Cardiac Fibrosis

Tenasin-C May Accelerate Cardiac Fibrosis by Activating Macrophages via the Integrin αVβ3/Nuclear Factor–κB/Interleukin-6 Axis


Abstract—Tenasin-C (TN-C) is an extracellular matrix protein not detected in normal adult heart, but expressed in several heart diseases closely associated with inflammation. Accumulating data suggest that TN-C may play a significant role in progression of ventricular remodeling. In this study, we aimed to elucidate the role of TN-C in hypertensive cardiac fibrosis and underlying molecular mechanisms. Angiotensin II was administered to wild-type and TN-C knockout mice for 4 weeks. In wild-type mice, the treatment induced increase of collagen fibers and accumulation of macrophages in perivascular areas associated with deposition of TN-C and upregulated the expression levels of interleukin-6 and monocyte chemoattractant protein-1 as compared with wild-type/control mice. These changes were significantly reduced in TN-C knockout/angiotensin II mice. In vitro, TN-C accelerated macrophage migration and induced accumulation of integrin αVβ3 in focal adhesions, with phosphorylation of focal adhesion kinase (FAK) and Src. TN-C treatment also induced nuclear translocation of phospho-NF-κB and upregulated interleukin-6 expression of macrophages in an NF-κB–dependent manner; this being suppressed by inhibitors for integrin αVβ3 and Src. Furthermore, interleukin-6 upregulated expression of collagen I by cardiac fibroblasts. TN-C may enhance inflammatory responses by accelerating macrophage migration and synthesis of proinflammatory/profibrotic cytokines via integrin αVβ3/FAK-Src/NF-κB, resulting in increased fibrosis. (Hypertension. 2015;66:757-766. DOI: 10.1161/HYPERTENSIONAHA.115.06004.) • Online Data Supplement

Key Words: angiotensin II • cardiac fibrosis • hypertension • remodeling • tenasin-C

Cardiac fibrosis, like hypertrophy of cardiomyocytes, is an important factor for ventricular remodeling, causing myocardial stiffness and possibly leading to diastolic heart failure. Recently, a critical role of renin/aldosterone/angiotensin system (RAAS)–mediated inflammation in progression of cardiac fibrosis has been recognized.1,2 Based on results of rodent models with angiotensin or aldosterone treatment, it is proposed that overactivated RAAS triggers macrophage infiltration in perivascular regions in the myocardium, upregulating proinflammatory and profibrotic mediators, and finally resulting in excessive deposition of collagen fibers.1,2 Generally, the fibrotic process requires multiple steps involving synthesis, degradation, and interaction of many extracellular matrix (ECM) proteins, including tenasin-C (TN-C), a huge hexameric ECM glycoprotein highly expressed during embryonic development, cancer invasion, and wound healing at locations where tissue structures are dynamically remodeled. TN-C has a wide range of effects on cell activity via multiple cell surface receptors, including various integrins, and may play a significant role in tissue remodeling.3 In the heart, TN-C is sparsely detected in healthy adults, but is transiently expressed at restricted sites, closely associated with tissue injury and inflammation, in diverse heart diseases, such as myocardial infarction, myocarditis, and dilated cardiomyopathy (reviewed in Imanaka-Yoshida4). Thus, specific expression makes TN-C a diagnostic marker for active inflammation in the myocardium.5,6 Furthermore, the serum levels of TN-C are elevated, reflecting local expression in diseased hearts.7 Increasing numbers of reports have demonstrated that this parameter can be a predictive biomarker for adverse ventricular remodeling and worse patient outcome (reviewed in Imanaka-Yoshida4), suggesting that TN-C could play a significant role in ventricular remodeling. However, the detailed molecular mechanisms remain to be elucidated.

In fact, TN-C has been suggested to act as a profibrotic and proinflammatory modulator in various diseases, for
example, in pulmonary fibrosis, liver fibrosis, or arthritis.\textsuperscript{5–10}
In the hypertensive heart caused by angiotensin II (AngII) infusion, we have previously reported that TN-C is highly expressed in perivascular inflammatory/fibrotic lesions\textsuperscript{11,12} and deletion of TN-C reduced interstitial fibrosis after myocardial infarction.\textsuperscript{13} These findings suggest that TN-C may exacerbate fibrotic change in the myocardium.

We here aimed to study the role of TN-C in cardiac fibrosis and related molecular changes by targeted deletion in the AngII-induced hypertensive cardiac fibrosis model. The extents of fibrosis, macrophage infiltration, and proinflammatory cytokine/chemokine expression were compared in TN-C knockout and wild-type mice. Moreover, we examined the direct effects of TN-C on macrophages isolated from mouse peritoneal cavity and identified a signaling pathway to enhance cell migration and inflammatory cytokine expression. Our findings suggest that TN-C may activate macrophages and enhance fibrosis.

### Methods
An expanded Methods section is available in the online-only Data Supplement.

### Reagents and Antibodies
A detailed description of this section is included in the online-only Data Supplement.

### Experimental Animals and Blood Pressure
BALB/c background TN-C knockout (TNKO) and wild-type (WT) mice were used in all of the experiments.

