Thymosin-β₄ prevents cardiac rupture and improves cardiac function in mice with myocardial infarction

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Peng H, Xu J, Yang XP, Dai X, Peterson EL, Carretero OA, Rhaleb NE. Thymosin-β₄ prevents cardiac rupture and improves cardiac function in mice with myocardial infarction. Am J Physiol Heart Circ Physiol 307: H741–H751, 2014. First published July 11, 2014; doi:10.1152/ajpheart.00129.2014.—Thymosin-β₄ (Tβ₄) promotes cell survival, angiogenesis, and tissue regeneration and reduces inflammation. Cardiac rupture after myocardial infarction (MI) is mainly the consequence of excessive regional inflammation, whereas cardiac dysfunction after MI results from a massive cardiomyocyte loss and cardiac fibrosis. It is possible that Tβ₄ reduces the incidence of cardiac rupture post-MI via anti-inflammatory actions and that it decreases adverse cardiac remodeling and improves cardiac function by promoting cardiac cell survival and cardiac repair. C57BL/6 mice were subjected to MI and treated with either vehicle or Tβ₄ (1.6 mg·kg⁻¹·day⁻¹ ip via osmotic minipump) for 7 days or 5 wk. Mice were assessed for 1) cardiac remodeling and function by echocardiography; 2) inflammatory cell infiltration, capillary density, myocyte apoptosis, and interstitial collagen fraction histopathologically; 3) gelatinolytic activity by in situ zymography; and 4) expression of ICAM-1 and p53 by immunoblot analysis. Tβ₄ reduced cardiac rupture that was associated with a decrease in the numbers of infiltrating inflammatory cells and apoptotic myocytes, a decrease in gelatinolytic activity and ICAM-1 and p53 expression, and an increase in the numbers of CD31-positive cells. Five-week treatment with Tβ₄ ameliorated left ventricular dilation, improved cardiac function, markedly reduced interstitial collagen fraction, and increased capillary density. In a murine model of acute MI, Tβ₄ not only decreased mortality rate as a result of cardiac rupture but also significantly improved cardiac function after MI. Thus, the use of Tβ₄ could be explored as an alternative therapy in preventing cardiac rupture and restoring cardiac function in patients with MI. thymosin-β₄; mice; myocardial infarction; cardiac rupture; cardiac function

MYOCARDIAL INFARCTION (MI) is a leading cause of death in the industrialized world. MI is associated with high rates of acute death caused by ventricular wall rupture, arrhythmias, or cardiogenic shock and long-term complications such as heart failure (17, 34). The percentage of autopsy-proven rupture of total autopsied patients who died of acute MI has increased from under 10% during the 1980s (1, 36) to 12–65% in recent decades (2, 14, 22, 34, 43). This increased percentage of autopsy-confirmed rupture deaths indicates the lack of effective strategies for rupture prevention post-MI. Both clinical and experimental studies have provided strong evidence that 1)

wall rupture at an early stage of MI is mainly the consequence of excessive regional inflammation and degradation of the extracellular matrix (ECM) by proteinases, particularly matrix metalloproteinases (MMPs), resulting in infarct size extension, and 2) massive cardiomyocyte death associated with a significant decrease in capillary density causes cardiac dysfunction (6, 16, 24, 32, 47, 48).

Thymosin-β₄ (Tβ₄) is an endogenous 43-amino acid peptide found in the circulation and various organs, including the heart (29). Besides its known actin-sequestering activity (19), Tβ₄ has numerous biological functions, including promotions of cell migration, angiogenesis, cell survival, and tissue regeneration and inhibition of inflammation (8). In permanently ligated mouse and ischemia-reperfusion pig models, Tβ₄ stimulated myocardial cell migration, promoted angiogenesis and survival of cardiomyocytes, and decreased inflammation, thus improving cardiac function (4, 21). Therefore, we hypothesized that Tβ₄ prevents cardiac rupture and improves cardiac function post-MI via its anti-inflammatory, proangiogenic, and antiapoptotic actions. The murine model of acute MI shares both clinical and pathological features of post-MI with changes found in human hearts, including cardiac rupture and dysfunction (17). Using C57BL/6 mice, we examined whether Tβ₄ 1) reduces the incidence of cardiac rupture during acute MI with a focus on its effects on inflammatory cell infiltration, gelatinolytic activity, neovascularization, and myocyte apoptosis in infarct border regions and 2) improves cardiac remodeling and function during the chronic phase of MI.

