were significantly higher in the infarct zone in WT than TNF−/− mice on day 3; (3) matrix metalloproteinase-9 and −2 activity in the infarcted myocardium was significantly higher in WT than in TNF−/− mice on day 3; (4) on day 28 after MI compared with sham, there was a significant decrease in LV developed pressure (74%) and ±dP/dt max (68.3%/65.3%) in WT mice but a less significant decrease in ±dP/dt max (25.8%/28.8%) in TNF−/− mice; (5) cardiac collagen volume fraction was lower in WT than in TNF−/− mice on days 3 and 7 but higher on day 28 compared with TNF−/− mice; and (6) a reduction in myocyte apoptosis in TNF−/− mice occurred on day 28 compared with WT mice.

Conclusions—Elevated local TNF-α in the infarcted myocardium contributes to acute myocardial rupture and chronic left ventricle dysfunction by inducing exuberant local inflammatory response, matrix and collagen degradation, increased matrix metalloproteinase activity, and apoptosis. (Circulation. 2004;110:3221-3228.)

Key Words: myocardial infarction • tumor necrosis factor • metalloproteinases • remodeling

After acute myocardial infarction (MI), a host of molecular, cellular, and physiological responses are triggered in the heart in response to injury, which can lead to acute myocardial wall rupture, life-threatening arrhythmias, and, in survivors, remodeling and transition to heart failure. Even though the incidence of myocardial rupture after infarction is now likely much lower than the previously published rates of survivors, remodeling and transition to heart failure. Even though the incidence of myocardial rupture after infarction is now likely much lower than the previously published rates of survivors, remodeling and transition to heart failure. Even though the incidence of myocardial rupture after infarction is now likely much lower than the previously published rates of survivors, remodeling and transition to heart failure.
reference to local inflammation, matrix alterations, and rates of apoptosis.

Methods

Animals

Left ventricular (LV) MI was created in 12-week-old male TNF−/− and wild-type (WT) mice (C57BL/6-TNFtm1Gk1, Jackson Laboratory) by left anterior descending coronary artery ligation, as previously described by our laboratory.6

In experiment 1, TNF−/− (n=80) and WT mice (n=120) underwent coronary artery ligation and were monitored rigorously for morbidity and mortality. After death in each animal, autopsy was immediately performed to determine the cause of death, particularly with reference to cardiac rupture.

In experiment 2, TNF−/− and WT mice were randomized into sham-operated controls (n=10) or infarction group, with preassigned euthanasia on postoperative days 3, 7, 14, and 28 (n=10 surviving animals per time point). Hearts were harvested, rinsed with PBS, frozen, and stored at −80°C until analysis.

In experiment 3, TNF−/− and WT mice with MI (n=10 per group) or sham (n=10) were killed on day 28. Hearts were collected to determine cardiac function by the Langendorff technique.

For details regarding methodology of evaluation of LV function, cardiac morphometry, Western blot analysis, and in situ hybridization, please refer to the online-only Data Supplement.

Immunohistochemistry and Collagen Content

Cryostat sections (4 μm) were cut from hearts and immunolabeled with antibodies recognizing CD45 (BD PharMingen), matrix metalloproteinase (MMP)-9 (Chemicon), and NF-κB p65 (Santa Cruz). Protocol and tests for specificity are described elsewhere in detail.6 Quantification of immunoreactive cells (20 optical fields) was done with the use of a Quantimet 600 image analysis system (Leica).

To determine collagen content, sections (6 μm thickness) were cut and stained with sirius red 3BA in saturated picric acid solution.7 With the use of an image analysis system (Leica Q500, Leica Imaging Systems), these sections were analyzed morphometrically.

Fibrillar collagen was identified in the picrosirius-stained sections by its red appearance.

MMP Zymography

Extracted proteins in nonreducing conditions were mixed with matched volume of sample buffer (Invitrogen Novex), and equal amounts of protein (20 μg) were loaded in each lane of 10% zymogram gelatin gels (Novex). After electrophoresis, the gel was incubated in 2.5% Triton-X 100 and further incubated for 16 hours in 50 mmol/L Tris-HCl buffer, pH 7.5, containing 200 mmol/L NaCl and 10 mmol/L CaCl2 at 37°C, and the gels were stained with Coomassie blue and destained in 30% methanol/10% acetic acid. White bands on a blue background indicated zones of digestion with MMP.