### Immunohistochemistry and Immunofluorescence Staining
Immunohistochemistry and immunofluorescence staining were performed and positive signals were quantitated as previously described.\textsuperscript{14}

### Image Analysis and Quantitative Real-Time PCR
Image analysis of positive areas stained by Sirius red, collagen I, III, and Mac-3 were performed with Image J software, and mRNA levels of collagen I\textsubscript{1}, III\textsubscript{α1}, TN-C, and monocyte chemoattractant protein (MCP)-\textsubscript{1} were evaluated by quantitative real-time PCR using Taqman probes as previously described.\textsuperscript{11} Interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α mRNAs were quantified using appropriate probes of Quantitect Primer Assay (Qiagen), the LightCycler FastStart DNA Master\textsuperscript{R,15} SYBR Green I (Roche, Mannheim, Germany), and a LightCycler (Roche). GAPDH mRNA levels were also quantified as an internal control.

### Cell Isolation, Culture, and Treatment
Macrophages were collected from the mouse peritoneal cavity 3 days after intraperitoneal injection of thioglycolate broth solution and plated to culture dishes.\textsuperscript{19} Adhesive cells were serum-starved (0.1% FBS) for 16 h for analysis by Western blotting and quantitative PCR. Cardiac fibroblasts were obtained from ventricles of BALB/c mice as described previously.\textsuperscript{16}

### Western Blot Analysis and Immunoprecipitation
A detailed description of this section is included in the online-only Data Supplement.

### Macrophage Migration Assays
Migration assays were performed as previously described with slight modification.\textsuperscript{12} A detailed description of this section is included in the online-only Data Supplement.

### Statistical Analysis
All data are expressed as mean±standard deviations (SDs). Differences in data between 2 groups were determined by t test. Comparisons between multiple groups were assessed by 1-way analysis of variance, followed by the Dunnett’s test from GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA). A value of P<0.05 was considered significant.

### Results

#### AngII Infusion Elevates Blood Pressure and Induces Cardiac Hypertrophy
AngII infusion elevated blood pressure in both WT and TNKO mice from the second week onward (Figure S1A in the online-only Data Supplement). There was no significant difference in systolic blood pressure between WT and TNKO mice. Increases in body weight were similar in all experimental groups during the study, and there were no statistically significant intergroup differences (Figure S1B).

AngII treatment increased the heart to body weight ratio in both WT and TNKO mice. There was no significant difference between WT and TNKO mice infused with AngII (Figure S2A). The breadth of the left ventricular myocytes of AngII-treated WT and TNKO mice tended to increase compared with that of the nontreatment group (Figure S2B). Expressions of atrial natriuretic peptide and brain natriuretic peptide in AngII treatment were also upregulated in the saline drinking WT and TNKO mice (Figure S2C and S2D). There were no significant differences between WT versus TNKO mice treated with AngII in all experiments regarding cardiac hypertrophy.

#### Deletion of TN-C Attenuates AngII-Induced Cardiac Fibrosis
TN-C deposition was immunohistochemically detected in perivascular regions in AngII-treated mice (Figure 1A). No TN-C immunoreactivity was observed in any tissue sections of TNKO mice. Quantitative RT-PCR analysis showed that TN-C mRNA in AngII-treated mice was markedly upregulated when compared with the saline-treated WT case (Figure 1B).

Amounts of collagen fibers were evaluated by histochemistry with Sirius red staining and immunohistochemistry for collagen I and III. AngII treatment of WT mice significantly increased the collagen fibers at perivascular area extending between individual myocytes (Figure 1C). Necrosis of cardiomyocytes and scar formation were not observed in our mouse models. Quantitative analysis of fibrotic areas demonstrated that perivascular fibrosis in AngII-treated WT mice was more extensive than in saline-treated WT mice (10.29±5.09 versus WT/Sa: 4.60±1.56, respectively, P<0.001), whereas fibrosis of AngII-treated TNKO mice was less prominent (5.68±2.36 versus WT/AngII: 10.29±5.09, respectively, P<0.01; Figure 1D). Immunohistochemistry for collagen I (Figure S3A and S3B) and III (Figure S3C and S3D) showed a similar pattern to that with Sirius red staining. Expression levels of collagen I\textsubscript{1} and III\textsubscript{α1} mRNAs in ventricular tissues were significantly increased in both AngII-treated WT and TNKO mice compared with control values, although there was no significant difference between WT and TNKO mice treated with AngII (Figure S4A). Significant change of mRNA levels for collagen III\textsubscript{α1} was not detected in any of the groups (Figure S4B), probably because of the small size of the fibrotic lesions in...
our experimental model. Renal fibrosis was not histologically observed among all groups (data not shown).

**TN-C Deletion Attenuates Cardiac Inflammation Induced by AngII**

We next investigated whether deletion of TN-C influences the inflammatory response in the mouse heart. To evaluate macrophage infiltration, histological sections from WT and TNKO mice after either saline or AngII treatment were immunostained with anti-Mac-3 antibodies (Figure 2A). AngII infusion resulted in increased Mac-3–positive cell infiltration in WT mice (50±15.13 versus WT/Sa: 6±2.55 cells/section, \(P < 0.001\); Figure 2B). In AngII-treated TNKO mice, infiltrating macrophages were significantly reduced as compared with the AngII-treated WT mice (28±11.45 versus WT/AngII: 50±15.13, respectively, \(P < 0.01\)).