MATERIALS AND METHODS

Animals

All animal experiments conducted in this study were approved by the Institutional Animal Care and Use Committee of Henry Ford Hospital. Twelve-week-old male C57BL/6 mice (26–27 g, The Jackson Laboratory, Bar Harbor, ME) were housed in vented cages with a 12:12-h light-dark cycle and fed ad libitum. The dose and drug delivery route were based on previous studies of Tβ₄ biodistribution (29) and the role of Tβ₄ in cardioprotection and repair in a murine model of acute MI (4).

Experimental Design

The present study focused on the detrimental effects of MI on the heart and the protective cardiac effects of Tβ₄ after MI. Due to the absence of any biological effect of Tβ₄ when administered at a dose of 1.6 mg·kg⁻¹·day⁻¹ for 7 days or 5 wk in a sham-operated (sham) group (pilot study), we decided not to include a sham MI + Tβ₄ group in the experimental design and statistical analysis. Thus, animals were allotted to the following three groups: 1) sham, 2) MI + vehicle, and 3) MI + Tβ₄.
Protocol 1 (7-day protocol): effect of Tβ4 on cardiac rupture. Mice were anesthetized with pentobarbital sodium (50 mg/kg ip) and an Alzet osmotic minipump (Durect, Cupertino, CA) containing either saline or Tβ4 (1.6 mg·kg⁻¹·day⁻¹, RegeneRx Biopharmaceuticals, Rockville, MD) was implanted intraperitoneally. To ensure that Tβ4 reached steady high circulating levels by the time of MI, 7 days after minipump implantation, mice were anesthetized with pentobarbital sodium (50 mg/kg ip), and MI was surgically induced by ligating the left anterior descending coronary artery as previously described (55). Animals were divided into the following three groups: 1) sham (n = 8), 2) MI + vehicle (n = 64), and 3) MI + Tβ4 (n = 22). Treatments were continued for 7 days. Mice were assessed for 1) cardiac remodeling and function by echocardiography and 2) changes in infiltrating neutrophils and macrophages, gelatinolytic activity, CD31 expression (angiogenesis), apoptotic myocytes, and ICAM-1 and p53 expression in the myocardium.

Protocol 2 (5 wk protocol): effect of Tβ4 on cardiac function. Mice were anesthetized with pentobarbital sodium (50 mg/kg ip) and subjected to MI as described above in protocol 1. The osmotic minipump containing Tβ4 was implanted immediately after MI surgery as described above in protocol 1. The following three groups were used: 1) sham (n = 10), 2) MI + vehicle (n = 21), and 3) MI + Tβ4 (n = 24). Treatment lasted for 5 wk. Cardiac remodeling and function were evaluated by echocardiography and interstitial collagen, and capillary density measured in the left ventricle (LV) by histochchemical staining.

Due to the small size of the mouse LV, some mice were used only for one or two analytic parameter(s) as indicated in the RESULTS.

Cardiac Rupture

In protocol 1, an autopsy was performed on each animal that was found dead after MI. The presence of a large amount of blood in the chest cavity and the perforation in the infarct-free wall indicated death due to cardiac rupture.

Cardiac Function by Echocardiography

Cardiac geometry and function were determined in awake mice at 7 days or 5 wk post-MI. LV diastolic dimensions (LVDd), LV diastolic areas (LVAd; obtained using two-dimensional echocardiography from the parasternal short-axis view), diastolic posterior wall thickness (PWTd), LV ejection fraction (EF), and shortening fraction (SF) were measured using a Doppler echocardiograph with a 15-MHz linear transducer (Acuson c256, Mountain View, CA) as previously described (54).

Histopathological Analysis

At the end of the experiments (7 days or 5 wk), mice were weighed and injected with an overdose of pentobarbital sodium (100 mg/kg ip). The heart was stopped at diastole by an intraventricular injection of 50 μl of 15% KCl, rapidly excised, and weighed. The LV, including the septum, was sectioned transversely into three slices from the apex to base. Slices were rapidly frozen in isopentane precooled in liquid nitrogen and then stored at −70°C until use. Cryosections of the LV (6 μm) were used for histological experiments. For the histology/morphology experiments detailed below, images of the sections were captured using a microscope (IX81, Olympus American, Melville, NY) equipped with a digital camera (DP70, Olympus American) and quantified with Microsuite Biological imaging software (Olympus).

