Increased Connective Tissue Growth Factor Expression in a Rat Model of Chronic Heart Allograft Rejection

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Background/Purpose: Chronic rejection limits the long-term success of cardiac transplantation and the underlying cause of the disease is unknown. Connective tissue growth factor (CTGF) is considered as a mitogenic and chemotactic factor for fibroblasts, and is associated with cell proliferation and collagen synthesis. We evaluated the expression of CTGF in a rat model of heart allograft chronic rejection.

Methods: Intra-abdominal heterotopic heart transplantation was performed from 20 Wistar rats to 20 Sprague-Dawley (SD) rats that received cyclosporine, mycophenolate mofetil and methylprednisolone as immunosuppression. Ten heart allografts were explanted at 2 and 8 weeks postoperatively for analysis of morphologic changes. The hearts from 10 normal Wistar rats served as a control group. Coronary artery density, luminal loss of myocardial coronary arteries, and myocardial fibrosis were measured. The expression of CTGF was studied by immunohistochemistry. Correlation between CTGF expression and development of cardiac allograft vasculopathy (CAV) or fibrosis was studied.

Results: Allografts harvested at 8 weeks postoperatively showed more coronary intimal proliferation, fibrosis and CTGF expression compared with the 2-week allografts ($p < 0.05$) and the controls ($p < 0.01$), but the coronary artery density was lower than in the control group ($p < 0.05$). However, the control group showed negligible CTGF expression. There were strong negative correlations between the gray value of CTGF protein expression and cardiac fibrosis and coronary intimal occlusion ($r = -0.734, -0.713; p < 0.01$), which demonstrated that CTGF protein expression was positively correlated with cardiac fibrosis and coronary intimal occlusion.

Conclusion: CTGF is expressed in cardiomyocytes in CAV. Increased expression of CTGF in cardiac allografts is associated with development of CAV and fibrosis formation. [J Formos Med Assoc 2009;108(3):240–246]

Key Words: connective tissue growth factor, fibrosis, graft rejection, heart transplantation

Cardiac transplantation has been established as an effective treatment for end-stage cardiac diseases. However, despite the fact that advances in immunosuppressive therapy and patient management during the past decade have improved short-term survival following heart transplantation, mortality after the first year has remained unchanged.1 Late graft loss in living patients is predominantly caused by chronic rejection. Chronic rejection is a progressive, irreversible disease that is characterized by deteriorating graft function, interstitial fibrosis and occlusion of luminal structures such as arteries and epithelial-lined conduits.2 Chronic rejection of cardiac allografts is manifested by the development of cardiac allograft vasculopathy (CAV) and interstitial
The classic vascular lesions of CAV are characterized by diffuse, concentric intimal thickening composed primarily of modified smooth muscle cells (SMCs). CAV may affect vessels along their entire length, and intimal thickening may thus be seen in large and small arteries. It is not only limited to coronary arteries but may be present in coronary veins in explanted human hearts. Despite intense investigation, the etiology and associated pathology of chronic rejection are still not clear. Results from many different laboratories have identified several major cytokines that may contribute to this process, and the most important of these is transforming growth factor-β (TGF-β). TGF-β not only has a fibrogenic effect, but also has several major physiologic roles, including suppression of the immune system, that are viewed as beneficial in inflammation, transplantation and epithelial proliferation. As a result, it is largely assumed that, from clinical and pharmaceutical standpoints, long-term inhibition of TGF-β may be a less suitable strategy to prevent chronic rejection after heart transplantation.

Connective tissue growth factor (CTGF) is a recently recognized downstream effector of TGF-β. CTGF is a 38-kDa, heparin-binding, cysteine-rich protein that can induce cell proliferation, collagen synthesis and chemotaxis in a variety of cells including endothelial cells, SMCs and fibroblasts. The CTGF promoter contains Smad binding elements and a unique TGF-β response element, CTGF mediates many of the fibrogenic activities of TGF-β and plays a more specific role as a fibrogenic cytokine, but CTGF alone is not involved in the immunomodulatory effects of TGF-β. Thus CTGF might be used as a potential target for preventing chronic rejection, while sparing the anti-inflammatory and anti-proliferative activities of TGF-β. More recently, CTGF has been found to be overexpressed in chronic allograft nephropathy. However, few studies have focused on its role during the process of heart allograft fibrosis and CAV.

Hence, to further explore the relationship between CTGF and chronic heart allograft injury, we tested CTGF expression by immunohistochemical staining, using a rat heart transplant model.

**Methods**

**Animals**

Twenty inbred male SD rats and 30 inbred male Wistar rats were purchased from the laboratory of the Second Xiangya Hospital, Changsha, Hunan, China. The rats were 6–8 weeks of age and weighed 250–300 g and were housed under specific pathogen-free conditions in the Unit for Laboratory Animal Medicine at the Second Xiangya Hospital. The animals were fed with regular rat food and tap water ad libitum. The Wistar rats served as heart graft donors and the SD rats as the recipients. The study was approved by the institutional ethics committee on animal research.