TN-C deposition was observed in the immediate vicinity of the macrophages in the perivascular lesions of AngII-treated WT mice (Figure 2C). Quantitative RT-PCR revealed that mRNA levels of MCP-1 (WT/AngII: 3.20±0.53 versus TNKO/AngII: 2.10±0.49, \(P < 0.05\); Figure 2D) and IL-6 (WT/AngII: 1.85±0.39 versus TNKO/AngII: 0.72±0.36, \(P < 0.01\); Figure 2E) were significantly higher in AngII-treated WT mice than in TNKO mice. Levels of the TNF-\(\alpha\) and IL-1\(\beta\) did not differ between WT and TNKO mice treated with AngII (Figure 2F and 2G).

**TN-C Enhances Macrophage Migration In Vitro**

To test the direct effects of TN-C on macrophage motility, a transwell migration assay of macrophages collected from the peritoneal cavity was performed using membrane inserts with 8-\(\mu\)m pores. TN-C on upper surface membrane significantly accelerated migration of WT macrophages to lower chamber in addition of MCP-1 (\(P < 0.001\); Figure 3). TN-C coating alone did not induce migration. On the other hand, coated TN-C significantly accelerated macrophage migration to MCP-1–supplemented lower chambers. There were no differences in migration between the WT and TNKO macrophages.

**TN-C Induces Expression of Cytokines and Nuclear Factor-\(\kappa\)B p65 Activation in Macrophages In Vitro**

We examined whether TN-C may transduce signaling to the nuclear factor (NF)-\(\kappa\)B proinflammatory pathway in macrophages. TN-C treatment induced phosphorylation of p65, a major component of NF-\(\kappa\)B, at serine 536 from 5 to 10 minutes after treatment (Figure 4A). Immunofluorescence showed translocation of p65 to nuclei in TN-C–treated cells, whereas staining was diffusely distributed in the cytoplasm of vehicle-treated cells (Figure 4B).

Next we investigated the effect of TN-C on induction of IL-6, TNF-\(\alpha\), and IL-1\(\beta\) mRNA expression. Quantitative PCR showed remarkable upregulation of IL-6 mRNA (39.90±18.10-fold, \(P < 0.01\)) by TN-C treatment. TNF-\(\alpha\) and IL-1\(\beta\) mRNA were also upregulated (21.84±3.69-fold, \(P < 0.001\) and 6.11±2.14 fold, \(P < 0.01\), respectively), but interferon-\(\gamma\) did not significantly increase after the treatment (data not shown). Pretreatment with the NF-\(\kappa\)B inhibitor parthenolide almost restored cytokine expression to the control levels (IL-6: 1.44±0.80-fold, \(P < 0.01\); TNF-\(\alpha\): 4.60±0.90-fold, \(P < 0.001\); IL-1\(\beta\): 1.21±0.31-fold, \(P < 0.01\); Figure 4C).

IL-6 is known as a profibrotic cytokine in the heart.\textsuperscript{18} We confirmed that treatment with IL-6 significantly accelerated mRNA expression of collagen type I at 6 h in cardiac fibroblasts isolated from a mouse heart. (\(P < 0.01\); Figure S5).
Several integrins are known to work as receptors for TN-C. 19 We examined which might be involved in activation of NF-κB and cytokine production induced by TN-C in macrophages. Integrins are heterodimeric proteins of α and β subunits. Among 18 types of α and 8 types of β subunits, αV and β1 are relatively common components of integrin heterodimers. First, an αV antagonist, cyclic RGDfV peptide (cyRGDfV), and a neutralizing antibody against β1 were tested. Western blotting analysis of TN-C–treated cells showed significant reduction of NF-κB phosphorylation on pretreatment with the αV antagonist, whereas anti-β1 antibodies exerted only limited effects (Figure 5A), implying that integrin heterodimers containing αV subunits might primarily transduce signals from TN-C. Integrin subunits of αV and β3 were detected in macrophage lysates by immunoblotting (Figure 5B). Immunoprecipitation using β3 antibodies showed large amounts of coprecipitated αV subunits in TN-C–treated cell lysates, but little in the control lysate. TNKO, TN-C knockout mice; and WT, wild-type mice.

Integrin αVβ3 Is a Major Functional Receptor in Inflammatory Responses Induced by TN-C

Several integrins are known to work as receptors for TN-C. 19 We examined which might be involved in activation of NF-κB and cytokine production induced by TN-C in macrophages. Integrins are heterodimeric proteins of α and β subunits. Among 18 types of α and 8 types of β subunits, αV and β1 are relatively common components of integrin heterodimers. First, an αV antagonist, cyclic RGDfV peptide (cyRGDfV), and a neutralizing antibody against β1 were tested. Western blotting analysis of TN-C–treated cells showed significant reduction of NF-κB phosphorylation on pretreatment with the αV antagonist, whereas anti-β1 antibodies exerted only limited effects (Figure 5A), implying that integrin heterodimers containing αV subunits might primarily transduce signals from TN-C. Integrin subunits of αV and β3 were detected in macrophage lysates by immunoblotting (Figure 5B). Immunoprecipitation using β3 antibodies showed large amounts of coprecipitated αV subunits in TN-C–treated cell lysates, but little in the control lysate. Immunofluorescence of integrin αVβ3 demonstrated that exposure to TN-C leads to clustering of integrin αVβ3 in focal adhesions at the cell periphery (Figure 5C).

