Soluble TNF receptors prevent apoptosis in infiltrating cells and promote ventricular rupture and remodeling after myocardial infarction

Yoshiya Monden a, Toru Kubota a,⁎, Takaki Tsutsumi a, Takahiro Inoue a, Shunichi Kawano a, Natsumi Kawamura a, Tomomi Ide a, Kensuke Egashira a, Hiroyuki Tsutsui b, Kenji Sunagawa a

a Department of Cardiovascular Medicine, Kyushu University Graduate School of Medical Sciences, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan
b Department of Cardiovascular Medicine, Hokkaido University Graduate School of Medicine, Kita-15, Nishi-7, Kita-ku, Sapporo 060-8638, Japan

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Abstract

Objective: Tumor necrosis factor (TNF-α) induced in damaged myocardium has been considered to be cardiotoxic. However, the negative results of RENEWAL and ATTACH prompt us to reconsider the role of TNF-α in cardiovascular diseases. The present study aimed to evaluate the effects of soluble TNF receptor treatment on myocardial infarction (MI).

Methods: An adenovirus encoding a 55-kDa TNF receptor-IgG fusion protein (AdTNFR1) was used to neutralize TNF-α, and an adenovirus encoding LacZ (AdLacZ) served as control. In the pre-MI treatment protocol, mice were given an intravenous injection of AdTNFR1 or AdLacZ 1 week before left coronary artery ligation to induce MI. In the post-MI treatment protocol, mice were treated with AdTNFR1 or AdLacZ 1 week after left coronary ligation.

Results: Treatment with AdTNFR1 neutralized bioactivity of TNF-α that was activated after MI and prevented apoptosis of infiltrating cells in infarct myocardium. However, pre-MI treatment with AdTNFR1 promoted ventricular rupture by reducing fibrosis with further activation of matrix metalloproteinase (MMP)-9. Post-MI treatment with AdTNFR1 exacerbated ventricular dysfunction and remodeling, with enhanced fibrosis of non-infarct myocardium with further MMP-2 activation.

Conclusions: Both pre- and post-MI treatments with AdTNFR1 were deleterious in a mouse MI model. Thus, TNF-α may play not only toxic but also protective roles in MI.

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Keywords: Apoptosis; Cytokines; Infarction; Matrix metalloproteinases; Remodeling

1. Introduction

Tumor necrosis factor-α (TNF-α) is a proinflammatory cytokine that exerts a wide range of biological activities [1]. TNF-α is induced in the failing human heart [2], and considered to be cardiotoxic, because in vitro studies have shown that TNF-α suppresses cardiac contractility [3], provokes myocardial hypertrophy [4] and induces apoptosis in cardiac myocytes [5]. It also has direct effects on the matrix and collagen framework, and is a potential major contributor to cardiac remodeling [6,7]. However, in anti-cytokine clinical trials, the use of either a soluble TNF receptor (RENEWAL) [8] or an anti-TNF antibody (ATTACH) [9] was not beneficial to patients with heart failure. Especially, patients who received the high dose (10 mg/kg) of infliximab (anti-TNF antibody) were more likely to die or be hospitalized for heart failure than patients in the placebo group. High doses of anti-TNF antibodies might have exacerbated the clinical condition of patients with moderate-to-severe chronic heart failure. These results suggest that TNF-α may not be exclusively toxic but may be partially protective in cardiovascular diseases.

Accumulated evidence indicates that cytokines are important mediators of wound healing and remodeling after myocardial infarction (MI). However, the roles of these
cytokines in MI remain controversial. Blockade of these cytokines has been reported to be beneficial [10–13], deleterious [14,15], or bidirectional [16,17]. Therefore, the present study was designed to assess the role of TNF-α induction in the process of wound healing and cardiac remodeling after MI. We used soluble TNF receptors to block the bioactivity of TNF-α [18]. Our results indicated that treatment with soluble TNF receptors prevented apoptosis, but significantly promoted ventricular rupture and remodeling with further activation of matrix metalloproteinases (MMPs) after MI. These results support the hypothesis that TNF-α may play not only toxic but also protective roles in MI.

