Characterization of fibrosis-promoting factors and siRNA-mediated therapies in C-protein-induced experimental autoimmune myocarditis

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ABSTRACT
Due to poor proliferation abilities of cardiomyocytes, the repair process in the heart after insults is often associated with fibrosis formation. In this study, we characterized inflammation and/or fibrosis-related molecules in the heart with experimental autoimmune carditis. Immunohistochemical examinations revealed that expression of tenascin-C (TNC), matrix metalloproteinase-9 (MMP-9), transforming growth factor-β1 (TGF-β1), connective tissue growth factor (CTGF) and α smooth muscle cell actin (αSMA) peaked at 2 weeks post-immunization but only TGF-β1 expression was sustained at 8 weeks. Administration of siR-NAs for MMP-2 (siMMP-2) and for MMP-9 (siMMP-9) alone did not modulate inflammation and fibrosis. In contrast, simultaneous administration of siMMP-2 and siMMP-9 significantly reduced inflammation and fibrosis. Of note, siRNA treatment for TGF-β1, which is an anti-inflammatory cytokine, increased inflammation and decreased fibrosis. These findings suggest that in case of diseases characterized by initial inflammation and subsequent fibrosis, immunotherapies should target inflammation, not fibrosis, because the latter therapies exacerbate inflammation.

1. Introduction

More than 750,000 new cases are diagnosed as heart failure in the United States and dilated cardiomyopathy (DCM) accounts for 25% of cases [1]. Myocarditis is suspected as a common precursor of DCM. Autoantibodies may play an important role of this process [2,3]. Inflammation caused by either autoimmune mechanisms or infection is one of the factors that promotes fibrosis formation of the lesions. In previous studies [4,5], we have demonstrated that cardiac C-protein that localizes in thick filaments of cardiac muscles [6] has a strong carditis-inducing ability. Experimental autoimmune carditis (EAC) that is induced in Lewis rats by immunization with C-protein is a T cell-mediated disease, but various types of autoantibodies are involved for the shift from EAC to DCM [5]. In addition, it was shown that several chemokines play an important role in disease progression [4].

Matrix metalloproteinases (MMPs) form a family of enzymes that mediate various functions in tissue destruction, remodeling and immune responses by hydrolyzing components of the extracellu-
were conducted in accordance with the Guideline for the Care and Use of Animals (Tokyo Metropolitan Institute of Medical Science, 2011). The experimental protocols were approved by the Animal Use and Care Committee of the Tokyo Metropolitan Institute of Medical Science.

2.2. Preparation of recombinant C-protein fragments and synthetic peptides

The preparation of recombinant C-protein was precisely described previously [4]. PCR products corresponding to cardiac C-protein Fragments 2 (CC2) were inserted into a cloning vector, pCR4 Blunt-TOPO, in the Zero Blunt TOPO Kit (Invitrogen) and clones with correct sequences were subcloned into the pQE30 expression vector (QIAGEN). Recombinant CC2 produced in transformed Escherichia coli was isolated under denaturing conditions and purified using Ni-NTA Agarose (QIAGEN).

2.3. EAC induction and tissue sampling

Lewis rats were immunized once on day 0 with CC2 with complete Freund’s adjuvant (CFA) (Mycobacterium tuberculosis 2.5 mg/ml) in the hind footpads. At the time of immunization, rats received an intraperitoneal injection of 1 μg pertussis toxin (PT) (Seikagaku Corp.). Although evaluation of EAC was mainly based on histological examinations of the heart performed at 1, 2, 4, 6, 8 and 12 weeks post-immunization (Fig. 1A), the clinical score was also recorded: grade 1, dyspnea; grade 2, dyspnea plus ruffling of fur; and grade 3, moribund condition or dead. As shown in a previous study [4], more than 60% of rats died by 12 weeks post-immunization.

