Upregulation of anticoagulant proteins, protein S and tissue factor pathway inhibitor, in the mouse myocardium with cardio-specific TNF-α overexpression

Yoshihiro Higuchi,1 Toru Kubota,2 Masamichi Koyanagi,1 Toyoki Maeda,1 Arthur M. Feldman,3 and Naoki Makino1

1Department of Cardiovascular and Geriatric medicine, Kyushu University Beppu Hospital, Japan; 2Department of Cardiology, Fukuoka City Hospital, Fukuoka, Japan; and 3Temple University School of Medicine, Philadelphia, Pennsylvania

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The development and progression of heart failure have been recognized to be associated with an inflammatory process because of the associated activation of proinflammatory cytokines, in particular IL-6, IL-1β, and TNF-α (13, 32). In addition, inflammation is strictly correlated with clotting activation, introducing a hypercoagulable state (27, 50). A hypercoagulable and inflammatory state is present in a large percentage of patients with heart failure and correlates with the severity of heart failure and with the systolic ventricular function (33). During the inflammation-induced activation of coagulation, anticoagulant mechanisms can be impaired (9, 26).

The thrombomodulin and protein C (TM/PC) pathway is the most important natural anticoagulant system (10, 36, 46). The membrane glycoprotein TM is an anticoagulant component of the endothelium and forms a 1:1 stoichiometric complex with thrombin to enhance the rate of PC activation by thrombin. Activated PC cleaves and inactivates activated factors V and VIII, thereby exerting potent negative feedback control on the generation of thrombin. The activated PC is substantially potentiated by protein S (PS), while the activated PC-independent anticoagulant effect of PS is extended via direct interaction with factors Xa and Va and phospholipids (6).

Recent experimental and clinical data have indicated that not only the TM/PC pathway but also the PS/tissue factor pathway inhibitor (TFPI) system function as potent natural anticoagulants. To investigate the balance between procoagulant and anticoagulant activities in the failing heart, we measured the cardiac expression of tissue factor (TF), type 1 plasminogen activator inhibitor (PAI-1), TM, PC, PS, and TFPI by RT-PCR and/or Western blot analysis in male transgenic (TG) mice with heart-specific overexpression of TF-α (40-kd TF) and anti-inflammatory cytokines (17). The expression of TF was upregulated in the myocardium of a 24-wk-old TG (end-stage HF) but not in that of 4-wk-old TG (early decompensated HF) compared with the wild-type mice. Both factors were also upregulated in the infarcted myocardium at 3 days after coronary ligation in the wild-type mice. The expression of TNF-α is recognized as a hypercoagulable state. However, the natural anticoagulation systems in the failing heart have not been studied. Recent experimental and clinical data have indicated that not only the thrombomodulin (TM)/protein C (PC) pathway but also the protein S (PS)/tissue factor pathway inhibitor (TFPI) system function as potent natural anticoagulants. To investigate the balance between procoagulant and anticoagulant activities in the failing heart, we measured the cardiac expression of tissue factor (TF), type 1 plasminogen activator inhibitor (PAI-1), TM, PC, PS, and TFPI by RT-PCR and/or Western blot analysis in male transgenic (TG) mice with heart-specific overexpression of TF-α (40-kd TF) and anti-inflammatory cytokines (17). The expression of TF was upregulated in the myocardium of a 24-wk-old TG (end-stage HF) but not in that of 4-wk-old TG (early decompensated HF) compared with the wild-type mice. Both factors were also upregulated in the infarcted myocardium at 3 days after coronary ligation in the wild-type mice. The expression of TM was downregulated in the TG heart, and PC was not detected in the hearts. The transcript levels of PS orphan receptors, Mer and Tyr3, but not Axl, were significantly upregulated in the TG heart. Double immunohistochemical staining revealed that myocardial infiltrating CD3-positive T cells may produce PS in the TG myocardium. In conclusion, the PS/TFPI was upregulated in the myocardium of a different etiological model of HF, thus suggesting a role for the PS/TFPI system in the protection of the failing heart under both inflammatory and hypercoagulable states.

natural anticoagulant; heart failure; inflammatory cardiomyopathy; myocardial infarction

Address for reprint requests and other correspondence: Y. Higuchi, Dept. of Cardiovascular and Geriatric Medicine, Kyushu Univ. Beppu Hospital, Japan, 4546 Tsurumihara, Beppu 874-0838, Japan (e-mail: yhiguchi@beppu.kyushu-u.ac.jp).
tial infiltrates and fibrosis in the ventricles, impairment of cardiac function with biventricular dilatation, and congestive heart failure resulting in premature death (24, 25, 28). Besides anticoagulant proteins, we also evaluated the myocardial expression of PS-related proteins, C4BP and growth arrest-specific gene 6 (Gas6; Ref. 37), and PS orphan receptors (17). Furthermore, an immunohistochemical analysis was performed to identify the cellular source of the PS.