**Heterotopic cardiac transplantation**

The rat model of heterotopic cardiac transplantation was performed according to the method of Ono and Lindsey, with some modifications. Briefly, rats were anesthetized with intraperitoneal injection of 5% chloral hydrate. The recipient abdomen was opened before the donor heart was explanted, in order to reduce the ischemic time. Immediately after explantation, the ascending aorta and pulmonary artery of the donor heart were anastomosed to the infrarenal abdominal aorta and inferior vena cava of the recipient in an end-to-side fashion. The donor heart was perfused with recipient rat’s blood and resumed contraction. Total procedure time averaged 58 minutes. Graft survival was monitored by daily abdominal palpation. Penicillin was administered at a dose of 10 × 10^4 U by intramuscular injection to the recipient immediately after transplantation and lasted for 3 days. The immunosuppressive regimen consisted of intramuscular cyclosporine (10 mg/kg/day), and mycophenolate mofetil (40 mg/kg/day) and methylprednisolone (3 mg/kg/day) by gavage from the second day after transplantation until specimens were harvested.
The control group received the same immuno-suppressive regimen as the allograft recipients.

**Grouping and acquisition of samples**

Ten transplants were harvested at 2 and 8 weeks postoperatively. Hearts from 10 normal Wistar rats served as a control group. The left ventricular wall tissues taken from the transplants immediately after sacrifice were fixed in 10% buffered formalin solution, routinely embedded in paraffin, and then serially sectioned at a thickness of 4 μm.

**Cardiac histology**

The paraffin sections were stained with hematoxylin and eosin (HE) for examination of pathologic morphology and stained with Van Gieson stain to delineate the internal elastic lamina. The mean total number of coronary artery vessels in five randomly chosen fields of view (50× magnification) was defined as myocardial coronary artery density. The Northing Medical Image Analysis Management System, Mias System 4.1 (Beijing University of Aeronautics & Astronautics, China), was used to measure the area surrounded by the internal elastic lamina and vessel lumen. Each artery was assessed for percentage of intimal proliferation (percentage of luminal occlusion), defined as the intimal area divided by the overall area inside the elastic lamina (intimal area plus luminal area).19 Graft interstitial fibrosis was estimated from sections stained with HE and scored semiquantitatively: 0, no fibrosis; 1, mild fibrosis; 2, moderate fibrosis; and 3, intense interstitial fibrosis.20 All analyses were performed in a blinded manner by two independent observers.

**Immunohistology**

The streptavidin–biotin–peroxidase complex (SABC, SA1052) immunohistochemistry kit was purchased from Boster Biological Technology Co., Ltd. (Wuhan, China). Formalin-fixed, paraffin-embedded sections were deparaffinized and rehydrated with distilled water. Endogenous peroxidase was blocked using blocking buffer (3% H₂O₂) and antigen retrieval was performed in a pressure sterilizer. After the primary rabbit anti-CTGF antibody (diluted 1:100) was added and set for 1 hour in a wetting case at 37°C, the slides were washed with phosphate-buffered saline (PBS), and incubated with biotinylated goat anti-rabbit secondary antibody for 15 minutes in a wetting case at 37°C. Then, the slides were incubated with SABC for 20 minutes in a 37°C water bath. After washing with PBS, the slides were incubated with 3,3′-diaminobenzidine. Then, the slides were counterstained with hematoxylin before mounting and evaluation. Negative controls were performed using PBS instead of CTGF antibody. Semiquantitative scoring of CTGF expression was analyzed on a digitally acquired image by the Mias System 4.1. From the image, the expression of CTGF was obtained by determining the average gray scale of masculine grana. The smaller the gray scale value, the deeper the masculine coloration, which signifies a larger amount of CTGF protein expression. For every slide, eight integral and non-overlapping visual fields were chosen at random to obtain the semiquantitative scores. The mean score of the eight fields gray scale was considered to represent the extent of CTGF expression.

**Statistical analysis**

All data are shown as mean±standard error of the mean. Analyses were performed using SPSS version 11.0 (SPSS Inc., Chicago, IL, USA) for Windows. Comparison of the percentage of intimal occlusion, cardiac vessel density and CTGF expression between the three groups was performed using ANOVA. Differences in myocardial fibrosis were determined using the Kruskal–Wallis test. Tests of association between levels of CTGF expression and myocardial fibrosis or high-grade CAV (>50% occlusion) were performed using Spearman correlation tests. A value of p<0.05 was considered statistically significant.