![Fig. 1](image)

**Fig. 1.** Fibrosis formation during the course of EAC. Before (N, naive) and after CC2 immunization (1, 2, 4, 6, 8 and 12 weeks); heart tissue was examined for fibrosis after elastic van Giessen staining (A). Histology of 4 weeks (B) and 12 weeks (C) are shown. Bar = 200 μm. Each section was then graded and the kinetics of fibrosis is depicted in D. Each bar represents the mean value ± SD. Four rats were examined at each time point.

2.4. Histological grading of inflammation and fibrosis and immunohistochemistry

Under deep anesthesia, rats were sacrificed at the indicated time points and hearts were processed for histological examinations. Inflammatory lesions in the heart were evaluated using hematoxylin and eosin (H&E)-stained sections according to the following criteria: grade 1, rare focal inflammatory lesions; grade 2, multiple isolated foci of inflammation; grade 3, diffuse inflammation involving the outer layer of the muscle; grade 4, grade 3 plus focal transmural inflammation; grade 5; diffuse inflammation. The extent of fibrosis revealed by elastic van Giessen (EVG) or Azan staining was graded into five categories: grade 0, normal connective tissue distribution; grade 1, rare scattered foci of fibrosis; grade 2, multiple isolated foci of fibrosis; grade 3, fibrosis involving the outer layer of the muscle; grade 4, grade 3 plus partial transmural fibrosis; and grade 5, diffuse fibrosis.

Single immunoperoxidase staining was performed using paraffin-embedded sections. After dewaxation and rehydration, endogenous peroxidase was blocked by incubating sections in 0.3% hydrogen peroxide. Then, sections were incubated with antibodies against tenascin-C (TNC) (IBL, Gunma, Japan), matrix metalloproteinase (MMP)-9 (Daichi Fine Chemical, Toyama, Japan), TGF-β1 (Assay BioTech), connective tissue growth factor (CTGF) (GeneTex) or α-smooth muscle cell actin (SMA) (American Research Products, Belmont, MA, USA) overnight, followed by appropriate biotinylated secondary antibodies (Vector, Burlingame, CA, USA) and horseradish peroxidase (HRP)-labeled Vectastain Elite ABC Kit (Vector). HRP binding sites were detected in 0.005% diaminobenzidine and 0.01% hydrogen peroxide. The number and extent of positive cells revealed by immunohistochemistry was graded into five categories: grade 0, no positive cells or normal staining pattern; grade 1, rare scattered foci of positive cells; grade 2, multiple isolated foci of positive cells; grade 3, positive cell cluster involving the outer or inner layer of the muscle; grade 4, grade 3 plus partial transmural positive cell cluster; and grade 5, diffuse positive cell cluster.

2.5. Treatment of EAC with siRNAs

Stealth™ siRNA targeting the indicated molecules is a 25-bp duplex oligonucleotide and was designed and synthesized by Invitrogen Life Technologies.

siCHECK vectors (Promega) containing the target gene were prepared according to the manufacturer’s instructions. Briefly, target genes were amplified using rat spleen cDNA with specific primer pairs containing appropriate restriction enzyme sites and KOD-plus-DNA polymerase (TOYOBO). After the enzyme treatment, the target gene fragments were inserted into psiCHECK™-1 Vector. Then, siCHECK vector containing the target gene and siRNA were cotransfected to HEK293 cells. The silencing efficiency was assayed with the Dual-Luciferase Reporter Assay System (Promega). The efficiency of the knockdown of the indicated siRNA was calculated as percent of firefly luciferase activity/renilla luciferase activity of siRNA compared to that of the control (Negative Universal Control, Invitrogen). Among three candidate siRNA for each molecule, the most efficient one was selected for further experiments.