MATERIALS AND METHODS

Animals and tissue preparation. Male TG mice and their wild-type littermates at age 24 wk were used for the investigation except for time-course experiment (4- and 12-wk-old TG and wild type) and MI mice (8- to 10-wk-old type). About 50% of the mice died from congestive (decompensated) heart failure by 24 wk of age (19). A decompensated state was defined in mice with dilated hearts with reduced fractional shortening (43). The overexpression of the transgene (murine TNF-α) is driven by the α-myosin heavy chain promoter, which restricts the expression of the protein to cardiac myocytes. The TG mice were identified by PCR with a sense primer (5′-CCA CAT TCT TCA GGA TTC TCT-3′) specific to the α-myosin heavy chain promoter exon 2 and an antisense primer (5′-CAG CCT TGT CCC TTG AAG AGA-3′) specific to the TNF-α cDNA nucleotides 579 to 599 (23). All mice were of the same FVB genetic background. Freshly isolated mouse tissues were either fixed in 4% neutral buffered paraformaldehyde or snap frozen in liquid nitrogen for later analysis. The experimental protocol conforming to the Guide for the Care and Use of Laboratory Animals published by National Institutes of Health (NIH Publication No. 85–23, revised 1996) and was approved by the Ethics Committee on Animal Experiments of Kyushu University Graduate School of Medical Sciences.

Clotting and bleeding times. Plasma from citrated blood (3.2% citrate, 1/10) was extracted by 15-min centrifugation (2500 g at 4°C) and stored immediately at −80°C until the analysis. The prothrombin time (PT) and activated partial thromboplastin time (aPTT) were assessed by an analyzer (CA1500; Sysmex, Kobe, Japan) using the appropriate reagents. To determine the tail bleeding time, the tail was transected 5 mm from the tip, and it was immediately immersed in PBS, and the time until the cessation of bleeding was recorded.

ELISA. The protein levels were assessed using ELISA kits for mouse TNF-α and IL-β (Quantikine, R&D Systems) according to the manufacturer’s instructions. The results are expressed as picograms of the target proteins per milligram of left ventricle (LV) or per milliliter of serum. The minimum detectable concentrations of mouse TNF-α and IL-1β by ELISA are typically <5.1 and 3.0 pg/ml, respectively.

Semiquantitative RT-PCR. Total RNA was extracted from the tissues using the RNA-Bee-RNA isolation reagent according to the manufacturer’s protocol (Tel-Test, Friendswood, TX). The application of DNase was performed during RNA extraction to avoid DNA contamination. The RT-PCR (using the BcaBEST RNA PCR kit ver. 2.0) were carried out overnight at 4°C. A primary antibody was polyclonal goat anti-TF (1:250), rat anti-PC (1:300), rabbit anti-PS (1:200), goat anti-Gas6 (1:350), goat anti-C4BP (1:200), goat anti-C4BP (1:250), or goat anti-actin (1:1,000) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), or with rat anti-TFPI (1:1,000), rat anti-PAI-1 (1:500), or rat anti-TM (1:200) antibodies (R&D Systems). Detection was performed with horseradish peroxidase (HRP)-conjugated secondary antibodies (Chemicon, Temecula, CA) and ECL Western blotting detection reagents (GE Healthcare, Little Chalfont, UK). The average densitometric analysis of the bands was carried out using the ImageJ software package (National Institutes of Health). The relative protein expressions levels were normalized to those of α-actin.