Fig. 1. Multi-probe RNase protection assay for proinflammatory cytokines and chemokines in infarct myocardium on day 3 after MI: a representative assay (a) and summarized data of densitometric analysis (b). Each value is normalized to that of GAPDH in each template set as an internal control. Cytotoxicity assay for TNF-α in infarct myocardium using WEHI cell line (c). Electrophoretic mobility shift assay for activation of NF-κB in infarct myocardium (d). Values are mean±SD. TNFR1 (−) indicates pre-MI treatment with AdLacZ; TNFR1 (+), pre-MI treatment with AdTNFR1; Sham, sham-operated mice; MI, coronary ligated mice. *p<0.05 vs. Sham/LacZ mice, †p<0.05 vs. MI/LacZ mice.
2. Methods

2.1. Animal model

An MI model was produced in male ICR mice (8–10 weeks old, weighing 35–40 g) by ligating the left coronary artery [10,16,19]. Sham operation without coronary artery ligation was also performed. This study was reviewed by the Committee of the Ethics on Animal Experiment, Kyushu University Graduate School of Medical Sciences and conducted in compliance with the Guideline for Animal Experiment of Kyushu University and the Japanese Law (No.105) and Notification (No. 6). The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.2. Experimental design

To block the activity of TNF-α, 10⁹ pfu of replication-deficient recombinant adenovirus was used, which encodes the extracellular domain of the human 55-kDa TNF receptor (soluble TNFR1) coupled with a mouse IgG heavy chain (AdTNFR1), and has been proven to suppress myocardial inflammation secondary to TNF-α overexpression in our previous study [18]. An adenovirus encoding LacZ (AdLacZ) served as control. To determine the effects of soluble TNF receptors on early ventricular rupture and late remodeling after MI, two independent protocols were performed. In the pre-MI treatment protocol, four experimental groups were studied: Sham/LacZ (n=63), Sham/TNFR1 (n=63), MI/LacZ (n=155), and MI/TNFR1 (n=161). AdTNFR1 or AdLacZ was injected intravenously 1 week before left coronary artery ligation, and the effects of TNF-α blockade on early ventricular rupture were examined on days 1, 3 and 14 after MI. In the post-MI treatment protocol, four experimental groups were studied: Sham/LacZ (n=19), Sham/TNFR1 (n=18), MI/LacZ (n=36), and MI/TNFR1 (n=38). AdTNFR1 or AdLacZ was injected intravenously 1 week after left coronary artery ligation, and the effects of TNF-α blockade on late remodeling were examined on day 28 after MI. For both protocols, the mice were randomly assigned independently to the four experimental groups. Due to the high mortality in MI groups, the number of MI mice used was 2–3 times greater than the number of Sham mice.

2.3. Enzyme-linked immunosorbent assay (ELISA)

Human TNFR1 protein levels were assessed by ELISA (Quantikine, No. DRT100, R&D Systems) [18].

2.4. RNase protection assay (cytokine gene expression)

Multi-probe RNase protection assays (RPA) were performed according to the manufacture’s protocol (RiboQuant,
Table 2
Characteristics of animal models (day 14)

<table>
<thead>
<tr>
<th></th>
<th>Sham/LacZ (n=9)</th>
<th>Sham/TNFR1 (n=10)</th>
<th>MI/LacZ (n=24)</th>
<th>MI/TNFR1 (n=14)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Echocardiographic data (under anesthesia)</td>
<td></td>
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<td></td>
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<tr>
<td>Heart rate (bpm)</td>
<td>466±15</td>
<td>463±14</td>
<td>459±47</td>
<td>469±55</td>
</tr>
<tr>
<td>LV EDD (mm)</td>
<td>3.4±0.3</td>
<td>3.4±0.3</td>
<td>5.5±0.3*</td>
<td>5.5±0.4*</td>
</tr>
<tr>
<td>LV ESD (mm)</td>
<td>2.2±0.3</td>
<td>2.3±0.3</td>
<td>4.8±0.3*</td>
<td>4.8±0.4*</td>
</tr>
<tr>
<td>Fractional shortening (%)</td>
<td>34.6±2.1</td>
<td>34.4±2.1</td>
<td>12.5±2.1*</td>
<td>13.4±3.0*</td>
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<tr>
<td>Infarct wall thickness (mm)</td>
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<td>–</td>
<td>0.27±0.05</td>
<td>0.24±0.05</td>
</tr>
<tr>
<td>Non-infarct wall thickness (mm)</td>
<td>0.80±0.00</td>
<td>0.79±0.04</td>
<td>0.93±0.08*</td>
<td>0.88±0.08*</td>
</tr>
</tbody>
</table>

| Organ weight data |                |                   |                |                |
| Body wt (g)       | 39.5±1.7       | 38.1±2.5          | 37.3±3.7       | 35.7±3.7       |
| Lung wt/Bd wt (mg/g) | 5.16±0.32    | 5.39±0.39         | 10.31±4.00*    | 9.89±2.77*     |
| Pleural effusion (%) | –             | 95.5              | 100            |                |
| Infarct area (%)  | –             | –                 | 58.6±2.2       | 57.5±1.6       |

LV, left ventricular; EDD, end-diastolic diameter; ESD, end-systolic diameter; wt, weight. Values are mean±SD.