For treatment of EAC with siRNAs, we employed ALZET osmotic pump system (DURECT Corporation, CA, USA). ALZET 2004 (0.25 μl/h for 4 weeks) pumps that had been set to release siRNAs at a rate of 30 μg/day were implanted intraperitoneally one day before immunization. This time point was chosen to minimize the effect of surgery.
2.6. Western blotting

Heart tissues were homogenized in 50 mM Tris–HCl (pH 7.5) containing 50 mM Tris–HCl (pH 7.5) containing 150 mM NaCl, 10 mM CaCl2, 0.05% Brij 35 with protease inhibitor cocktail (Merck Japan). After centrifugation, supernatants were harvested and 100 μg of protein was run on an appropriate gel and transferred to the PVDF membrane (Merck-Millipore). After blocking with 5% nonfat milk, the blots were incubated with anti-MMP-2, anti-MMP-9 or anti-TGF-β1 antibody (Assay Biotechnology, CA, USA) at 4 °C overnight, followed by incubation with Mouse TrueBlot ULTRA HRP anti-mouse IgG or Rabbit TrueBlot HRP anti-rabbit IgG (eBioscience, CA). The blots were developed by enhanced chemiluminescence reagents (ECL Plus; GE Healthcare Japan) according to the manufacturer’s instructions.

2.7. Gelatin zymography

Gelatin zymography was performed using the Novex In-gel Zymography System (Life Technologies Japan) according to the manufacturer’s instructions. Heart tissues were homogenized in 50 mM Tris–HCl (pH 7.5) containing 150 mM NaCl, 10 mM CaCl2, 0.05% Brij 35 with protease inhibitor cocktail (Merck Japan). After centrifugation, supernatants were harvested and 100 μg of protein was mixed with Novex Tris–Glycine SDS sample buffer and run on Novex 10% Zymogram Gelatin Gel, followed by incubation with Zymogram Renaturing Buffer and then with Zymogram Developing Buffer. After an overnight reaction, the gel was stained with SimplyBlue Safestain (Life Technologies Japan).

2.8. Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) followed by a pos-hoc (Fisher’s PLSD) test. P-values less than 0.05 were considered significant.

3. Results

3.1. Fibrosis formation in the heart with autoimmune inflammation

Rats were immunized with cardiac C-protein Fragments 2 (CC2) and PT to induce cardiac EAC and hearts were taken at 1, 2, 4, 6 and 8 weeks for histology (Fig. 1A). Then, we evaluated the degree of cardiac fibrosis at these time points according to the grading system (Fig. 1A). We did not employ quantitative analysis by measuring the area of fibrosis because case to case variations were so large that image analysis did not always reflect the severity of fibrosis. As shown in Fig. 1B, fibrosis was most extensive at 4 weeks post-immunization (Fig. 1B) and was diffusely observed throughout the myocardium. At 12 weeks, fibrosis was mainly found around the pericardium (Fig. 1C). Fibrosis grading revealed that the fibrosis score peaked at 4 weeks and maintained at a high level thereafter till 12 weeks (Fig. 1D). In contrast, inflammation peaked at 2–4 weeks, declined thereafter and became minimal at 10–12 weeks as reported previously [10].

3.2. Immunohistochemical examination of Tenascin-C (TNC), TGF-β1, CTGF and MMP-9

The presence or absence of immunoreactivities of fibrosis-related molecules at various time points was examined by immunohistochemistry. Under normal conditions (not shown) and on day 7 post-immunization (Fig. 2A), no positive staining for TNC was found in all the areas examined. At 2 weeks, strong and diffuse immunoreactivities were present mainly in parenchymal cells, probably cardiomyocytes (Fig. 2B). At 4 weeks, immunoreactivities became localized around blood vessels (Fig. 2C) and became very faint at 8 weeks (Fig. 2D).

In the heart of naïve animals, only blood vessels were positively stained for TGF-β1 (Fig. 3A) Some CTGF-positive cells were present in the endocardium (Fig. 3B). During early stages of the disease, i.e., at 1 and 2 weeks post-immunization, TGF-β1 immunoreactivities were localized around blood vessels (Fig. 2C) and became very faint at 8 weeks (Fig. 2D).
were very similar to those of CTGF (Fig. 3C–F) although blood vessels were negative for CTGF at all the stages. At 1 week, a few TGF-β1- and CTGF-positive cells (Fig. 3C and D, respectively) were localized around small inflammatory foci. At 2 weeks when inflammation was maximal, a considerable number of infiltrating cells were positive for TGF-β1 (E) and CTGF (F). At 4 and 8 weeks, while TGF-β1 immunoreactivities became very faint including those on blood vessels (G), a large number of CTGF-positive cells that possessed a morphological feature of fibrocytes were present in the parenchyma (H). Bar = 100 μm.