Furthermore, 3 inhibitors of αVβ3 integrin, neutralizing anti-β3 antibody, cyRGDfV, and PI1 (HSDVHK), all significantly abrogated the NF-κB phosphorylation (Figure 5D) and IL-6 upregulation (Figure 5E) induced by TN-C, indicating that integrin αVβ3 is a functional receptor of macrophages for TN-C mediating the inflammatory signaling.
TN-C Enhances Focal Adhesion Kinase and Src Phosphorylation via Integrin αVβ3

Integrins regulate focal adhesion kinase (FAK)-Src family kinase activation triggering intracellular signaling to regulate cell behavior (reviewed in Cabodi et al[20]). Immunofluorescence with antibodies specific to phosphorylated FAK at Tyr (Y)925 and to phosphorylated Src at Y416 (Y416 in human Src and Y423 in mouse) demonstrated that TN-C treatment induces increased phosphorylation of FAK and Src in lamellipodia of the cells, accompanied by nuclear translocation of NF-κB. This was abolished by treatment with P11, an integrin αVβ3 inhibitor (Figure 6A and 6B). Western blotting of macrophage lysates also confirmed enhanced phosphorylation of FAK at Y925, but not at Y397, and of Src at Y416 after TN-C treatment, which were inhibited by P11 (Figure 6C and 6D). Furthermore, inhibitors of Src, PP2, significantly abrogated the enhanced phosphorylation of NF-kB and IL-6 upregulation (Figure 6E) and IL-6 upregulation (Figure 6F) induced by TN-C.

TN-C Enhances Macrophage Migration via Integrin αVβ3/Src In Vitro

We examined whether integrin αVβ3 and Src also mediate enhancing migration of macrophage by TN-C. Integrin αVβ3 inhibitors (anti-β3 neutralizing antibody and P11) and PP2 effectively inhibited the TN-C–enhanced migration of macrophages (Figure 7). However, anti-integrin β1–neutralizing antibody revealed no difference in the combination of MCP-1 and TN-C, indicating that integrin αVβ3/Src is a main pathway of enhancing macrophage migration.

Perivascular Macrophages in AngII-Treated Hearts Feature Integrin αVβ3 Expression and NF-κB Activation

Participation of integrin αVβ3 and NF-κB signaling of macrophages in inflammation and fibrosis enhanced by TN-C was verified in hearts of AngII-treated mice in vivo. On immunofluorescence, triple-positive macrophages for Mac-3, integrin αVβ3, and phosphorylated NF-κB were visible in perivascular regions of AngII-treated hearts of WT mice, whereas only a few macrophages positive for Mac-3, but negative for the other markers, were found in TNKO hearts (Figure 8). TN-C surrounding the macrophages could be responsible for driving their activation in perivascular regions in the AngII-treated heart.

Discussion

The present study clearly demonstrated that TN-C accelerates AngII-induced cardiac fibrosis by enhancing inflammatory responses. Deletion of TN-C significantly lessened cardiac fibrosis and macrophage infiltration and downregulated cytokines/chemokine expression in AngII-treated mice. In vitro studies showed that TN-C accelerated migration and expression of proinflammatory/profibrotic cytokines by macrophages via integrin αVβ3/FAK-Src and NF-kB.

Generally, fibrosis is closely related to inflammation and indeed considered as the end product of inflammatory reactions in various disorders. Cardiac fibrosis is defined as increase of fibrillar collagen in the myocardium, classified into replacement (=secondary) and reactive (=primary) types.[21] With replacement fibrosis, necrosis of myocytes elicits acute inflammation to remove damaged tissue, and dropout myocardial cells...
are replaced with collagen fibers. In the case of reactive fibrosis, collagen fibers among individual cells increase without loss of cardiomyocytes. RAAS-mediated inflammation is implicated in progression of this type of fibrosis. Administration of AngII or aldosterone to experimental animals induces vascular inflammation, accumulation of macrophages in perivascular areas, upregulation of various cytokines/chemokines through the NF-κB signaling pathway, and increase of synthesis and accumulation of various ECM molecules by fibroblasts.

TN-C has been proposed to be involved in fibrosis mostly based on highly upregulated expression in the early stages of fibrotic lesions. Recent studies have further shown that deletion of TN-C attenuates interstitial fibrosis after myocardial infarction, liver fibrosis, and lung fibrosis and reduces matrix synthesis in injured aortic walls. Conversely, locally applied TN-C promoted collagen fiber deposition in aneurysmal cavities in a rat model. Moreover, TN-C accelerates recruitment of myofibroblasts, the major source of collagen during fibrotic processes. These findings suggest that TN-C may work as a profibrotic molecule.