2.8. Infarct size and pathological analysis

Infarct size was determined by methods described previously for mice [10,16,19]. Briefly, the LV was cut from apex to base into 4 transverse sections. Infarct length was measured along the endocardial and epicardial surfaces from each of the LV sections, and the values from all specimens were summed. Total LV circumference was calculated as the sum of endocardial and epicardial segment lengths from all LV sections. Infarct size (in percent) was calculated as total infarct circumference divided by total LV circumference. Picrosirius red staining was performed to observe interstitial collagen fibers and determine collagen volume fraction [10,19]. Collagen volume fraction was measured at 6 fields for each heart. Myocardial infiltration was quantified by determining nuclear density (nuclei/mm2) on hematoxylin and eosin stained sections [18]. Because it is difficult to differentiate inflammatory cells from myocytes and/or fibroblasts, all nuclei were included. In each mouse, six independent high-power fields were analyzed and averaged. To further determine the number of macrophages, an immunohistochemical analysis using a specific antibody against mouse Mac-3 (macrophage marker, BD Pharmingen) was performed.

2.9. Apoptosis

Apoptosis was evaluated by a ligation-mediated PCR fragmentation (DNA laddering) assay (Maxim Biotech Inc) [20]. In addition, LV tissue sections were stained for terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) to detect apoptotic cell [20]. The number of TUNEL-positive nuclei was counted, and the data were normalized per total nuclei identified as hematoxylin-positive staining in the same sections.

2.10. MMP zymography

Gelatin zymography was performed as previously described [19]. The zymograms were digitized, and the size-fractionated bands, which indicated the MMP proteolytic levels, were measured as the integrated optical density in a rectangular region of interest.

2.11. Double immunohistochemical staining for MMP-9 and Mac-3

Double immunohistochemical staining for MMP-9 (Santa Cruz Biotech.) and Mac-3 (macrophage marker, BD Pharmingen) in infarct myocardial sections were performed by routine protocols at our laboratory to localize MMP-9 with potential MMP-producing cells [21].

2.12. Statistical analysis

The results are presented as mean±SD. Statistical comparisons were performed using ANOVA with Students–Newman–
Keuls post hoc test or unmatched Student’s *t*-test where appropriate. When the Levene test for homogeneity of variance revealed significant differences between groups, nonparametric tests (Kruskal–Wallis, the Mann–Whitney *U* test) were performed on the variables. Survival analysis was performed by the Kaplan–Meier method, and between-group difference in survival was tested by the log-rank test. Differences were considered significant at a *p* value less than 0.05.

3. Results

3.1. Pre-MI treatment protocol

3.1.1. TNF-α in infarct myocardium

Plasma levels of human TNFR1 10 days after inoculation with AdTNFR1 (or 3 days after MI or sham operation) were 512.7±93.6 (SD) μg/ml, which were similar to our previous report [18]. Multi-probe RPA was used to evaluate expression of proinflammatory cytokines and chemokines in infarct myocardium 3 days after MI or sham operation (Fig. 1a). Transcript levels of TNF-α, IL-1β, IL-6, TGF-β, MCP-1, and RANTES were significantly up-regulated in infarct myocardium, and were not affected by treatment with TNFR1 (Fig. 1b). However, as summarized in Fig. 1c, cytotoxic activity of TNF-α, which was significantly increased in infarct myocardium, was significantly attenuated by treatment with TNFR1. To examine the downstream signals of TNF-α, activation of NF-κB was evaluated in infarct myocardium on day 3 using EMSA. As shown in Fig. 1d, increased activation of NF-κB in infarct myocardium was attenuated by TNFR1 treatment (*n*=4 per group).