Fig. 3. Immunohistochemical examination of TGF-β1 (A, C, E and G) and CNTF (B, D, F and H). Under normal conditions, only blood vessels were positively stained for TGF-β1 (A). Some CTGF-positive cells were present in the endocardium (area encompassed by dotted line in B). At 1 week, a few TGF-β1- and CTGF-positive cells (indicated by arrows in Fig. 3C and D, respectively) were localized in and around small inflammatory foci. At 2 weeks when inflammation was maximal, a considerable number of infiltrating cells were positive for TGF-β1 (E) and CTGF (F). At 4 and 8 weeks, while TGF-β1 immunoreactivities became very faint including those on blood vessels (G), a large number of CTGF-positive cells that possessed a morphological feature of fibrocytes were present in the parenchyma (H). Bar = 100 μm.

Before EAC induction, αSMA was localized around blood vessels (Fig. 4A). As early as 1 week post-immunization when inflammatory foci were small in size and scarce, a large number of cells with morphological features of myofibroblasts became positive for
a SMA (Fig. 4B). At 2 weeks (Fig. 4C) and 4 weeks (Fig. 4D), severe inflammatory cell infiltration was observed. a SMA-positive myofibroblasts were diffusely distributed among inflammatory foci.

3.3. Semiquantitative analysis of positive staining revealed by immunohistochemistry

We also performed semiquantitative analysis using positive-cell scoring shown in the materials and methods (Table 1). Since positive cells were not evenly distributed and greatly varied from case to case, exact quantitation i.e., counting of positive cells, was not appropriate for evaluation of the disease status. The staining pattern found in the heart of naïve rats was scored as grade 0.

Among fibrosis-related molecules examined in the present study, there were three types showing different kinetics. First, MMP-9 immunoreactivities were only found in the early stage. The robust increase of a SMA-positive staining, which is a marker for myofibroblast, was first found at 1 week post-immunization and gradually decreased in number by 8 weeks. Second, TNC was found at the peak of inflammation and showed high levels at later stages. Third, the increase of TGF-β and CNTF immunoreactivities was found at 1 week, peaked at 2 weeks and gradually declined by the end of the examination. These findings suggest that molecules that are directly involved in fibrosis formation maintain a high level at later stages when fibrosis is evident (Table 1).

3.4. Modulation of EAC with siRNA for MMP-2, MMP-9 and TGF-β1

We finally performed a treatment experiment using siRNA to elucidate the role of fibrosis-related molecules. With the computer

| Table 1 |
| Summary of immunostaining for TNC, MMP-9, TGF-β1, CTGF and aSMA. |

<table>
<thead>
<tr>
<th>Fibrosis score</th>
<th>TNC</th>
<th>MMP-9</th>
<th>TGF-β1</th>
<th>CTGF</th>
<th>aSMA</th>
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<tbody>
<tr>
<td>Naive</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1 W</td>
<td>1</td>
<td>1.3 ± 0.6</td>
<td>1.7 ± 0.6</td>
<td>4.3 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>2 W</td>
<td>2</td>
<td>3.7 ± 0.6</td>
<td>2.7 ± 0.6</td>
<td>4.7 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>4 W</td>
<td>4.5 ± 0.7</td>
<td>1.3 ± 0.6</td>
<td>2.7 ± 0.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8 W</td>
<td>3.3 ± 0.5</td>
<td>2.0 ± 1.0</td>
<td>1.7 ± 0.6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The number and extent of positive cells revealed by immunohistochemistry was graded into five categories: grade 0, no positive cells or normal staining pattern; grade 1, rare scattered foci of positive cells; grade 2, multiple isolated foci of positive cells; grade 3, positive cell cluster involving the outer or inner layer of the muscle; grade 4, grade 3 plus partial transmural positive cell cluster; and grade 5, diffuse positive cell cluster. Mean ± SD of three rats/group is shown. Each group consisted of 4–6 rats.