Infarct size. A 6-μM LV cryosection was cut from each slice and stained with Masson’s trichrome. Whole heart images of the sections were captured at low magnification and processed with Microsuite Biological imaging software. Endocardial and epicardial circumferences as well as the length of the scar were measured for each slice with Microsuite Biological imaging software (Olympus). The ratio of scar length to ventricular circumference of the endocardium and epicardium of the three slices was determined, averaged, and expressed as a percentage to define infarct size. Mice with infarct size < 20% were excluded.

Immunohistochemical staining for CD68-positive macrophages, lymphocyte antigen 6B.2 alloantigen-positive neutrophils, and CD31-positive endothelial cells in infarct border regions. LV cryosections were fixed in cold acetone for 10 min and rinsed in PBS for 5 min. Sections were incubated in 3% hydrogen peroxide in distilled water to quench endogenous peroxidase activity, rinsed in PBS, and preincubated with blocking serum (2% normal serum) for 30 min, after which sections were incubated at 4°C overnight with the following primary antibodies: 1) rat anti-mouse CD68 (a macrophage marker, 1:200, AbD Serotec, Raleigh, NC); 2) rat anti-mouse lymphocyte antigen 6B.2 alloantigen (a neutrrophil marker, 1:200, AbD Serotec); and 3) rabbit polyclonal antibody against CD31 (a marker for endothelial cells, 1:200, Abcam, Cambridge, MA). Each section was then washed three times in PBS and assayed with a Vectastain ABC kit and 3-amino-9-ethylcarbazole substrate (Vector Laboratories, Burlingame, CA). Sections were counterstained with hematoxylin to show the nucleus in blue. Images of the sections were captured at high magnification. Positive staining was identified by a reddish-brown color. The number of positive cells in both border zone and remote areas and capillaries in the border zone were counted and expressed as numbers of cells or vessels per square millimeter of the myocardium, respectively. We identified the infarct border area according to the following histological features under the microscope: 1) intensified infiltrating leukocytes, 2) disarray of myofibrils, 3) necrotic myocytes and fibrotic tissue/scars, and 4) visible alive cardiomyocytes.

Gelatinolytic activity. Gelatinolytic activity in the LV was determined in cryosections using in situ zymography (18). Briefly, DQ-gelatin (Molecular Probes, Eugene, OR) was dissolved at a concentration of 1 mg/ml in distilled water and then diluted 1:10 in 1% (wt/vol) low-gelling temperature agarose (Sigma, St. Louis, MO) in PBS. Subsequently, 25 μl of this mixture were placed on air-dried LV sections and incubated for 5 h at room temperature after placement of a 22 × 22-mm coverslip. The presence of autofluorescence in sections was tested by incubation in the agarose-containing medium that lacks DQ-gelatin. Specific in situ zymography was tested by preincubation of the sections with 20 mM EDTA in PBS at room temperature for 1 h and then incubation of the sections with incubation medium. With appropriate background and autofluorescence correction, the remote areas showed no fluorescence. Images of the sections showing the borders of infarction (an area showing bright green fluorescence within the alive myocardium) were captured and processed with ImageJ (National Institutes of Health, http://rsbweb.nih.gov/ij/) to assess fluorescence intensity. The intensity was expressed as arbitrary units (mean tissue pixel intensity/tissue area).

In situ detection of cardiomyocyte apoptosis by TUNEL. LV cryosections were double labeled by immunohistochemistry and TUNEL to identify α-actinin (sarcomeric, a marker of cardiomyocytes) and apoptotic cells, respectively. Briefly, 4% formaldehyde-fixed cryosections were pretreated with protease K (1:50) for 15 min at room temperature. Sections were incubated at 4°C overnight with monoclonal antibody (Pan-α-actinin) and goat anti-rat IgG antibody (Jackson Immunoresearch Laboratories, West Grove, PA). The TUNEL assay was performed using a TdT-Fluor in situ apoptosis detection kit (Trevigen, Gaithersburg, MD). Sections were counterstained with 4′,6-diamidino-2-phenylindole to show nuclei in blue. Images of the sections showing the myocardium were captured and processed with Microsuite Biological imaging software to assess the area of the myocardium. TUNEL-positive cardiomyocytes with bright green nuclei surrounded by red stainings (α-actinin) were counted and expressed as numbers of TUNEL-positive myocytes per millimeter square.