Andria analyst software (Bio-Rad).

Fibrillar collagen was identified in the picrosirius-stained sections by its red appearance.

Apoptosis Index Determination

The terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) assay was used to monitor the extent of DNA fragmentation as a measure of apoptosis in cryostat sections. The assay was performed according to the recommendations of the manufacturer (Boehringer Mannheim). Fluorescein-conjugated dUTP incorporated in nucleotide polymers was detected and quantified by fluorescence microscopy. TUNEL–positive nuclei were distinguished from the TUNEL–negative nuclei by counterstaining with Hoechst 33258 and were counted after being photographed.

The count was done blindly. The percentage of nuclei labeled by TUNEL per unit of cells stained with Hoechst nuclear dye reflected the apoptotic index.

Statistical Analysis

Statistical analysis of cardiac rupture rate, cardiac function, in situ hybridization, MMP activity, collagen content findings, and cell number was performed with the use of ANOVA with subgroup testing. Values are expressed as mean±SEM, with P<0.05 considered significant.

Results

Contribution of TNF in Acute Phase After MI

**TNF−/− Improved Survival Rate and Decreased Cardiac Rupture**

Ligation of the left anterior descending coronary artery produced large infarcts averaging 45±6.4% of LV wall in both WT and TNF−/− mice. Survival rate after MI was compared between WT and TNF−/− mice. In WT mice, 31.6% survived within the first week after MI, whereas 84% of TNF−/− mice survived (Figure 1A). Among the WT mice, 53.3% died of fatal cardiac rupture of the LV wall (accounting for 95% of the acute deaths), in contrast to only 2.5% of TNF−/− mice that died of cardiac rupture (P<0.05) (Figure 1B).

**TNF−/− Reduced Tissue Inflammation**

As shown in Figure 2A, nuclear immunolocalization of activated NF-κB was not observed in sham-operated WT and TNF−/− hearts (a, c). However, after MI, inflammatory cells, interstitial cells, and myocytes were positively labeled by antibody against activated NF-κB (b, d) in the infarcted myocardium in WT mice on day 3. In contrast, a significant reduction (47%) in the immunostaining of activated nuclear NF-κB (P<0.01) was observed in the day 3 infarcted myocardium of the TNF−/− mice (Figure 2B).

Figure 1. A, Kaplan-Meier survival curves in WT (n=120) and TNF−/− (n=80) mice after MI. *P<0.001 vs WT. B, Rupture rate of mice. *P<0.001 vs WT.
Inflammatory cytokines interleukin-1 (IL-1) and interleukin-6 (IL-6) were detected by Western blot analysis (Figure I online). The relative levels of IL-1 and IL-6 proteins in the infarcted myocardium increased on days 3 and 7 in both groups (Figure IA). Between WT and TNF-α mice, IL-1 and IL-6 expression in TNF-α mice was significantly decreased by 36% (IL-1) and 50% (IL-6) at day 3 and 54% and 32% at day 7, respectively (P<0.01) (Figure IB).

Representative immunofluorescent images of tissue sections from WT and TNF-α mice stained for CD45 (Figure 3A) indicated a large inflammatory infiltrate of cells not only in the infarcted zone but also in the noninfarcted region. In contrast, no evidence of inflammatory cell infiltrate was observed in the noninfarcted myocardium of both WT and TNF-α mice with MI. The changes in MMP-2 activity were not statistically different between TNF-α and WT mice.

**TNF-α Decreased MMP Expression and Activation**

As shown by immunofluorescence microscopy (Figure 4A), the number of MMP-9–positive cells was predominately localized to the peri-infarct and infarcted regions in both WT and TNF-α mice. However, the prevalence of MMP-9–positive cells was reduced in TNF-α mice (b) compared with WT mice (a). This was concordant with zymography data that indicated that MMP activity was increased in the infarcted heart on day 3 and peaked before cardiac rupture. Compared with the WT sham group, which displayed low levels of cardiac MMP-9, MMP-9 activity was significantly increased in the peri-infarct, infarcted, and noninfarcted myocardium of WT mice with MI. In TNF-α mice with MI, MMP-9 activity was significantly less (44%) than that in WT mice (P<0.05) (Figure 4B, 4C). The changes in MMP-2 activity were not statistically different between TNF-α and WT mice.