| Table 2 |
| Treatment of EAC with siRNA for MMP-2, MMP-9 and TGF-β1. |

<table>
<thead>
<tr>
<th>siRNA</th>
<th>Inflammation</th>
<th>Fibrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-2</td>
<td>3.9 ± 0.5</td>
<td>2.5 ± 0.4</td>
</tr>
<tr>
<td>MMP-9</td>
<td>3.9 ± 1.0</td>
<td>4.6 ± 1.1</td>
</tr>
<tr>
<td>MMP-2 plus MMP-9</td>
<td>0.6 ± 0.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.4 ± 0.3&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>4.9 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.9 ± 0.9&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Saline</td>
<td>3.8 ± 1.0&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>3.4 ± 0.5&lt;sup&gt;ad&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

ALZET osmotic pumps 2004 (0.25 µl/h for 4 weeks) containing the indicated siRNA were set to release siRNA at a rate of 30 µg/day and implanted intraperitoneally one day before immunization. Histological examination for inflammation and fibrosis was performed at 4 weeks post-immunization. Each group consisted of four rats.

<sup>a</sup> Significantly different between the two groups (p = 0.000351).
<sup>b</sup> Significantly different between the two groups (p = 0.044141).
<sup>c</sup> Significantly different between the two groups (p = 0.001009).
<sup>d</sup> Significantly different between the two groups (p = 0.008082).

Fig. 4. SMA staining of the heart after CC2 immunization. Before (A) and after (B–D) immunization, paraffin-embedded sections of hearts taken at 1 week (B), 2 weeks (C) and 4 weeks (D) were stained for SMA. Under normal conditions, immunoreactivities are localized in the perivascular area. At 1 week post-immunization, when inflammatory foci are not yet formed in the heart, many SMA-positive cells are scattered throughout the parenchyma (B). At 2 and 4 weeks, immunoreactivities become diffuse and less intense (C and D). Bar = 100 µm.
search, three candidate siRNAs for each target molecule were selected and prepared. The silencing efficiency of siRNA was evaluated using the Dual-Luciferase® Reporter Assay System and the results are shown in Fig. 5A–C. The most efficient one was used for further studies. For in vivo administration, osmotic pumps containing the indicated siRNA were set to release siRNA at a rate of

![Graphs and images showing luciferase activity and gel zymography results]
30 μg/day and implanted intraperitoneally one day before immunization. Then, hearts were removed on day 28 and examined in the presence or absence of inflammation and fibrosis. The results are summarized in Table 2. Administration of siRNA for MMP-2 (siMMP-2) and for MMP-9 (siMMP-9) alone did not modulate either inflammation or fibrosis compared with saline controls. In sharp contrast, simultaneous administration of siMMP-2 and siMMP-9 significantly reduced the severities of inflammation and fibrosis.

In order to evaluate in vivo suppression effects of siRNAs, we also performed gel zymography for MMP-2 and MMP-9 and Western blotting analysis for TGF-β1. As shown in Fig. 5D, gelatinase activities of MMP-2 and MMP-9 was suppressed by siRNAs (Fig. 5D). The combination treatment with siMMP-2 and siMMP-9 suppressed the gelatinase activities almost completely (Fig. 5F and G). Western blotting revealed that TGF-β1 expression was significantly inhibited by siTGF-β1 treatment (Fig. 5E and H).

Pathology of EAC treated with siTGF-β1 was interesting (Fig. 6A–C). At 4 weeks post-immunization, inflammation of siRNA-treated rats became severer (Fig. 6A) and fibrosis formation was very mild (blue staining in B) compared with that of control animals (see panel B of Fig. 1). TGF-β1 immunostaining revealed a smaller number of positive infiltrating inflammatory cells (C). A, H&E staining; B, azan staining; C, TGF-β1 immunostaining, Bar = 100 μm.