Interstitial collagen fraction and capillary density. LV sections were cut from each frozen slice and stained separately with fluoros-
cein-labeled peanut agglutinin (Vector Laboratories) and rhodamine-labeled *Griffonia simplicifolia* lectin I (Vector Laboratories) to determine the interstitial collagen fraction (ICF) and capillary density in the heart, as we have previously described (25). Twelve fields were randomly chosen from each specific region, including noninfarct and infarct border areas.

**Western Blot Analysis**

About 20 mg of snap-frozen LV tissue from the base of the heart were thawed in 250 μl lysis buffer (Cell Signaling Technology, Danvers, MA) containing protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN) to which 1 mM PMSF was added right before use. Small pieces were disrupted and homogenized with a Polytron, with samples kept at 4°C throughout all procedures. Homogenized LV samples were centrifuged at 14,000 g for 10 min at 4°C, and supernatants containing total LV tissue lysates were collected. Protein in the supernatant was measured with Coomassie reagent (Thermo Scientific, Rockford, IL). The 60-μg aliquots of protein were kept at −72°C.

**Tβ4 content in the LV.** LV lysates were separated by electrophoresis on 4–20% Tris-glycine gels (Invitrogen, Carlsbad, CA) and electrotransferred to nitrocellulose membranes (0.2-μm pore size) at a constant voltage of 100 V for 40 min at 4°C. Tβ4 was detected by immunoblot analysis of the membranes overnight at 4°C with a rabbit polyclonal antibody against Tβ4 (FL-44, 1:500, Santa Cruz Biotechnology) and enhanced chemiluminescence reagent (Amer sham Biosciences, Piscataway, NJ). After the detection of Tβ4, the membrane was reblotted with a rabbit monoclonal antibody against GAPDH (1:3,000, Cell Signaling Technology). Band intensity was quantified by densitometry, Tβ4 was normalized to GAPDH, and the results are expressed as fold increases compared with sham groups.

**ICAM-1 and p53 protein expression.** LV lysates were subjected to Western Blot Analysis with 10% SDS-PAGE under reducing conditions and electrotransferred to nitrocellulose membranes (0.45-μm pore size). ICAM-1 and the transcriptional factor p53 were detected by immunoblot analysis of the membranes overnight at 4°C with a mouse antibody against ICAM-1 (0.1 μg/ml, R&D Systems, Minneapolis, MN) and p53 (1C12, 1:1,000, Santa Cruz Biotechnology), respectively. The rest of the immunoblot procedure was the same as that described above for Tβ4 detection. Both ICAM-1 and p53 were normalized to GAPDH, and the results are expressed as fold increases compared with sham groups.

**Statistical Analysis**

Binary data (cardiac rupture) are expressed as proportions, and groups were compared using a χ²-test for two-by-two tables. Continuous data are expressed as means ± SE, and groups were compared using a two-sample Wilcoxon test. A nonparametric method was chosen as the variances differed substantially between groups. In all settings where multiple testing was used, Hochberg’s method was used to determine significance. Adjusted P values of <0.05 were considered significant.

**RESULTS**

**Protocol 1**

**Incidence of rupture after MI.** All 86 mice with MI (MI + vehicle group, n = 64, and MI + Tβ4 group, n = 22) survived 24 h after MI surgery. Seven days after MI, 56.3% of vehicle-treated mice (36 of 64 mice) and 22.7% of Tβ4-treated mice (5 of 22 mice) died due to LV rupture (Fig. 1A), and in Tβ4-treated animals, Tβ4 content was significantly higher compared with nontreated groups (P < 0.01; Fig. 1B). Thus, a significantly lower incidence of LV rupture was observed in the Tβ4-treated group. The time window of rupture was 3–6 days after MI in both groups.

**Cardiac remodeling and function on day 7 after MI.** Echo-cardiographic data from surviving mice are shown in Table 1. MI caused LV chamber dilatation, as shown by increased LVDd and LVDa. Cardiac function as measured by EF and SF was markedly reduced by MI. Both MI-induced LV chamber dilatation and cardiac dysfunction were not changed by 7-day Tβ4 treatment. Mice with MI had increased LV weight, which was not affected by 7-day treatment with Tβ4 (Table 1). No significant difference in infarct size was noted between vehicle- and Tβ4-treated groups at 7 days post-MI (Table 1).