3.1.2. Increased ventricular rupture with TNFR1 treatment

Survival analysis was performed in four groups of mice, including Sham/LacZ, Sham/TNFR1, MI/LacZ, and MI/TNFR1. Mice that died within 12 h after the operation were excluded, because early operative mortality was not different between MI/LacZ (23.2%) and MI/TNFR1 mice (21.8%). No mice died after sham operation. In contrast, as shown in Fig. 2a, 19 of 43 MI/LacZ and 29 of 43 MI/TNFR1 mice died by the end of 2 weeks after coronary ligation. Statistical analysis indicated that pre-MI treatment with soluble TNF...
receptors significantly increased the mortality after MI ($p<0.05$). The cause of death was classified as either congestive heart failure or ventricular rupture, because no mice died without congestion (pleural effusion and increased lung weight) or blood clot in the pericardial sac. Although mortality presumably due to congestive heart failure was not different between MI/LacZ and MI/TNFR1 mice (Fig. 2b), mortality due to ventricular rupture was significantly higher in MI mice treated with TNFR1 (Fig. 2c). To elucidate the mechanisms by which pre-MI TNFR1 treatment promotes ventricular rupture, the following studies were performed.

### 3.1.3. No differences in hemodynamic parameters or infarct size

Hemodynamic parameters and infarct size on days 3 and 14 after MI are summarized in Tables 1 and 2, respectively. Echocardiography revealed that both end-diastolic and end-systolic dimensions as well as non-infarct wall thickness increased progressively after MI, with significant decreases in fractional shortening and infarct wall thickness. These changes in echocardiographic parameters were not affected by treatment with TNFR1. Furthermore, infarct size was similar in MI/LacZ and MI/TNFR1 mice. Because neither
heart rate nor systemic blood pressure was affected by TNFR1 treatment, increased ventricular rupture in MI/TNFR1 mice was not attributable to increased afterload or wall stress.

3.1.4. Reduced apoptosis and retention of infiltrating macrophages

DNA laddering assay indicated that apoptosis, which increased substantially in infarct myocardium, was markedly decreased by TNFR1 treatment (Fig. 3a). TUNEL staining was performed to identify apoptotic cells on day 3. TUNEL-positive cells were mostly infiltrating mononuclear cells besides neutrophils and myocytes (Fig. 3b). As summarized in Fig. 3c, treatment with TNFR1 significantly reduced TUNEL-positive cells in infarct myocardium.

Hematoxylin and eosin staining was performed to evaluate infiltration of inflammatory cells. Marked infiltration of inflammatory cells was observed in infarct myocardium

![Fig. 5. Collagen volume analysis of the infarct myocardium on day 3 after MI: representative micrographs of Picrosirius red staining (a) and summarized data for collagen volume fraction (b). Gelatin zymography for MMP-2 and MMP-9 in infarct myocardium on day 3 after MI: representative gel (c) and summarized data for densitometric analysis (d). Each value is expressed as the ratio to the average of MMP-2 in Sham/LacZ mice. Double immunohistochemical staining for mouse Mac-3 and MMP-9 in infarct myocardium on day 3 after MI (e). Values are mean±SD. Arrows indicate both Mac-3- and MMP-9-positive cells; TNFR1 (−), pre-MI treatment with AdLacZ; TNFR1 (+), pre-MI treatment with AdTNFR1; Sham, sham-operated mice; MI, coronary ligated mice. *p<0.05 vs. Sham/LacZ mice, †p<0.05 vs. MI/LacZ mice.]
on day 1, and subsequently subsided on day 3 (Fig. 4a). Although inflammatory cell infiltration was not significantly different between MI/LacZ and MI/TNFR1 mice on day 1, significantly more infiltrating cells remained in MI/TNFR1 myocardium on day 3, which might reflect reduced apoptosis by TNFR1 treatment (Fig. 4b). Immunohistochemical staining revealed that most of these retained infiltrating cells on day 3 were macrophages (Fig. 4c). As summarized in Fig. 4d, TNFR1 treatment significantly increased the number of macrophages in infarct myocardium on day 3.

3.1.5. Degradation of extracellular matrix with further activation of MMP-9

Collagen was visualized in LV cross-section using Picrosirius red staining (Fig. 5a). Collagen volume fraction was not affected by MI, but was significantly reduced in MI/TNFR1 mice on day 3 (Fig. 5b).

To further elucidate the mechanisms of reduced myocardial fibrosis in MI/TNFR1 mice, the activities of MMP-2 and -9 were evaluated in infarct myocardium on day 3 using gelatin zymography (Fig. 5c). The activities of MMP-2 and -9 increased significantly after MI (Fig. 5d). Treatment with TNFR1 mice on day 3 (Fig. 5b).