**Collagen mRNA Expression**

Type I collagen mRNA level was assessed by quantitative in situ hybridization (Figure II online). Low-level type I collagen gene expression was observed in the normal myocardium. After MI, type I collagen mRNA was markedly increased at the site of MI on day 3, reached a peak at day 7, and declined thereafter but remained higher than controls in both WT and TNF-α mice. No significant difference in type I collagen mRNA was observed at the infarct site between WT and TNF-α mice at all time points evaluated (Figure IIIB).

**TNF-α Protected Against Collagen Degradation**

The picrosirius red staining revealed that collagen fraction was decreased at the infarct site at day 3 after MI in both WT and TNF-α groups compared with sham groups. The total collagen fractions were 2.3±1.2% in WT, 4.3±0.6% in TNF-α, and 6.6±2.1% in sham-operated mice. The infarcted WT myocardium at days 3 and 7 after MI had a collagen fraction of 9.6±2.3%.
fraction that was significantly less than that in TNF⁻/⁻ mice (Figure 5A, 5B).

**Contribution of TNF in Chronic Phase After MI**

**LV Morphology**

There were no apparent differences in the infarct size of both WT and TNF⁻/⁻ groups at day 3 or 28 (Figure 6A). Furthermore, no apparent differences in infarct/circumferential fiber length were observed among the groups. Conversely, the LV circumferential length and diameter of WT mice tended to be increased compared with TNF⁻/⁻ mice. The septal thickness in WT mice showed a significant decrease compared with TNF⁻/⁻ mice (Figure 6B).

**TNF⁻/⁻ Improved Cardiac Function**

On day 28 after MI, cardiac function, including LV peak pressure and ±dP/dt, were assessed in the isolated heart (Figure 6C). In WT and TNF⁻/⁻ animals after MI, there was a highly significant decrease in ±dP/dt (68.3%/65.3%) in WT mice compared with sham controls, but this was less apparent in TNF⁻/⁻ mice (25.8%/28.8%). There was also a significant reduction in LV peak pressure (65.3%; P<0.05) in WT mice, with much less reduction (25.8%) in TNF⁻/⁻ mice.

**TNF⁻/⁻ Prevented Collagen Deposit in Noninfarcted Region After MI**

In both groups, collagen continued to accumulate at the site of infarction from weeks 1 to 4 after MI. In the infarct zone itself, the collagen deposition was not different between the 2 groups. However, increased collagen deposition was observed in WT myocardium remote from the site of infarction, including the septum, right ventricle, endocardium, and pericardium. In WT mice, multiple patchy foci of fibrosis were observed on microscopy in the remote zone, but this was conspicuously absent in TNF⁻/⁻ mice (Figure 5A, b, d). Quantitative analysis also showed increased collagen volume in the noninfarcted region (P=0.065) in WT mice compared with TNF⁻/⁻ mice (Figure 5B).

**TNF⁻/⁻ Reduced Apoptosis After MI**

There was no difference in the percentage of TUNEL-positive apoptotic cells observed in the infarcted zone of TNF⁻/⁻ mice compared with WT. However, in the noninfarcted zone, as shown in Figure 7, a significant decrease in the number of apoptotic cells was observed in TNF⁻/⁻ mice at day 28 compared with WT animals. This is consistent with the improved ventricular function in the TNF⁻/⁻ mice.
Cardiac repair after infarction is a highly complex process, involving diverse inflammatory and growth factor signaling pathways, extracellular matrix remodeling, and cell deaths and possibly progenitor cell mobilization and proliferation. In addition to intrinsic cardiac TNF-α production, inflammatory cells such as monocyte-derived macrophages release large quantities of cytokines. Previous studies have demonstrated that cardiac-specific expression of TNF-α results in myocardial inflammation, cardiac hypertrophy, progressive dilatation, and increased apoptosis, which lead to heart failure and death. Our study demonstrates that in a mouse model in which the TNF-α gene has been ablated, the extent of local leukocyte and macrophage infiltration in the infarcted myocardium is significantly reduced. This is consistent with a reduced expression of IL-6 in infarcted myocardium in TNF−/− mice, in which IL-6 mediates transendothelial migration and adhesion of neutrophils to infarct region.