In addition, there is a growing body of evidence that TN-C may modulate inflammatory responses. Experiments using TNKO mice indicated that TN-C augments inflammatory responses. Whole mount immunostaining for TN-C showed that TN-C is highly expressed in inflammatory cells, such as macrophages and neutrophils, at the site of injury. Furthermore, mice deficient in TN-C have reduced infiltration of inflammatory cells and decreased expression of proinflammatory cytokines. These findings suggest that TN-C may play a key role in the modulation of the inflammatory response during fibrosis.

**Figure 5.** Integrin αVβ3 is involved in NF-κB activation and IL-6 expression. A, Immunoblot phosphorylation of NF-κB after TN-C stimulation was analyzed in the presence of 100 nM cyRGDfV and 10 μg/mL anti-β1 antibodies for 30 min. Addition of cyRGDfV clearly inhibited the phosphorylation. Integrin αV subunit regulates NF-κB phosphorylation after TN-C stimulation. B, Immunoblotting of αV and β3 subunits in macrophage lysates. The samples were immunoprecipitated with anti-integrin β3 or nonimmune (n.i.) antibodies (control lanes) and subjected to Western blotting. Immunoprecipitation of lysates from TN-C–treated macrophages showed αV coprecipitation with β3. C, Immunofluorescence images of integrin αVβ3 20 min after 10 μg/mL TN-C stimulation. TN-C–treated cells show intense staining of αVβ3 in focal adhesions. D and E, Western blot analysis of NF-κB phosphorylation (D) and quantitative PCR analysis of IL-6 mRNA expression (E) after 10 μg/mL TN-C stimulation in the presence of αVβ3 inhibitors, 10 μg/mL of a neutralizing anti-integrin β3 antibody, 100 nmol/L cyRGDfV, and 10 μg/mL P11. TN-C stimulation enhanced phosphorylation of NF-κB and IL-6 upregulation. When macrophages were pretreated with inhibitors 1 h before TN-C incubation, both NF-κB phosphorylation and IL-6 upregulation were blocked. Each bar represents a mean±SD for 3 independent experiments. IL indicates interleukin; NF-κB, nuclear factor; and TN-C, tenascin-C.
responses in hepatitis and autoimmune arthritis and induces production of various proinflammatory cytokines by macrophages and fibroblasts.8,9,27

We have previously reported that TN-C is detected in perivascular inflammatory lesions of the heart in AngII-treated mice11 and rats.12 In the present study, we confirmed that AngII infusion induced marked accumulation of macrophages in perivascular areas in wild-type mice and that TN-C was deposited around macrophages. AngII treatment also elevated mRNA levels of MCP-1 and IL-6 in the myocardium, whereas deletion of TN-C significantly reduced these changes. AngII upregulates MCP-1 expression in various cells28 and also acts as a strong inducer of TN-C in fibroblasts.11,12 Therefore, we hypothesized that TN-C synthesized by perivascular fibroblasts may affect macrophages infiltrating perivascular areas triggered by MCP-1.

In vitro, TN-C accelerated migration of mouse peritoneal macrophages to MCP-1 and also enhanced NF-κB activation in macrophages within 5 to 10 minutes. Furthermore, TN-C upregulated mRNA levels of IL-6 in a NF-κB–dependent manner. IL-6 and other members of the interleukin family are known to be profibrotic cytokines during progression of fibrosis in the heart and kidney.18,29 IL-6 stimulates collagen synthesis by myofibroblasts.18,29 We also confirmed that recombinant IL-6 upregulated collagen I mRNA expression of cardiac fibroblasts in culture. Increased level of IL-6 was detected in the mouse heart undergoing fibrosis. Taken together, in the AngII-treated heart, TN-C may enhance macrophage activity and upregulate IL-6 secretion, which in turn increases synthesis of collagen, leading to acceleration of fibrosis.

We also tried to identify receptors for TN-C on macrophages. Multiple cell-surface receptors, including integrins α9β1,27 αVβ1,30 αVβ3,11 and toll-like receptor 4,32 bind to the respective domains of TN-C and transmit multiple signals depending on the cell type and the context.19 Several studies have indicated that integrin αVβ3 is highly expressed on activated or growing cells in inflamed tissues.33 We found
that perivascular macrophages in AngII-treated mouse hearts expressed integrin \(\alpha V\beta 3\). Therefore, we expected that integrin \(\alpha V\beta 3\) might be the receptor for TN-C in this context. To test this possibility, we first examined the effect of integrin \(\alpha V\) antagonists or anti-integrin \(\beta 1\) antibodies on NF-\(\kappa B\) phosphorylation induced by TN-C because the great majority of integrin dimers include an \(\alpha V\) or \(\beta 1\) subunit. We found that inhibition of the \(\alpha V\) subunit significantly suppressed NF-\(\kappa B\) phosphorylation induced by TN-C, whereas the anti-\(\beta 1\) subunit showed weak effect, suggesting that \(\alpha V\)-mediated signaling is primarily involved in the activation of macrophages by TN-C. To identify the partner of the \(\alpha V\) subunit, further inhibitory experiments were executed with anti-integrin \(\beta 3\) antibodies and found that NF-\(\kappa B\) p65 phosphorylation and IL-6 expression were inhibited. Moreover, we confirmed that an \(\alpha V\beta 3\) antagonist and Src inhibitor also gave similar results.