3.2. Post-MI treatment protocol

3.2.1. Exacerbation of cardiac dysfunction and remodeling after MI

Post-MI treatment protocol was conducted to evaluate effects of TNFR1 on ventricular remodeling, because pre-MI treatment significantly increased ventricular rupture and precluded late phase analysis. In this study, 102 mice underwent coronary ligation, and 28 died within 7 days. The

<table>
<thead>
<tr>
<th>Characteristics of animal models (day 28)</th>
<th>Sham/LacZ</th>
<th>Sham/TNFR1</th>
<th>MI/LacZ</th>
<th>MI/TNFR1</th>
</tr>
</thead>
<tbody>
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<td><strong>Echocardiographic data (under anesthesia)</strong></td>
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<td></td>
</tr>
<tr>
<td>N</td>
<td>19</td>
<td>18</td>
<td>32</td>
<td>29</td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>465±11</td>
<td>461±13</td>
<td>471±16</td>
<td>467±14</td>
</tr>
<tr>
<td>LV EDD (mm)</td>
<td>3.9±0.3</td>
<td>4.0±0.3</td>
<td>5.6±0.3*</td>
<td>6.0±0.4†</td>
</tr>
<tr>
<td>LV ESD (mm)</td>
<td>2.5±0.2</td>
<td>2.6±0.2</td>
<td>4.8±0.2*</td>
<td>5.3±0.5†</td>
</tr>
<tr>
<td>Fractional shortening (%)</td>
<td>35.6±1.4</td>
<td>35.7±1.9</td>
<td>14.1±0.9*</td>
<td>11.7±1.9†</td>
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<tr>
<td>Non-infarct wall thickness (mm)</td>
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<td>0.95±0.06*</td>
<td>1.06±0.07†</td>
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<tr>
<td>N</td>
<td>6</td>
<td>6</td>
<td>12</td>
<td>11</td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>434±21</td>
<td>435±22</td>
<td>429±25</td>
<td>434±24</td>
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<tr>
<td>Mean aortic pressure (mm Hg)</td>
<td>81.8±4.3</td>
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<td>79.2±4.5</td>
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<td>LV EDP (mm Hg)</td>
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<td>2.1±1.0</td>
<td>10.1±2.5*</td>
<td>14.5±3.3‡</td>
</tr>
<tr>
<td>LVdP/dt max (mm Hg/s)</td>
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<td>7084±422</td>
<td>5363±794*</td>
<td>4482±568‡</td>
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<td>LVdP/dt min (mm Hg/s)</td>
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<td>4944±264</td>
<td>3864±602*</td>
<td>3219±424‡</td>
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<tr>
<td>N</td>
<td>19</td>
<td>18</td>
<td>32</td>
<td>29</td>
</tr>
<tr>
<td>Body wt (g)</td>
<td>42.0±2.1</td>
<td>41.9±2.4</td>
<td>40.7±2.5</td>
<td>40.6±3.1</td>
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<tr>
<td>Lung wt/Body wt (mg/g)</td>
<td>4.88±0.30</td>
<td>4.84±0.26</td>
<td>8.50±2.70*</td>
<td>10.13±2.78‡</td>
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<tr>
<td>Pleural effusion (%)</td>
<td>0</td>
<td>0</td>
<td>71.9</td>
<td>93.1</td>
</tr>
<tr>
<td>LV wt/Body wt (mg/g)</td>
<td>2.68±0.14</td>
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<td>3.33±0.29†</td>
</tr>
<tr>
<td>LV area (%</td>
<td>13</td>
<td>12</td>
<td>20</td>
<td>18</td>
</tr>
<tr>
<td>Infarct area (%)</td>
<td>–</td>
<td>–</td>
<td>57.8±3.1</td>
<td>56.0±3.4*</td>
</tr>
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</table>

LV, left ventricular; EDD, end-diastolic diameter; ESD, end-systolic diameter; EDP, end-diastolic pressure; wt, weight. Values are mean±SD. *P<0.05 vs. Sham/LacZ. †P<0.05 vs. MI/LacZ.

surviving mice (n=74) were randomly assigned to AdLacZ or AdTNFR1 injection on day 7. Four of 36 MI/LacZ and 9 of 38 MI/TNFR1 mice died of congestive heart failure by the end of 4 weeks after ligation (Fig. 6, p=0.16). None of the sham-operated mice died. Plasma levels of human TNFR1 3 weeks after inoculation with AdTNFR1 (or 4 weeks after MI or sham operation) were 71.1±28.5 (SD) μg/ml, which were similar to our previous report [18]. Percent infarct area was not different between MI/LacZ and MI/TNFR1 mice on day 28 (Table 3).