Immunohistochemical analysis. All samples were fixed in 4% neutral buffered paraformaldehyde and embedded in paraffin. Three micrometer-thick cryostat sections were used for immunostaining. The sections were deparaffinized in xylene, dehydrated in ethanol, and treated with 3% H2O2 in methanol for 10 min at room temperature to eliminate the endogenous peroxidase activity. After blocking the nonspecific binding with 10% normal goat serum in PBS for 10 min at room temperature, the reactions with the primary antibodies were carried out overnight at 4°C. A primary antibody was polyclonal goat anti-mouse TF (Santa Cruz Biotechnology). These sections were then allowed to react with the EnVision+Dual Link System-HRP (Dako, Tokyo, Japan) for 60 min. Color development was carried out in a 0.5% solution of 3,3-diaminobenzidine (EnVision+Kit-HRP, Dako). The sections were weakly counterstained with Mayer’s hematoxylin and were assessed and photographed using light microscopy and were evaluated for the reaction as a brown-colored precipitate at the antigen site. Double immunohistochemical staining for PS (ProteinTech Group) and CD3 (T-cell marker, Santa Cruz Biotechnology) or Mac-3 (macrophage marker, AbD Serotec) in myocardial sections was performed using sequential protocols at our laboratory to localize PS with potential PS-producing cells (34). A duplicate sample was prepared as above but omitting the primary antibody to control for any nonspecific signal.

Creation and analyses of myocardial infarction model mice. We generated MI in male wild-type mice (n = 25; 8–10-wk-old and 20–26 g in weight) by ligating the left coronary artery as described in a previous study (34). A sham operation without coronary ligation was also performed as a control (n = 20). Three days after MI, the myocardial tissues contained.
with MI were carefully dissected into an infarcted LV with the peri-
infarct rim (a 0.5- to 1-mm rim of normal-appearing tissue). These tissues
were snap frozen for RNA analysis. Three whole hearts were also excised
3 days after MI, and the LV was cut from apex to base into four
transverse sections for triphenyl tetrazolium chloride or Masson’s
trichrome staining. The infarct sizes (in percent) were calculated as the
total infarct circumference divided by the total LV circumference using
Masson’s trichrome sections, as described previously (34). The survival
analysis was performed in both the sham (n = 17) and MI mice (n = 17).
Animals were followed for up to 14 days after surgery.

Statistical analyses. Student’s t-test was used to compare con-
tinuous variables. A one-way ANOVA using Bonferroni’s multiple-
comparison test was appropriately used to examine the differences
among subgroups. The survival analysis was performed by the Ka-
plan-Meier method. The results are presented as the means ± SD. The
analyses were performed using the SPSS software package for Win-

Fig. 1. Inflammatory and hypercoagulable states in the myocardium of transgenic (TG) male mice at the age of 24 wk. A and B: representative micrographs of hematoxylin-eosin staining (A) and immunohistochemical staining for tissue factor (brown staining; B) of the myocardium of TG and wild-type (WT) mouse. Arrows indicate increased tissue factor (TF) expression near the cellular infiltrates. Scale bar = 100 μm. C, D, and E: protein expression levels of TF (C), tissue factor pathway inhibitor (TFPI; D), and type 1 plasminogen activator inhibitor (PAI-1; E) were determined by a Western blot analysis in the hearts of TG and WT mouse. Data are represented as the relative ratio to actin. Values are means ± SD (n = 5 to 6; *P < 0.05 relative to WT).
RESULTS

Hypercoagulable state in the TG mouse myocardium. TG mice at 24 wk of age presented a diffuse interstitial infiltration in the myocardium (Fig. 1A). As shown in Table 1, the concentrations of TNF-α and IL-1β were significantly increased not only in their myocardium (P < 0.001) but also in their serum (P < 0.05), compared with their wild-type littermates. It is worth noting that all of the individual mouse serum samples measured less than the lowest mouse TNF-α standard, 23.4 pg/ml.

The immunohistochemical analysis showed that the TF protein expression was increased within the entire whole myocardium, especially around the cellular infiltrates, in the TG heart (Fig. 1B). A Western blotting analysis was used to investigate the effects of TNF-α overexpression on the expression levels of the TF, TFPI, and PAI-1 proteins in the myocardium. The Western blot quantification by densitometry using actin as a calibrator showed respective 3.8-, 8.4-, and 8.9-fold increases in the amount of TG myocardial proteins compared with the wild type (Figs. 1C-E). TNF-α overexpression in the heart led to a significant increase in not only the myocardial TF and PAI-1 but also TFPI protein levels, suggesting that the hypercoagulable state in the myocardium can be counterbalanced at least in part by the upregulation of the TFPI. We also demonstrated increases in the levels of mRNA expression of these genes (see Fig. 6).