**Inflammatory cell infiltration in the LV.** Immunohistochemical experiments showed markedly increased infiltration of both macrophages and neutrophils in the LV infarct border 7 days after MI (Fig. 2A). Numbers of macrophages were almost doubled compared with numbers of neutrophils in the MI + vehicle group. Tβ4 significantly reduced numbers of infiltrating macrophages (Fig. 2B) and neutrophils (Fig. 2C).
examined whether inhibited macrophage infiltration by Tß4 was associated with reduced expression of ICAM-1 in the LV. We found that ICAM-1 expression was significantly decreased in Tß4-treated mice compared with vehicle-treated mice (Fig. 2D).

**Gelatinolytic activity in the border of infarct area.** In situ zymography analysis revealed that gelatinolytic activity was not detectable in the normal myocardium (Fig. 3A). However, it increased markedly in the infarct border on day 7 post-MI, which was significantly inhibited in animals treated with Tß4 (Fig. 3).

**Angiogenesis.** Numbers of CD31-positive capillaries were markedly reduced in the infarct border in the MI + vehicle group compared with the sham group, whereas Tß4 treatment significantly increased numbers of capillaries, indicating an enhanced angiogenic response (Fig. 4).

**TUNEL-positive cardiomyocytes.** TUNEL-positive cells were barely found in the myocardium of sham mice, as shown in Fig. 5A. Numbers of TUNEL-positive cardiomyocytes were remarkably increased post-MI, and positive myocytes were mainly located near the infarct area. Tß4 treatment for 7 days markedly reduced TUNEL-positive cells (Fig. 5C).

The tumor suppressor protein p53 mediates cell apoptosis through its activity as a transcription factor (49). p53 expres-

### Table 1. Infarct size, LV weight, and echocardiographic measurements 7 days post-MI

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sham Group</th>
<th>MI + Vehicle Group</th>
<th>MI + Tß4 Group</th>
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</thead>
<tbody>
<tr>
<td>Infarct size, %</td>
<td>N/A</td>
<td>37.4 ± 4.0</td>
<td>42.3 ± 4.0</td>
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<tr>
<td>LV diastolic dimension, mm</td>
<td>2.60 ± 0.08</td>
<td>5.10 ± 0.04*</td>
<td>5.24 ± 0.50†</td>
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<td>LV diastolic area, mm²</td>
<td>4.94 ± 0.58</td>
<td>23.06 ± 4.08*</td>
<td>25.07 ± 4.31†</td>
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<td>Diastolic posterior wall thickness, mm</td>
<td>0.82 ± 0.02</td>
<td>0.82 ± 0.03</td>
<td>0.85 ± 0.01</td>
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<tr>
<td>Ejection fraction, %</td>
<td>75.16 ± 3.25</td>
<td>27.54 ± 3.87*</td>
<td>30.64 ± 3.52†</td>
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<tr>
<td>Shortening fraction, %</td>
<td>51.88 ± 2.12</td>
<td>18.61 ± 2.89*</td>
<td>27.18 ± 4.31†</td>
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<tr>
<td>LV weight, mg/10 g</td>
<td>3.25 ± 0.03</td>
<td>24.75 ± 0.03</td>
<td>27.54 ± 0.84</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>3.87* 4.08‡</td>
<td>42.30 ± 0.05</td>
<td>42.30 ± 0.05</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>2.89* 3.52†</td>
<td>27.18 ± 0.03</td>
<td>27.18 ± 0.03</td>
</tr>
<tr>
<td>LV thickness, mm</td>
<td>1.74* 2.95*</td>
<td>49.41 ± 2.95*</td>
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<tr>
<td>Neutrophils</td>
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<td>Diastolic posterior wall</td>
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<td>thickness, mm</td>
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</table>

Values are means ± SE; n = 8–10 animals/group. Animals were allotted to the following three groups: 1) sham operated (sham), 2) myocardial infarction (MI) + vehicle, and 3) MI + thymosin-ß4 (Tß4). LV, left ventricular; N/A, not applicable. *P < 0.005 and †P < 0.05 vs. the sham group.