Echocardiography showed that cardiac dimensions of surviving mice on day 28 were significantly higher in MI/LacZ mice compared to Sham/LacZ or Sham/TNFR1 (Table 3). MI/TNFR1 mice showed significantly more cavity dilatation with exacerbation of contractile dysfunction compared with MI/LacZ. Pressure measurement with a Millar catheter showed no significant differences in heart rate and aortic blood pressure among 4 groups. However, LV end-diastolic pressure, which increased significantly in MI/LacZ mice, was further

![Fig. 6](https://academic.oup.com/cardiovascres/article-abstract/73/4/794/317624)
augmented significantly in MI/TNFR1 mice. Both LV $dP/dt_{\text{max}}$ and LV $dP/dt_{\text{min}}$, which decreased significantly in MI, were further lowered significantly by post-MI treatment with soluble TNF receptors. Although body weight was similar among 4 groups, LV weight/body weight ratio increased significantly in MI and further exacerbated with TNFR1 treatment. Along with increased LV end-diastolic pressure, lung weight/body weight ratio also increased significantly in MI/LacZ mice with further increment by TNFR1 treatment. These results suggest that the post-MI treatment with soluble TNF receptors exacerbates ventricular remodeling and pulmonary congestion after MI.

3.2.2. Enhanced fibrosis in non-infarct myocardium with further activation of MMP-2

Collagen was visualized in LV cross-section using Picrosirius red staining (Fig. 7a). As summarized in Fig. 7b, collagen volume fraction, which increased in non-infarct myocardium of MI/LacZ mice, was further augmented significantly by TNFR1 treatment. To further elucidate the mechanisms of increased myocardial fibrosis in MI/TNFR1 mice, MMP-2 and -9 activities were evaluated in non-infarct myocardium on day 28 using gelatin zymography (Fig. 7c). As summarized in Fig. 7d, MMP-2 activity increased significantly...
after MI and was further activated by TNFR1 treatment, although MMP-9 activity was not altered. These results indicate that post-MI treatment with soluble TNF receptors exacerbates ventricular dysfunction and remodeling, with enhanced fibrosis and further activation of MMP-2 in non-infarct myocardium.

4. Discussion

Proinflammatory cytokines including TNF-α have been implicated in the pathogenesis of cardiovascular diseases [6,7]. However, the roles of these cytokines in myocardial infarction remain controversial. In the present study, we evaluated the effects of soluble TNF receptor treatment on MI. Treatment with soluble TNFR1 neutralized the bioactivity of TNF-α that was activated after MI, and prevented apoptosis of infiltrating cells in the infarct myocardium. However, pre-MI treatment with soluble TNFR1 promoted ventricular rupture by reducing fibrosis with further activation of MMP-9. Furthermore, post-MI treatment with soluble TNFR1 exacerbated ventricular dysfunction and remodeling, and enhanced fibrosis in non-infarct myocardium with further activation of MMP-2. Because both pre- and post-MI treatments with soluble TNFR1 were deleterious in a mouse model of MI, TNF-α may play some protective roles in MI.

We used AdTNFR1 to block the effects of TNF-α after MI. We have previously confirmed the efficacy of AdTNFR1 treatment in transgenic mice with cardiac-specific overexpression of TNF-α [18]. Injection of 10⁹ pfu of AdTNFR1 increased plasma levels of soluble TNFR1 substantially and ameliorated myocardial inflammation induced by TNF-α overexpression for 6 weeks [18]. In the present study, proinflammatory cytokines and chemokines including TNF-α, IL-1β, IL-6, TGF-β, MCP-1, and RANTES were up-regulated in infarct myocardium 3 days after coronary ligation. Pre-MI treatment with AdTNFR1 increased plasma levels of soluble TNFR1 as previously reported [18]. Although AdTNFR1 treatment did not affect the transcript levels of proinflammatory cytokines and chemokines, it significantly blocked the bioactivity of TNF-α in infarct myocardium. These results indicate that treatment with AdTNFR1 neutralizes the effects of TNF-α induced after MI. Furthermore, induction of proinflammatory cytokines and chemokines in infarct myocardium is not solely mediated by TNF-α.