Coagulation activity of plasma and bleeding time. The PT and aPTT assays are globally accepted parameters for assessing changes in coagulation. No changes were observed between TG and wild-type mice, neither for PT nor aPTT (Table 2). In addition, we determined the bleeding time in TG and wild-type mice to confirm whether there were differences in bleeding. The bleeding time is largely dependent on both the number and function of platelets. No differences in the bleeding times were found between TG and wild-type mice (Table 2).

Expression of TM and PC. The level of TM mRNA was significantly lower in the hearts of the TG mice than in those of the wild-type mice (Fig. 2A). A Western blot analysis demonstrated that the TM protein was also downregulated in the TG mouse hearts (Fig. 2B). The densitometric analysis showed that the protein level of ventricular TM in the TG heart was almost half that of the wild-type heart tissue specimens. Neither PC mRNA nor protein was detected in the mouse heart (Fig. 2, C and D), consistent with the study of Yamamoto and Loskutoff (51). The liver was used as a control, because the liver is the main site of the synthesis of PC.

Table 1. *TNF-α and IL-1β protein levels by ELISA in 24-wk-old mice

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<tr>
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<th>WT (n = 5)</th>
<th>TG (n = 5)</th>
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<tr>
<td>Ventricle, pg/mg</td>
<td></td>
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<tr>
<td>TNF-α</td>
<td>1.3 ± 1.1</td>
<td>181.2 ± 36.9†</td>
</tr>
<tr>
<td>IL-1β</td>
<td>3.0 ± 1.2</td>
<td>99.3 ± 29.9†</td>
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<tr>
<td>Serum, pg/ml</td>
<td></td>
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<tr>
<td>TNF-α</td>
<td>6.8 ± 1.3</td>
<td>21.0 ± 1.3†</td>
</tr>
<tr>
<td>IL-1β</td>
<td>6.0 ± 1.4</td>
<td>9.1 ± 1.9†</td>
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Values are means ± SD; n = no. of mice. WT, wild-type mice; TG, transgenic mice. *P < 0.05 vs. WT. †P < 0.001 vs. WT.

Table 2. Systemic coagulation activity in 24-wk-old mice

<table>
<thead>
<tr>
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<th>WT (n = 3)</th>
<th>TG (n = 3)</th>
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<tr>
<td>PT, s</td>
<td>7.7 ± 0.2</td>
<td>7.9 ± 0.5</td>
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<tr>
<td>aPTT, s</td>
<td>22.5 ± 0.8</td>
<td>20.7 ± 1.1</td>
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<td>Bleeding time, s</td>
<td>361.7 ± 81.1</td>
<td>333.7 ± 83.6</td>
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Values are means ± SD; n = no. of mice. PT, prothrombin time; aPTT, partial prothrombin time.

Expression of PS and related proteins, Gas6, and C4BP. The expression levels of PS mRNA and protein in the TG myocardium were increased to twice and 3.5-fold the level in the wild-type mice, respectively (Figs. 3, A and B). The myocardial protein expression levels of Gas6, which is a PS homologue with a 44% identical amino sequence, were not significantly different between the TG and wild-type mice. The high-molecular weight C4BP is generally composed of seven identical α-chains and a single β-chain. The protein levels of C4BPα and C4BPβ were measured separately, using antibodies that specifically recognize C4BPα and C4BPβ. The C4BPα protein was found to be upregulated in the TG heart (Fig. 3D); however, we detected no C4BPβ protein by a Western blot analysis in either the wild-type or the TG mouse hearts (data not shown), consistent with the fact that murine C4BP lacks C4BPβ (38). The PS antibody used in this study probably recognized only free PS, because murine PS could not exist as a complex form with C4BPβ. The upregulation of the PS-related complement regulator C4BPα may have a protective role against complement activation in damaged heart tissue (44).