Activation of ICAM-1 signaling plays an important role in the recruitment of inflammatory immune cells, including macrophages (12). Tß4 exerts anti-inflammatory actions by inhibiting proinflammatory mediators in vivo (35, 46). Thus, we further

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**Fig. 2.** A: representative images of immunohistochemical staining for macrophages (top) and neutrophils (bottom) in the region of the infarct border at 7 days post-MI. B and C: quantitative analysis of CD68-positive cells (a marker for macrophage) and lymphocyte antigen (Ly) 6B.2-positive cells (a marker for neutrophils), respectively, showing that Tß4 significantly reduced infiltrating macrophages and neutrophils. D: ICAM-1 (80 kDa) expression in the myocardium was significantly increased at 7 days post-MI, which was almost prevented by Tß4. n = 5–7 animals/group. *P < 0.01; †P < 0.05; ‡P < 0.01.
sion in the myocardium was barely detected by Western blot analysis. MI markedly induced p53 protein expression at 7 days post-MI in the MI + H11001 vehicle group, and this expression was partially but significantly downregulated by T/H9252 treatment (Fig. 5D).

**Protocol 2**

Cardiac remodeling and function 5 wk after MI. Echocardiographic data are shown in Figs. 6 and 7. MI caused LV chamber dilatation, as detected by increase in LVDd and LVAd. Cardiac function, as assessed by EF and SF, was markedly reduced by MI. However, by week 5, Tβ4 treatment significantly prevented LV dilation and improved cardiac function, as evidenced by increased EF and SF in the MI + Tβ4 group (Figs. 6, A–C, and 7). PWTd was similar among sham (0.83 ± 0.02 mm), MI + vehicle (0.85 ± 0.02 mm), and MI + Tβ4 groups (0.85 ± 0.02 mm). Untreated mice with MI had increased LV weight, which was not affected by Tβ4 with 5-wk treatment (Fig. 6D).

Infarct size was similar between vehicle-treated (38.9 ± 3.1%) and Tβ4-treated (34.4 ± 4.0%) groups at 5 wk post-MI.

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**Fig. 3.** A: representative images of in situ zymography for gelatinolytic activity in the region near the infarct border. No clear gelatinolytic activity was detected in the sham heart. B: semiquantitative analysis of gelatinolytic activity showing that MI increased gelatinolytic activity, which was significantly blunted by Tβ4. n = 4–6 animals/group. *P < 0.005; †P < 0.05; ‡P < 0.01.

**Fig. 4.** A: representative images of capillary vessels as indicated by positive staining for CD31 in the region of the infarct border. B: quantitative analysis of numbers of capillary vessels showing that capillary vessels were markedly reduced after MI but significantly increased with Tβ4 treatment. n = 5–6 animals/group. *P < 0.005; †P < 0.05; ‡P < 0.01.
Capillary density and collagen deposition. Capillary density was significantly lowered in the MI + vehicle group compared with the sham group; 5-wk treatment with TB4 led to a significant increase in capillaries in the myocardium (Fig. 8, A, top, and B). MI also caused cardiac fibrosis, as indicated by increased myocardial ICF in the MI + vehicle group. TB4 significantly inhibited collagen deposition in the heart after MI, which was significantly downregulated by TB4 treatment. *P < 0.005; †P < 0.005; ‡P < 0.005.

**Discussion**

The major findings of the present study are as follows: 1) mice treated with TB4 exhibited a reduced mortality rate as a result of decreased cardiac rupture during the acute MI; 2) MI was associated with an excessive inflammatory response, as demonstrated by the increases in neutrophil and macrophage infiltration and ICAM-1 expression, enhanced gelatinolytic activity, reduced numbers of capillaries, and increased cardiomyocyte apoptosis in the heart; 3) these excessive inflammatory responses were partially prevented by TB4; and 4) TB4 treatment for 5 wk ameliorated LV dilatation and improved cardiac function, and it reduced interstitial fibrosis and increased capillary density in the myocardium.