Inflammatory cell infiltration in turn facilitates the expression and activation of MMPs. The latter is believed to play an important role in balancing extracellular matrix production and degradation in the myocardium. MMPs contribute to the degradation of the resident collagen network in the infarcted myocardium within days after MI. Intriguingly, our MMP-9 data indicate that it was localized mainly to leukocytes, supporting the notion that infiltrating inflammatory cells are an important source of MMPs in the infarcted myocardium. Other types of cells can also synthesize MMPs. TNF−/− mice expressed much lower levels of MMPs before cardiac rupture, along with a lower expression of IL-1.

Myocardial fibrillar collagen content depends on the balance between collagen synthesis and degradation. Type I collagen accounts for 85% of total collagen in the myocardium. Type I collagen gene expression was activated on day 3, and collagen deposition was evident on day 7. Cardiac rupture occurs mainly on days 4 and 5 after MI and therefore is primarily associated with MMP-induced collagen degradation. This concept is supported by a recent study by Peuhkurinen et al, who demonstrated that increased matrix degradation or a block in collagen synthesis was associated with an increased risk of cardiac rupture in patients. In our study there was no actual significant difference in collagen gene expression in the infarcted myocardium within the first week after MI between WT and TNF−/− mice. These findings suggest that overexpression and activities of MMPs induced by TNF at the site of MI may increase the degradation of existing collagen in the early stage of infarction, contributing to cardiac rupture. After week 1, newly formed collagen at the site of MI protects the heart from further cardiac rupture.

Early inflammatory response may have long-term consequences in ventricular function and remodeling. Our study indeed demonstrated that after MI, LV function in TNF−/− mice was significantly improved compared with WT mice. Morphometry also confirmed less LV chamber dilatation in the TNF−/− mice but no difference in infarct size. Potential mechanisms may include less apoptosis and fibrosis in the remote zone secondary to less inflammatory cytokine activation. We were surprised to see numerous patches of fibrosis in the noninfarcted myocardium at day 28 in the WT mice. Increased collagen deposition in the interstitium of the
noninfarcted region may contribute to changes in ventricular compliance, resulting in increased stiffness and altered LV performance. These findings suggest that TNF-α-induced inflammatory responses stimulate diffuse myocardial fibrosis beyond the site of infarction, contributing to chronic ventricular dysfunction.

The aforementioned observation adds to currently existing understanding of the mechanisms by which TNF-α can directly depress cardiac function or indirectly depress it via nitric oxide pathways. Previous studies have demonstrated that TNF-α can directly decrease calcium release within the myocytes, possibly mediated by sphingomyelin pathways. Indirect myocardial depression via upregulation of the inducible form of nitric oxide synthase can in turn induce desensitization of myofilaments to intracellular calcium, as well as modulate the contractile response to adrenergic stimulation. These may be initially protective mechanisms attempting to decrease the amount of mechanical work output by the heart during acute MI. However, chronically this could increase wall stress, leading to further TNF-α production and secondary matrix and myocyte remodeling and aggravated mechanical dysfunction.

A final contributing mechanism to the ventricular dysfunction may be the increased incidence of myocyte apoptosis in WT mice. In our study apoptotic myocytes were observed in the remote, noninfarcted myocardium but were seen very rarely in TNF−/− mice. The observation that TNF-α−/− reduced the incidence of apoptosis in ventricular myocytes after infarction, despite evidence elsewhere, was unexpected and counterintuitive. This suggests that TNF-α likely activates...
NF-kB operates as a proapoptotic or antiapoptotic factor depending on the context of cell type because elevated TNF-α may have an alternative role as a stress response factor. Moreover, elevated TNF-α levels have been detected in endothelial cells enriched with activated NF-κB. This notion is supported by recent studies in which endothelial cells defective in NF-κB activation showed less fibrosis and apoptosis in the remote myocardium away from the infarct zone, contributing to the improved ventricular function. However, despite these fundamental insights into TNF contributions to cardiac remodeling after MI, the best means of modulating the TNF effect clinically remains a challenge.

Acknowledgments

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