Immunohistochemical staining of PS, CD3, and Mac-3. Initially, liver cells were stained as a control because the liver is the main site of synthesis for PS. The PS antibody strongly stained liver cells in both the TG (Fig. 4A) and wild-type mice (data not shown). Double staining showed that the infiltrating cells with strong PS immunoreactivity also had immunostaining for CD3 while no CD3-positive cells were observed in the wild-type control myocardium (Fig. 4B). In contrast CD3, Mac-3-positive cells were identified in the TG myocardium but double staining for both PS and Mac-3 were not observed. The cells with a simple PS staining had similar morphological characters with PS- and CD3-double-positive cells observed in the TG myocardium (Fig. 4B). These findings suggest that infiltrating T cells but not macrophages are one of the major sources of PS in the TG myocardium.

mRNA expression of Tyro3, Axl, and Mer receptor tyrosine kinases. PS and its structural homologue, Gas6, can interact with the TAM family of receptor tyrosine kinases, which include Tyro3, Axl, and Mer (16). To investigate the expression of the TAM receptor transcripts in the murine myocardium, the mRNA expression of Tyro3, Axl, and Mer in the TG and wild-type hearts was measured by semiquantitative RT-PCR (Fig. 5). The expression of actin mRNA was employed as an internal control. A statistical analysis indicated that there were 2.2- and 1.8-fold increases in the Tyro3 and Mer mRNA levels, respectively, in the TG hearts compared with the wild-type hearts (Fig. 5, A and C). However, the mRNA expression level of Axl was similar between the TG and wild-type hearts (Fig. 5B).

Time-course experiments in the different stages of heart failure. To elucidate the influences of the stage of heart failure on the
expression of both anticoagulant (PS and TFPI) and procoagulant (TF and PAI-1) genes, RT-PCR was performed in the TG mice at 4 and 12 wk, as well as 24 wk of age. RT-PCR showed that these genes were comparable between TG and wild-type mice at 4 wk of age (Fig. 6). At 12 wk of age, the mRNA expression levels were increased in TG mice, but the differences were not statistically significant compared with wild-type mice (P < 0.05). At 24 wk of age, the transcripts of these genes were upregulated significantly compared with wild-type mice (P < 0.05), which is consistent with the results of Western blot analysis as shown in Figs. 1 and 3.

**DISCUSSION**

This study found that 1) cardiac TNF-α overexpression increased the mRNA and protein levels of TF and PAI-1, as well as those of the proinflammatory cytokines TNF-α and IL-1β, indicating the induction of the coagulation cascade and impairment of fibrinolysis, leading to the promotion of coagulation in the myocardium. 2) The downregulation of the mRNA and protein levels of TM and that lack of detectable PC indicated that the TM/PC anticoagulant pathway does not play a major role in the TG myocardium. 3) The mRNA and protein levels of PS and TFPI were significantly increased in TG myocardium compared with wild-type mice. The similar results were observed in the infarcted myocardium compared with sham-operated myocardium. The transcriptional levels of the PS orphan receptor tyrosine kinases Mer and Tyro3, but not Axl, were upregulated in the TG heart. 4) Both procoagulant (TF and PAI-1) and anticoagulant (PS and TFPI) factors were upregulated in the myocardium of 24-wk-old TG (end-stage heart failure), but not in that of 4-wk-old TG (early decompensated heart failure) compared with the wild-type mice.
These results indicated that the PS/TFPI pathway may function as an anticoagulant system in failing myocardium. 5) The double immunohistochemical staining revealed that PS- and CD3-double-positive infiltrating cells were observed in the TG myocardium (Fig. 4). The distribution of TF was predominantly observed surrounding the infiltrating cells (Fig. 1), indicating that infiltrating T cells may produce PS under inflammatory and hypercoagulable states.

As a proinflammatory cytokine, TNF-α alters the expression of a variety of hemostatic and fibrinolytic genes, leading to an increase in the prothrombotic potential. TF plays a central role in the activation of coagulation by binding and activating factor VII, which activates the coagulation network to generate thrombin. The present study demonstrated that the expression of the TF protein in the TG heart was upregulated, suggesting that there was activation of the coagulation cascade in the TG heart. The immunohistochemical analysis demonstrated that TF was most highly expressed in the areas surrounding the infiltrating cells in the TG myocardium. These findings are consistent with the study by Antoniak et al. (3), which demonstrated that TF upregulation correlated positively with the infiltration of CD3-positive or Mac-3-positive cells and with myocardial TNF-α expression in a mouse model of viral myocarditis. PAI-1 plays an important role in the regulation of fibrinolysis by binding to and rapidly inactivating both tissue-type and urokinase-type PAs (47). The elevation of PAI-1 is known to be associated with an increased risk for thrombotic disorders. A recent study (52) demonstrated that PAI-1 is also a determinant of cardiac fibrosis. Therefore, the upregulated PAI-1 in TG heart may be in part reflected by the development of interstitial fibrosis in the myocardium.