MI evokes intense inflammatory responses both systemically and within the infarcted myocardium. Although inflammatory responses are essential for post-MI wound healing and scar formation, excessive inflammatory responses and ECM remodeling can lead to harmful consequences (15, 16), such as cardiac rupture, maladaptive LV remodeling, and cardiac dysfunction. Clinical studies have shown that hearts from patients with cardiac rupture revealed a higher density of inflammatory cells and more abundant MMP-9 expression in the infarct myocardium compared with MI patients without cardiac rupture (23, 48). We and others (6, 51, 56) have reported that in murine models of acute MI, cardiac rupture mainly occurs at...
3–5 days post-MI and that neutrophils constitute the majority of infiltrating cells within the first 1–2 days after MI, which are followed by a marked accumulation of macrophages on days 3–4. The peak times of rupture occurrence and macrophage infiltration in the infarcted myocardium overlap (at days 3–5 post-MI), indicating macrophages as the major inflammatory cell population correlated to the risk of cardiac rupture. Here, we report, for the first time, that Tβ4 significantly reduced the incidence of cardiac rupture, which can be attributed to its anti-inflammatory action, as evidenced by the reduced numbers of infiltrating macrophages and neutrophils in the infarct border.

Pronounced inflammatory cell infiltration is always associated with upregulated expression of proinflammatory cytokines and mediators and with enhanced expression and activities of gelatinolytic proteinases (13). MMP-2 and MMP-9 are the typical gelatinolytic proteinases. As demonstrated by Heymans et al. (20), deletion of MMP-9 significantly lowered cardiac rupture in mouse models of MI. Similarly, MMP-2 expression in the heart was also elevated after MI; disruption of MMP-2 decreased mortality caused by post-MI cardiac rupture compared with wild-type mice (27). Together, these clinical and animal experimental results suggest that excessive leukocyte infiltration and gelatinolytic proteinase expression are closely

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**Fig. 6.** A: representative M-mode echocardiography performed on conscious mice given either vehicle or Tβ4 for 5 wk after MI. B and C: LV diastolic dimension (LVDd; B) and LV diastolic area (LVAd; C). LV chamber dilation in mice with MI was significantly prevented by Tβ4 treatment. D: LV weight-to-body weight ratio (LVW/BW) in mice with or without Tβ4 treatment after MI. n = 10–14 animals/group. *P < 0.005; †P < 0.05; ‡P < 0.005.

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**Fig. 7.** A and B: LV ejection fraction (EF; A) and shortening fraction (SF; B) in mice treated with or without Tβ4 post-MI, showing significantly increased cardiac function by five-wk Tβ4 treatment. n = 10–12 animals/group. *P = 0.0001; †P < 0.005; ‡P < 0.005.
THYMOSIN-β4 PROTECTS THE HEART AFTER MI IN MICE

Fig. 8. A: representative images of capillaries (red stained, top) and interstitial collagen (green stained, bottom) in the LV 5 wk after MI. B and C: quantitative analysis of capillary density (B) and interstitial collagen fraction (ICF; C) showing that MI caused a decrease in capillary density and an increase in ICF and that these adverse effects were significantly prevented by Tβ4. n = 5–8 animals/group. *P < 0.005, vehicle-treated vs. sham groups; †P < 0.01; ‡P < 0.05.