Cardiac rupture is an acute fatal complication that occurs during the early phase after MI. Disorganized infarct healing and the resultant deficiency or disruption of extracellular matrix (ECM) at the infarct site may lead to myocardial rupture. In the present study, we have shown that pre-MI treatment with soluble TNFR1 increases ventricular rupture after MI without affecting systemic blood pressure or heart rate, but significantly reduced collagen content of infarct myocardium with further MMP-9 activation. Because targeted disruption of MMP-9 is known to prevent ventricular rupture after MI [22], further activation of MMP-9 may be the primary cause of increased ventricular rupture in this mouse MI model. Further activation of MMP-9 may be attributed to increased macrophages in soluble TNFR1-treated infarct myocardium, because macrophages produce substantial amounts of MMP-9 [21]. Increased macrophages may result from the anti-apoptotic effects of soluble TNFR1, because apoptosis of infiltrating cells was significantly attenuated by the treatment. These results suggest that TNF-α may be necessary for proper coordination of tissue repair processes after MI.

TNF-α is a potent inducer of apoptosis in a variety of cells including macrophages and myocytes [5,20]. In the present study, we have demonstrated that treatment with soluble TNFR1 decreases the number of apoptotic cells in infarct myocardium 3 days after MI, which is consistent with the blockade of TNF-α bioactivity by the treatment. Most of the apoptotic cells were interstitial infiltrating cells including macrophages, rather than myocytes. Because the number of infiltrating cells was not different between AdLacZ- and AdTNFR1-treated infarct myocardium 1 day after MI, the increase in relative number of macrophages in AdTNFR1-treated infarct myocardium after 3 days was probably due to decreased apoptosis of infiltrating cells. In contrast, Kurrelmeyer et al. [15] reported that targeted disruption of both TNFR1 and TNFR2 increased the size of infarct myocardium by enhancing apoptosis of cardiac myocytes within 24 h after MI, suggesting that TNF-α may protect cardiac myocytes from ischemic injury by preventing apoptosis. Furthermore, pre-MI treatment with TNF-α has been shown to ameliorate ischemic/reperfusion injury with induction of MnSOD [23]. These results suggest that the pathophysiological roles of TNF-α in the subacute phase of MI (more than 3 days) may be different from those in acute ischemia (within 24 h). TNF-α may protect cardiac myocytes from cell death in acute ischemia, but promote apoptosis of infiltrating cells to resolve inflammation in the subacute phase.

Myocardial infarction leads to complex structural alterations in both infarct and non-infarct myocardium, resulting in progressive dilatation and dysfunction of the ventricle (remodeling). TNF-α is induced in the failing human heart [2]. Its role in the progression of ventricular remodeling is inferred from findings that TNF-α suppresses cardiac contractility [3], provokes myocardial hypertrophy [4], and induces apoptosis in cultured cardiac myocytes [5]. However, in the present study, treatment with soluble TNFR1 further exacerbated ventricular dysfunction and remodeling even when given after the acute phase. These results suggest that TNF-α may protect infarct and non-infarct myocardium from the progression of remodeling. The dynamics of synthesis and breakdown of ECM proteins play an important role in post-MI LV remodeling. In particular, increased expression and activation of MMPs have been implicated in this process [21]. Several studies have demonstrated that MMPs are involved not only in cardiac rupture [19,22] but also in LV remodeling and failure [24,25]. Among the known MMPs, MMP-2 and MMP-9 have been shown to play an important role in post-MI remodeling [19,24]. Although MMP-9 is
mainly expressed in infiltrating inflammatory cells such as neutrophils and macrophages, MMP-2 is ubiquitously distributed in cardiac myocytes and fibroblasts and is up-regulated after MI. In the present study, we have demonstrated that post-MI treatment with soluble TNFR1 further activates MMP-2 in non-infarct myocardium, while the pre-MI treatment augments MMP-9 in infarct myocardium. Furthermore, we have previously reported that targeted disruption of MMP-2 as well as MMP-9 attenuates not only ventricular rupture but also ventricular remodeling after MI [13]. Therefore, exacerbation of ventricular remodeling and dysfunction after TNFR1 treatment may be mediated by further activation of MMP-2, although the precise mechanism by which MMP-2 is further activated remains undetermined.