Furthermore, to investigate signaling molecules immediately downstream of integrin, we focused on FAK-Src. Adhesion of macrophages to TN-C led to phosphorylation of FAK (pY925)/Src (pY416), which was inhibited by the integrin \(\alpha V\beta 3\) antagonist. Therefore, our experimental data suggest that integrin \(\alpha V\beta 3\) can be a functional receptor in TN-C-mediated proinvasive and proinflammatory actions on macrophages, with involvement of FAK-Src and NF-\(\kappa B\) activation.

Several reports including ours\textsuperscript{11,14} demonstrated that inflammatory reactions are elicited in the perivascular region of myocardium. Various proinflammatory cytokines can increase the synthesis of TN-C,\textsuperscript{18} which may interact with integrin \(\alpha V\beta 3\) of macrophages, upregulating IL-6, a profibrotic cytokine, expression via FAK-Src through NF-\(\kappa B\) and augmenting inflammatory responses. IL-6 released from macrophages may stimulate cardiac fibroblasts and promote collagen synthesis, resulting in the cardiac fibrosis. In addition to AngII, various remodeling mediators, including proinflammatory cytokines, growth factors, and endothelin-1, can increase the synthesis of TN-C. Therefore, TN-C may be a key molecule to modulate inflammatory/fibrotic signaling by creating a positive feedback loop during ventricular remodeling.

**Perspectives**

The present study clearly demonstrates that TN-C can be a proinflammatory/profibrotic molecule and play an important role in cardiac fibrosis. Uregulated TN-C in the AngII-induced hypertensive heart enhanced accumulation and synthesis of inflammatory cytokines and chemokines of macrophages, which, in turn, stimulated fibroblasts to synthesize collagen. Deletion of TN-C attenuated fibrosis, as well as inflammation, in the heart. Therefore, TN-C might be a new biotarget to reduce cardiac fibrosis.
References


### Novelty and Significance

**What Is New?**
- We have found a novel molecular mechanism involved in augmentation of cardiac fibrosis triggered by renin/aldosterone/angiotensin system-mediated inflammation. An extracellular matrix protein, tenascin-C (TN-C), activates macrophages and increases proinflammatory/profibrotic cytokine production through integrin $\alpha$V$\beta$3/FAK-Src/NF-κB, leading to fibrosis.

**What Is Relevant?**
- Accumulating clinical studies have indicated that high serum levels of TN-C in heart disease could predict worse patient outcome. Our present study may explain one mechanism of how TN-C may exacerbate ventricular remodeling.

**Summary**
- TN-C, a huge hexameric extracellular matrix glycoprotein, is highly upregulated under various cardiovascular diseases. Accumulating data suggest that TN-C can be a predictive biomarker for worse patient outcome and play a significant role in the progression of ventricular remodeling. We investigated the role of TN-C in cardiac fibrosis and the underlying molecular mechanisms driving the inflammation. We found that deletion of TN-C significantly reduced interstitial collagen fibers and macrophage accumulation in the perivascular region and upregulated mRNA expression of interleukin-6 in an angiotensin II–induced hypertensive mouse heart, compared with those of wild-type mice. In vitro, TN-C upregulated the expression of interleukin-6 via integrin $\alpha$V$\beta$3/NF-κB axis. These results provide direct evidence that TN-C accelerates interleukin-6 production by macrophages via integrin $\alpha$V$\beta$3/NF-κB and worsens perivascular inflammation, thereby exacerbating fibrosis in a hypertensive mouse heart.

Tenascin-C May Accelerate Cardiac Fibrosis by Activating Macrophages via the Integrin αVβ3/Nuclear Factor-κB/Interleukin-6 Axis


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Tenascin-C may accelerate cardiac fibrosis by activating macrophages via the integrin αVβ3/NF-κb axis.

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A short title: TN-C modulates inflammation and fibrosis.