The major producer of PS and other factors of the coagulation system is the liver (48). PS is also synthesized at extrahepatic sites, including by endothelial cells (11, 18), megakaryocytes (40), and vascular smooth muscle cells (20, 41). The expression of PS has been also observed in the murine heart and cultured cardiomyocytes, which raises the possibility that PS contributes to the regional anticoagulant properties in the heart (41). TF and its inhibitor, TFPI, are the key proteins involved in the initiation of blood coagulation. Kereveur et al. (21) reported that cardiomyo-
cytes express TFPI and that its expression could be increased by IL-1β. In a recent study by Hackeng et al. (15), PS specifically inhibited TF activity by promoting the interaction between the full-length TFPI and factor Xa. Therefore, the upregulated PS/TFPI system in the TG heart may have an anticoagulant activity, at least in part, by inhibiting the TF activity.

Based on their LV chamber dimensions, systolic function, cardiac hypertrophy, and the presence of pleural effusion, male

Fig. 4. Protein S (PS) producing cells in the liver and heart of 24-wk-old TG mice. A: representative microphotographs of immunohistochemical staining with antibodies against PS (blue staining) in the liver cells. A negative control was also constructed without the primary antibody. B and C: representative microphotographs of double immunohistochemical staining for PS and CD3 (B) or PS and Mac-3 (C). Blue, PS; brown, CD3 or Mac-3. Black arrows indicate both PS- and CD3-positive cells. Arrowheads indicate Mac-3-positive cells showing no PS staining while the cells with a simple PS staining are indicated by white arrows. Scale bar = 10 μm.

Fig. 5. Differential mRNA expression level of Tyro3, Axl, and Mer receptor tyrosine kinase (TAM) family members in the heart tissues from TG and WT mice at the age of 24 wk. Representative images for Tyro3 (A), Axl (B), and Mer (C) are shown. Data measured by semiquantitative RT-PCR are represented relative to the WT expression, which was normalized to 1. Values are means ± SD (n = 4 in each). *P < 0.05 vs. WT.
TG mice displayed a variation in the time course of the development of heart failure, with hypertrophic/compensated heart failure observed in mice at 3 wk of age and younger, with early congestive (decompensated) failure observed at 4 wk of age, and congestive (decompensated) failure starting ~13 wk of age (43). About 50% of the TG mice died from congestive heart failure by 24 wk of age (19). We (24) previously reported that age did not appear to influence the cardiac TNF-α levels in the TG myocardium, since there was virtually no difference in the levels of TNF-α at 6, 12, and 24 wk. However, the time-course experiment demonstrated both procoagulant (TF and PAI-1) and anticoagulant (PS and TFPI) factors were upregulated in the myocardium of 24-wk-old TG but not in that of 4- and 12-wk-old TG myocardium. These findings sug-

Fig. 6. mRNA expression level of procoagulant and anticoagulant genes at the different time points [4, 12, and 24-wk (w) of age] in the heart tissues from TG and WT mice. Representative images for TF (A), PAI-1 (B), protein S (C), and TFPI (D) are shown. Data measured by semiquantitative RT-PCR are presented as the fold change normalized to actin compared with 4-wk-old WT control mice (defined as 1.0-fold). Values are the means ± SD (n = 3 in each). *P < 0.05 vs. WT.
suggested that these genes were influenced by the stage of heart failure but not directly by the levels of cardiac TNF-α.

TG hearts were characterized by a marked infiltration of inflammatory cells in the myocardium (24). In our previous study (43), the image analysis of myocardial sections from TG mice with early stage compensated heart failure showed widespread Mac-3 (macrophage), CD4 (T-lymphocytes), and CD45/B220 (B-lymphocytes) staining within the myocardium. Polymorphonuclear granulocytes were rarely seen in the TG myocardium (31). Interestingly, the presence of B-lymphocytes was dramatically reduced in the TG hearts of mice with decompensated (end-stage) heart failure (43). We therefore suggest that B lymphocytes and polymorphonuclear granulocytes contribute little, if any, to the production of PS in the TG hearts with end-stage heart failure. Given the increased infiltration of T cells and macrophages in the TG myocardium, we studied their histological localization in relation to PS. Double staining identified CD-3-positive T cells as one of the major sources of PS in the TG myocardium. PS has been reported to be inducible by the cytokine interleukin 4 (IL-4) in primary T cells (42). A previous study demonstrated that IL-4 was detected in the TG heart (43). These observations suggest that infiltrating CD3-positive T cells may produce PS stimulated by IL-4 in the TG myocardium.