The present findings contradict the results of Sun et al. [12] using TNF-α knockout mice. Targeted disruption of TNF-α by gene knockout significantly reduced acute cardiac rupture and improved chronic left ventricular dysfunction after MI, accompanied by a reduction of cardiac inflammatory cell infiltration, cytokine expression, and MMP-9 activity. Chronically, TNF-α knockout mice also showed less fibrosis and apoptosis in the myocardium remote from the infarct zone, which contributed to improved ventricular function [12]. These results are thoroughly opposite to ours. Although gene targeting completely eliminated TNF-α in myocardium, the absence of TNF-α during embryogenesis and development may have altered other signaling pathways to secure physiological growth of these mice. Therefore, the differences between the present study and the previous report may be attributed to the methods adopted to block the effects of TNF-α.

TNF-α initiates its biological effects by binding two distinct cell surface receptors with approximate molecular masses of 55 kDa (TNFR1) and 75 kDa (TNFR2) [1]. Both receptors are expressed in most cell types, including cardiac myocytes. Although most biological activities of TNF-α are signaled through TNFR1, the role of TNFR2 remains unclear. Targeted disruption of TNFR1 has been shown to reduce ventricular rupture and remodeling after MI [13]. Furthermore Higuchi et al. [26] have recently demonstrated that ablation of TNFR1 ameliorated heart failure and improved survival while ablation of the TNFR2 gene exacerbated heart failure and reduced survival of TNF-α transgenic mice, suggesting a cardioprotective role of TNFR2-mediated signaling. Therefore, exacerbation of ventricular rupture and remodeling observed in the present study may have derived from blockade of TNFR2-mediated signaling. Because the dissociation constants of TNFR1 and TNFR2 are 2–5 × 10^-10 and 3–7 × 10^-11, respectively [1], high levels of TNF-α interact with both TNFR1 and TNFR2, while low levels may only stimulate TNFR2 pathways. In other words, low levels of soluble TNF receptors may only block cytotoxic TNFR1, whereas high levels of soluble TNF receptors may also block the protective TNFR2. Because plasma levels of soluble TNF receptors in the present study (about 500 μg/ml) were more than 100 times higher than clinical plasma levels (approximately 0.3–3 μg/ml) [27], these high levels may block the protective TNFR2 resulting in deleterious results. Using lower doses of soluble TNF receptors may produce different results.

Another explanation for the negative results of the present study might be intrinsic toxicity of soluble TNF receptors [7]. Soluble TNF receptor also acts as a carrier protein that stabilizes TNF-α resulting in the accumulation of high concentrations of immunoreactive TNF-α [18]. Because binding of TNF-α–TNFR complexes is reversible [28], the increase in level of TNF-α–TNFR complex may lead to an increase in the duration of TNF bioactivity. Specifically, this might be the case for soluble TNFR2 fusion proteins, including etanercept that was used in RENEWAL [8]. Studies of the binding of TNF-α with soluble TNF receptor fusion proteins have shown that TNFR2 exchanges bound TNF-α about 50- to 100-fold faster than TNFR1 [29]. Thus, although both fusion proteins in equilibrium bind TNF-α with high affinity, the TNF-α–TNFR1 fusion protein complex is kinetically more stable than the TNFR2 fusion construct. Because the soluble TNF receptors used in the present study were TNFR1, the intrinsic toxicity might have been less than that if TNFR2 were used. To confirm this hypothesis, we measured the bioactivity of TNF-α in the myocardium, and found that the bioactivity was significantly reduced by the treatment. Therefore, this might not be the case in the present study.

Although the negative results of anti-TNF clinical trials in patients with heart failure prompted us to conduct the present study, there are several important aspects that are different between the present study and the clinical trials. First, acute myocardial infarction was used as a model of heart failure in the present study, while patients with chronic heart failure were recruited in the clinical trials. The roles of proinflammatory cytokines might be different in acute and chronic phases of myocardial infarction. Second, the dosage of soluble TNF receptors used in the present study was more than 100 times higher than that used in the clinical trials. Effects of high doses should be different from those of lower doses, which remain undetermined in this mouse MI model. Therefore, caution has to be exercised in interpreting the present results in association with the clinical trials.

In conclusion, treatment with soluble TNF receptors increases ventricular rupture and exacerbates cardiac remodeling in a murine model of MI. Because TNF-α seems to play both cytotoxic and protective roles in cardiovascular diseases, further studies are required to elucidate the optimal approach to modulate proinflammatory cytokines in clinical practice.

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References