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Supplemental Methods

Reagents and antibodies

Micro-osmotic pumps were purchased from ALZET (Cupertino, CA). Angiotensin II (AngII) was obtained from SIGMA (St. Louis, MO). The anti-collagen I antibody was purchased from Abcam (Cambridge, MA). Production of mouse monoclonal anti-TN-C antibody (4F10TT, IBL, Takasaki, Japan) was as previously described.\(^1\) The anti-Mac-3 antibody was obtained from Pharmingen (Cambridge, MA). FITC-conjugated anti-TN-C was from IBL. cyRGDFV (Cyclo [Arg-Gly-Asp-D-Phe-Val]) was from Peptide Institute (Osaka, Japan). P11 (HSDVHK) and PP2 were from Calbiochem (San Diego, CA). Anti-integrin\(\beta1\) and \(\beta3\) antibody were from BioLegend (San Diego, CA). Anti-integrin \(\alphaV+\beta3\) polyclonal antibody was from Bioss Biological Technology Ltd. (Beijing, China). The antibodies against NF-\(\kappa\)B, phospho-NF-\(\kappa\)B (Ser536), FAK, p-FAK (Tyr397, Tyr925), SRC, p-SRC (Tyr416), and Alexa 488-labeled anti-rat IgG (H+L) were purchased from Cell Signaling Technology (Beverly, MA). Alexa 546-labeled F(ab')\(_2\) fragment of goat anti-rabbit IgG (H+L) and Alexa 647-labeled goat anti-mouse IgG2b (\(\gamma2b\)) were from Life Technologies (Carlsbad, CA). Anti-integrin \(\alphaV\) (Q-20) and \(\beta3\) (H-96) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Rat anti-mouse CD51 (integrin \(\alphaV\)) used by immunoprecipitation was from eBioscience (San Diego, CA). Anti-mouse CD61 (integrin \(\beta3\)) used by immunoprecipitation and horse radish peroxidase (HRP)-conjugated anti-rabbit IgG Fab’ were from MBL (Nagoya, Japan). HRP-conjugated goat F (ab’) fragment anti-rat IgG (H+L) was from Beckman Coulter (Villepinte, France). Hoechst33342 was from Dojindo Laboratories (Dojindo, Kumamoto, Japan). Rhodamine phalloidin was from Invitrogen (Carlsbad, CA). The Integrin \(\alphaV\) (Q-20) and \(\beta3\) (H-96) antibodies were purchased from Santa Cruz Biotechnology. The following specific primers for real-time PCR were purchased from Qiagen (Valencia, CA): QuantiTect primer assay, QT01048355 for mouse IL-1\(\beta\), QT00098875 for mouse IL-6, QT00104006 for mouse TNF-\(\alpha\), QT01658692 for mouse
GAPDH.

Experimental Animals and Blood Pressure
The experiments, which were reviewed and approved by the Mie University Animal Experiment and Care Committee, were conducted in TN-C-null (-/-) female, 8-week-old BALB/c mice. Wild-type littermates were used as controls. All experiments were performed according to international guidelines. All animals were supplied with 1% NaCl drinking water and assigned to 1 of the following 4 groups: (1) vehicle WT control mice (n = 9); (2) Ang II-treated WT mice (n = 9) (3) TN-C knockout (TNKO) mice (n = 9); (4) Ang II-treated TNKO mice (n = 9). A micro-osmotic pumps containing 0.1 mL of the vehicle (0.9% NaCl: 99.7% acetic acid = 15:1, vol/vol) only or with 2.83 mg/mL of AngII was implanted subcutaneously into the interscapular regions of isoflurane-anesthetized mice, and AngII (560 ng/kg body weight/min) was infused for 4 weeks. The body weights and blood pressure of all mice were recorded weekly. Blood pressure was monitored with the BP-98A (Softron, Tokyo, Japan) tail cuff system. Experiments were initiated at the same time each day to allow for any diurnal variation in blood pressure.

Immunohistochemistry and immunofluorescence staining
Immunohistochemistry and immunofluorescence staining were performed and positive signals were quantitated as previously described. For indirect immunofluorescence staining, antigen retrieval was performed by autoclaving using HistoVT one (Nacalai Tesque, Kyoto, Japan). In brief, sections after antigen retrieval were incubated with either polyclonal rabbit anti-TN-C antibodies, or monoclonal anti-Mac-3 antibodies for identification of macrophages, followed by treatment with horse radish peroxidase (HRP)-conjugated or fluorescent dye-labeled secondary antibodies. For collagen I staining, the Benchmark® LT
auto-immunostaining system (Ventana Medical Systems, Tokyo, Japan) was used as described previously. With immunofluorescence staining, images were taken using a confocal laser-scanning microscope LSM 710 and ZEN 2009 software (Carl Zeiss, Oberkochen, Germany).

**Cell Isolation, Culture and Treatment**

One mL of 2.9% thioglycolate broth solution (Fluka, Bornem, Belgium) was injected into the peritoneal cavity of a 12-week-old female either TNKO or WT mice (BALB/c background). Three days after the injection, the abdominal cavity was washed with 3 ml of sterile Hanks’s solution and the solution was centrifuged at 1500 g for 2 min. Cells were resuspended in RPMI 1640 (Sigma) containing antibiotic and 10% heat-inactivated (56°C, 30 min) fetal bovine serum (FBS) (Roche, Mannheim, Germany), and plated to 24-well culture dishes (BD Falcon, Franklin Lakes, NJ) at a concentration of about 4×10⁵ cells/well for 2 h, and non-adhesive cells were removed. Adhesive cells were serum-starved (0.1% FBS) for 16 h for analysis by Western blotting. To analyze cytokine mRNA expression, cells were seeded in 12-well culture dishes (BD Falcon) at a concentration of about 1×10⁶ cells/well and allowed to rest for 2 h, and non-adhesive cells were removed. Cells were serum-starved (0.2% FBS) for 16 h and then treated with purified TN-C for 20 min, 30 min or 6 h and analyzed by immunofluorescence staining, western blotting or RT-PCR, respectively. Cardiac fibroblasts were obtained from ventricles of Balb/c mice as described previously and plated in MULTI-WELL 6-well plates (BD Falcon). After culture in serum-free IMDM media for 16 h, cells were treated with 5 ng/mL recombinant IL-6 (R&D Systems, Minneapolis, MN) for 6 h.