In summary, we made longitudinal examinations of localization of fibrosis-related molecules in the heart with EAC and found findings suggesting that TGF-β1 and CTGF play a key role in fibrosis formation in EAC and are potential targets of fibrosis-preventing immunotherapies (discussed in detail below).

We performed treatment experiments of EAC using siRNA for fibrosis-related molecules including MMP-2, MMP-9 and TGF-β1 to elucidate the pathomechanisms and to develop effective immunotherapies for autoimmune inflammation and subsequent fibrosis. MMPs have pro-inflammatory nature. In a previous study [10], we showed that minocycline, which inhibits MMP-9 activities more strongly than MMP-2, significantly suppressed EAC, but that an MMP-2-specific inhibitor, TISAM, did not affect the disease course. The present study clearly demonstrated that either MMP-2- or MMP-9-specific inhibition alone did not improve the severities of both inflammation and fibrosis. In sharp contrast, combination therapies with siRNA for MMP-2 and MMP-9 suppressed gelatinase activities almost completely and significantly ameliorated EAC pathology. TGF-β1 is an anti-inflammatory cytokine that promotes fibrosis formation. Treatment with siRNA for TGF-β1 revealed interesting findings. Inflammation in the heart of siRNA-treated rats became more severe and fibrosis formation was significantly suppressed compared with those of control animals. Since TGF-β1 knockout mice show extensive inflammation and necrosis in multiple organs [19], transient suppression of the same cytokine may also result in similar changes. Although suppression of TGF-β1 is reported to effectively inhibit cardiac fibrosis [20,21], it is not applicable to fibrosis accompanied with inflammation. Similarly, there are reports showing that CTGF suppression by siRNA effectively inhibited cardiac fibrosis in animal models [22,23]. In the latter case, fibrosis was induced without inflammation. Therefore, there is the possibility that treatment with siRNA for CTGF may exacerbate inflammation in EAC.

In summary, we made longitudinal examinations of localization of fibrosis-related molecules in the heart with EAC and found findings suggesting that TGF-β1 and CTGF play a key role in fibrosis formation. Since suppression of these molecules may exacerbate preceding inflammation in myocarditis, inhibition of the inflammatory process, e.g., treatment with siRNAs for MMP-2 plus MMP-9, would be the first choice for immunotherapies.

4. Discussion

For a long time, the heart has been considered as an organ in which cardiomyocytes cannot be replaced by newly formed cells in case of insults such as autoimmune inflammation or infarction. Fibrosis often replaces the area of necrosis. In the literature, TGF-β1 plays a central role in fibrosis formation. TGF-β1 stimulates αSMA-positive myofibroblasts (MFB) [11,12] to promote fibrosis [13]. CNTF has similar functions as TGF-β1 independently or after stimulation with TGF-β1 [14,15]. TGF-β1 also stimulates TNC [16], which in turn, activates MMP-9 [17] and MFB [8] to accelerate fibrosis formation. It was reported that MMP-9 activates TGF-β1 [18]. However, it remains unclear whether these molecular interactions take place together during the progression of a particular cardiac disease.

EAC is an autoimmune inflammation of the heart induced by immunization of cardiac specific antigen, CC2, followed by extensive fibrosis as shown in Fig. 1. In the present study using this model, we made longitudinal examinations of molecules that relate to fibrosis formation. Table 1 shows the summary of the findings. As demonstrated in Fig. 1, fibrosis formation peaked at 4 weeks. Immunohistochemical examinations revealed that all the molecules examined here except αSMA were stained most intensively at 2 weeks. At later stages (4 and 8 weeks), staining for TNC, MMP-9 and αSMA became faint. Positive cells for TGF-β1 and CTGF, both of which are strong fibrosis-promoting factors, were present till the end of examination. These findings suggest that TGF-β1 and CTGF play a key role in fibrosis formation in EAC and are potential targets of fibrosis-preventing immunotherapies (discussed in detail below).

Conflict of interest

The authors declare that no conflict of interest exists.

Acknowledgments

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References