The related TAM receptors are thought to be orphan receptors for PS and Gas6 (17). TAM receptors are increasingly being implicated in a host of discrete cellular responses, including cell survival, proliferation, migration, and phagocytosis (16). PS only binds to Tyro3 and Mer, while Gas6 binds to all three receptors (17). Mer is a key macrophage receptor and appears to be required for the ingestion of apoptotic cells (12, 45). PS and Gas6 may provide a possible recognition bridge between phagocytes and apoptotic cells, acting as so-called “binding molecules” (22). PS could bind and induce the phosphorylation of the Mer receptor tyrosine kinase in tissue macrophages (29), and a Northern blot analysis showed almost exclusive expression of Mer in the murine monocytic cell lineage (14). Therefore, the infiltrating monocytes and/or macrophages may be the cell source of the increased Mer expression in the TG heart. Prompt phagocytosis of apoptotic cells stimulated by PS prevents inflammatory and autoimmune responses to dying cells (2). The autoimmune response occurs concomitantly with cardiac decompensation and may participate in triggering the transition to heart failure in TG mice (43). These findings suggest that PS may contribute to the clearance of apoptotic cells and prevent autoimmune and inflammatory responses by interacting with the Mer receptor tyrosine kinase on tissue macrophages and/or monocytes in the TG heart.

Fig. 7. A: Representative images of sectioned triphenyl tetrazolium chloride (TTC)-stained myocardial infarction (MI) hearts on day 3 after MI (top left). Masson-trichrome-stainedLV cross-sections at the same level of the TTC-stained section (bottom left). Scale bar = 5 mm. Kaplan-Meier survival curves of MI (n = 17) and sham mice (n = 17) (right panel). B–E: differential mRNA expression levels of anticoagulant (PS and TFPI) and procoagulant (TF and PAI-1) genes in the heart tissues from MI and sham-operated mice 3 days after surgery. Representative images for TF (B), PAI-1 (C), PS (D), and TFPI (E) are shown. Data measured by semiquantitative RT-PCR are represented relative to the sham expression, which was normalized to 1. Values are means ± SD (n = 3 in each). *P < 0.05 vs. sham.
The mRNA expression levels of both anticoagulant (PS and TFPI) and procoagulant (TF and PAI-1) genes were upregulated in the infarcted myocardium, and these levels were consistent with the results of the TG myocardium of 12- or 24 wk-old mice. The increased expression of TF and PAI-1 mRNA has been reported in the ischemic hearts of rodents (8, 49). We suggest that the thrombogenic properties in the ischemic heart may be counterbalanced by the upregulated PS/TFPI system. In the present study, we used acute phase MI mice 3 days after ligation; however, the late phase analyses will be required to examine the role of PS/TFPI on the ventricular remodeling process in this MI model.

No functionally relevant alteration of the systemic coagulation activity was apparent in the blood of TG mice evaluated by PT, aPPT, and the bleeding time analysis. The minimal increase in the plasma levels of TNF-α in TG mice might have contributed, in at least part, to the lack of overall effects of upregulation of both an anticoagulant and a procoagulant pathway on the systemic coagulation activity. However, we did not evaluate the thrombin-antithrombin (TAT) complexes, which are globally accepted parameters for assessing changes in coagulation. An analysis of the coagulation parameters in congestive heart failure patients indicated that congestive heart failure leads to a hypercoagulable state, with high levels of thrombin formation markers, such as TAT complexes (5).

In conclusion, myocardial inflammation is highly associated with hypercoagulable state in the heart. The upregulated PS/TFPI in the myocardium may have a protective role as a novel anticoagulant and anti-inflammatory system in the failing heart. The more studies are needed to shed further light on the pathophysiological importance of the upregulated PS/TFPI system in the heart failure.

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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: Y.H. conception and design of research; Y.H. and M.K. performed experiments; Y.H., T.K., A.M.F., and N.M. analyzed data; Y.H. and T.K. interpreted results of experiments; Y.H., M.K., and T.M. prepared figures; Y.H. drafted manuscript; Y.H., T.K., A.M.F., and N.M. edited and revised manuscript; A.M.F. and N.M. approved final version of manuscript.

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