associated with cardiac rupture. Since Tβ4 has been shown to reduce inflammatory cell infiltration and downregulate inflammatory mediators (26, 45), these activities have been linked to its ability to decrease gelatinolytic proteinases and gene transcript levels of a variety of proinflammatory cytokines and chemokines. We tested whether Tβ4 prevented cardiac rupture after MI due to its anti-inflammatory actions. Indeed, Tβ4-treated mice had a low incidence of cardiac rupture and reduced inflammatory responses, as indicated by a decrease in inflammatory cell infiltration and gelatinolytic activity in the infarct border region. Our findings are consistent with both clinical and experimental results from other studies showing that excessive cardiac inflammation constitutes one of the central mechanisms of cardiac rupture pathogenesis. ICAM-1 is expressed on the membranes of leukocytes and endothelial cells, and it binds to integrins of type CD11a/CD18 (lymphocytes) or CD11b/CD18 (macrophages) (38, 53). ICAM-1 signaling can mediate the recruitment of inflammatory immune cells (12). In our murine model of MI, ICAM-1 expression in the myocardium was significantly increased, which was consistent with the clinical findings showing that serum levels of ICAM-1 in patients within the first week of MI were significantly higher than those in normal control subjects (11); this enhanced ICAM-1 expression was inhibited by Tβ4. The changes in ICAM-1 expression in our study corresponded to changes in macrophage infiltration, indicating the role of ICAM-1 in macrophage recruitment. Thus, we believe that Tβ4 may have decreased the numbers of infiltrating macrophages in part by decreasing local ICAM-1 levels post-MI. In clinical studies, proinflammatory cytokines, including IL-8, have been shown to be induced during MI through the NF-κB pathway in the myocardium (9) and to predict the development of heart failure after MI (10, 30). Importantly, Tβ4 inhibits NF-κB activation and thus the expression of the downstream IL-8 gene as well (35). Therefore, we can speculate that the anti-inflammatory effects of Tβ4 in the heart after MI could be due to its inhibition on NF-κB activation and thus the expression of proinflammatory cytokines/chemokines. Heart angiogenesis/neovascularization is implicated to play a role in improving prognosis post-MI (41, 42). Tβ4 has been shown to promote coronary vessel development and collateral growth not only during embryonic development but also in the adult epicardium by stimulating epicardial vascular progenitors, which migrate and differentiate into smooth muscle and endothelial cells (44). Indeed, in the present study, we showed that Tβ4 exerted a proangiogenic effect at an early stage of MI,
as evidenced by the increased numbers of CD31-positive capillaries in the infarct border. Since newly formed capillaries are an important component of tissue granulation and repair, the enhanced angiogenic/vasculogenic response may contribute greatly to the reduced incidence of cardiac rupture in the acute phase of MI and also later on to the improved cardiac remodeling and function.

Both experimental (3, 39) and human (31, 40) studies have detected increased numbers of TUNEL-positive cardiomyocytes in post-MI hearts, as early as the initiation of acute MI, and as well as up to 6 mo post-MI, along with LV dilation and contractile dysfunction. This implies that myocyte death may have an important pathogenic role in early and late LV remodeling and dysfunction after MI (39, 50). The tumor suppressor protein p53 regulates cell cycle activity and apoptosis through its activity as a transcription factor (49). Matsusaka et al. (28) has shown that p53−/− mice with MI exhibited improved survival entirely due to a reduced rupture death rate, associated with increased infarct wall thickness and markedly reduced numbers of apoptotic myocytes in the infarct region. Notably, these mice also had better preservation of LV function on day 3 after MI. These studies demonstrated that p53-dependent myocyte apoptosis is involved not only in early cardiac rupture (28) but also in late remodeling and dysfunction (39) after MI. Importantly, Tβ4 markedly decreased cardiomyocyte apoptosis, as assessed by TUNEL assay 24 h after ligation in an acute MI mouse model (4). Consistent with other studies, we observed notable apoptotic myocytes and high levels of p53 expression in the myocardium of mice 7 days after MI, which were partially prevented by Tβ4. The reduced incidence of cardiac rupture and restored cardiac function by Tβ4 in MI models in the present study might be due to negative regulation of p53 by Tβ4 and thus reduced myocyte apoptosis mediated by Tβ4.

Tβ4 treatment for 5 wk significantly ameliorated LV dilatation and improved cardiac function, as evidenced by the increase in SF and EF post-MI, which were associated with significantly increased numbers of capillary and decreased cardiac fibrosis in the LV. Our results are consistent with previously published data showing improved cardiac function as evaluated by echocardiography after 2- and 4-wk treatment with Tβ4 using the same model (4). As found in the present study and pilot studies, Tβ4 limited excessive inflammation and promoted cardiomyocyte survival and angiogenesis after ischemic heart injury (4, 7, 21). These studies demonstrated a strong rationale for the use of Tβ4 to prevent cardiac damage and promote regeneration after an infarction. Since N-acetyl-Lysyl-proline and/or indoleacetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP) in cardiac healing after ischemia have been shown to affect angiogenesis and survival/repair. The anti-inflammatory properties of Tβ4 aid in this process, as they reduce inflammation and promote the formation of new blood vessels. This is supported by the findings that Tβ4 treatment for 5 wk after MI significantly increased numbers of capillary and decreased cardiac fibrosis in the LV. This suggests that Tβ4 may be a potential therapeutic agent in the treatment of MI, as it can improve cardiac function and prevent cardiac rupture. As a result, the use of Tβ4 in clinical trials is justified, and further research is needed to explore its potential in the treatment of MI.