**Western Blot Analysis**

Cultured cells were lysed in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40 and SDS) (Nakarai Tesque, Kyoto, Japan) containing a protease inhibitor cocktail tablet...
(EDTA free; Roche, Mannheim, Germany) and a phosphatase inhibitor cocktail, PhosSTOP (Roche), in accordance with the manufacturer’s instructions. Lysates were added to Laemmli’s sample buffer and included proteins (10µg/lane) were separated on 2-15% SDS-polyacrylamide gels (ATTO, Tokyo, Japan) and transferred onto Immobilon membranes (Millipore, Billerica, MA). The membranes were treated with 5% bovine serum albumin (BSA) in Tris-buffered saline with 0.1% Tween-20 (TBS-T) including PhosSTOP to block non-specific reactions, and immunostained with primary antibodies. Subsequently, HRP-conjugated goat anti-rabbit secondary antibodies (SIGMA) and the ECL prime detection system (GE Healthcare, Uppsala, Sweden) were used for visualization.

**Immunoprecipitation**

To immunoprecipitate various integrin subunits, cells were lysed in ice-cold lysis buffer, 1% Triton X-100, 10% glycerol, 150 mM NaCl, with a proteinase inhibitor cocktail and PhosSTOP. The lysates were centrifuged at 15,000×g for 20 min and supernatants were pre-cleaned with Protein G Mag Sepharose (GE Healthcare) for 2 h, and then incubated with corresponding antibodies or non-immunized mouse IgG, as a negative control, at 4°C for 4 h. After samples were precipitated with protein G beads at 4°C for 16 h, the beads were washed three times with lysis buffer and proteins eluted by boiling in sample buffer with or without 2-mercaptopethanol for 10 min and separated by SDS-PAGE. The samples were subjected to Western blotting as described above.

**Macrophage Migration Assay**

Migration was measured in 24-well plates with a Cell Culture Insert, polyethylene terephthalate track-etched membranes with 8-µm pores (BD Falcon). A final concentration of 10 ng/mL of recombinant MCP-1 (PeproTech Inc., Rocky Hill, NJ) in 1% BSA in Hanks’s solution was seeded to the lower well, and 1 µg of purified TN-C was coated onto the upper
surfaces of the filters. Cells \( (7.5 \times 10^5 \text{ cells}) \) were added to the upper chamber in 300 \( \mu \text{L} \) DMEM containing 0.2\% fetal calf serum. After incubation at 37°C in a humidified environment for 3 h, the filters were removed. Then, the upper surfaces of the filters were scraped twice with cotton swabs to remove non-migrating cells. Cells that had migrated to the undersurface were washed with PBS, fixed, and stained with Diff-Quick solution (Sysmex, Kobe, Japan). The images of 25 serial high-power bright-fields (magnification\( \times 200 \)) were combined together using the MozaiX module within Zeiss AxioVision software (Carl Zeiss) and counted by ImageJ software.

**Supplemental References**

Figure S1: A and B, Overview of results for systolic blood pressure (A) and body weight (B) in each experimental group. Angiotensin II (AngII) infusion caused hypertension with significantly elevated systolic blood pressure (SBP) in both WT and TNKO mice. However, there is no significant difference in SBP between WT and TNKO mice with the infusion. Body weights are not different among the four groups. Values are means ± SD (n=9 each group). *P <0.05 vs WT group; †P <0.05 vs TNKO group.
Figure S2: Evaluation of cardiac hypertrophy. A and B, Ratio of heart weight to body weight (A) and the breadth of cardiomyocytes (B) were measured. The ratio after AngII-treatment was elevated but did not differ between WT and TNKO groups. C and D, Quantitative PCR analysis of mRNA expression of atrial natriuretic peptide (ANP) (C) and brain natriuretic peptide (BNP) (D) was also performed. Both ANP and BNP mRNA were up-regulated by AngII-treatment groups, but not did not differ between the WT and TNKO groups. Values are means ± SD (n=5 each group). *P <0.05, **P <0.01 and ***P <0.001 vs WT group. †P <0.05 vs TNKO group. N. S., not significant.
Figure S3: Collagen I and III in fibrotic lesion of Ang treated mouse heart. Representative photographs of immunostaining for collagen I and III (A and C). Positively stained area was quantified by image analyses (B and D) (n=9 for each group). **P < 0.01 vs WT group. Magnification: × 200.
Figure S4: A and B, Relative expression levels of collagen Iα2 (A) and IIIα1 (B) mRNAs, quantified by qRT-PCR. Data represent the means ± SDs of nine samples of each group. *P < 0.05 vs WT group. †P < 0.05 vs TNKO group.
**Figure S5**: IL-6 stimulated the expression of collagen I mRNA in cardiac fibroblasts. Addition of IL-6 (5 ng/mL) to the culture medium significantly up-regulated collagen I mRNA. Each bar represents a mean ± SD for 3 independent experiments. **P <0.01 vs non treatment cells**