**Results**

**Histology examination**

None of the biopsies in the control group demonstrated evidence of inflammatory or abnormal
fibrosis, with uniform staining of cardiac muscle, no granulocyte infiltration, and thin vessel walls (Figures 1A, D and G). However, with the characteristic presence of concentric intimal proliferation and inflammatory infiltration of the arterial intima, CAV was seen in the 2-week group (Figures 1B, E and H), and became even more severe in the 8-week group, with the vessel in a “triangular shape” (Figures 1C, F and I). Interstitial fibrosis was more obvious in the 8-week group; the collagen deposition was mainly in the interstitium, around the blood vessels and cardiac myocyte with fiber derangement. However, there were less inflammatory infiltration and lower coronary arterial density compared with the 2-week group. Cardiomyocyte apoptosis was seen in the 8-week group; with the reliquus cardiac myocyte, islands of myocardium are formed.

Comparison of the indexes among different groups

The allograft hearts in the 8-week group showed the lowest coronary arterial density (0.58 ± 0.22) and comparisons between the three groups showed significant differences between the control and 8-week group (p < 0.01) (Table). We identified increased fibrosis formation in the 2-week group (1.15 ± 0.36), but the highest fibrosis formation was in the 8-week group (2.75 ± 0.45). Statistical comparison showed significant differences

Figure 1. Light microscopy of rat heart grafts over time course of transplantation (original magnification, 200×). Sections were stained with hematoxylin and eosin (A–C) or Van Gieson stain (D–I). Panels A, D and G are the control group. Panels B, E and H are the 2-week group. Panels C, F and I are the 8-week group.
between the transplantation groups \((p < 0.05)\) and between the transplantation groups and the control group \((p < 0.05, p < 0.01)\). Similar findings were observed when we compared the percentage of intimal occlusion, with the 8-week group having the greatest degree of intimal occlusion compared with the other two groups \((p < 0.01)\).

**Increased expression of CTGF is associated with allograft fibrosis and CAV formation**

Hearts obtained from the control group showed negligible amounts or an absence of CTGF expression (Figure 2A). In contrast, immunoreactivity for CTGF localized to cardiomyocytes, thickened coronary artery wall and the interstitium was detectable in the 2-week group (Figure 2B), but was less obvious compared with that in the 8-week group (Figure 2C). Also, the mean gray scale value was highest in the control group, which signified a smaller amount of CTGF protein compared with that in the transplantation group \((p < 0.01)\) (Figure 3). There was also a significant difference concerning CTGF expression between the transplantation groups \((p < 0.01)\) (Figure 3). We subsequently evaluated whether myocardial fibrosis and high-grade CAV were associated with the expression of CTGF. To determine a possible association, we grouped all rat hearts, irrespective of study group, and found a significant negative association between myocardial fibrosis and the mean gray scale value \((r = -0.734, p < 0.01)\), which signified a strong positive correlation between CTGF protein expression and myocardial fibrosis. The same positive correlation was found between CTGF protein

### Table.

Comparison of indexes of rat hearts in the different study groups. Analyzed by ANOVA or Kruskall–Wallis test (fibrosis grade)

<table>
<thead>
<tr>
<th></th>
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<th>Coronary vessel density</th>
<th>Fibrosis grade (0–3)</th>
<th>Intimal occlusion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>10</td>
<td>3.57 ± 0.54</td>
<td>0.00 ± 0.01</td>
<td>0.07 ± 0.03</td>
</tr>
<tr>
<td>2-wk group</td>
<td>10</td>
<td>3.02 ± 0.43</td>
<td>1.15 ± 0.36*</td>
<td>27.58 ± 11.14*</td>
</tr>
<tr>
<td>8-wk group</td>
<td>10</td>
<td>0.58 ± 0.22*</td>
<td>2.75 ± 0.45*†</td>
<td>72.29 ± 20.57*†</td>
</tr>
</tbody>
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\*\(p < 0.05\) vs. control group; †\(p < 0.05\) vs. 2-week group.
expression and percentage of luminal occlusion, which was an indicator of CAV formation.

Discussion

Here, we showed a time-dependent increase in CTGF expression in a rat model of heterotopic heart transplantation. Additionally, we demonstrated a strong positive association between CTGF expression and CAV formation. These results indicate that CTGF plays an active role in allograft fibrosis formation and CAV pathology.

CAV may affect not only the coronary arteries, but also coronary veins of explanted human hearts.6 We observed that the coronary vessel density was greatly decreased in the 8-week compared with the 2-week or the control group, and a diffuse concentric intimal thickening, which meant that this model was sufficient for our study. We also found intimal proliferation in the 2-week group, with abundant lymphocyte and macrophage infiltration. This suggested that cardiac artery vasculopathy began to form during the cellular rejection phase. As acute moderate/severe cellular rejection has a cumulative impact on cardiac artery vasculopathy onset,21 we should treat cellular rejection more actively to abate chronic rejection.

We observed overexpression of CTGF in heart allografts in a time-dependent manner and a positive correlation between CTGF expression and fibrosis. CTGF was predominantly expressed in the cardiomyocytes, incrassated coronary vessel wall and myocardial interstitium in heart allografts. Koitabashi et al found that cardiomyocytes can secrete CTGF and thus promote cardiac fibroblast proliferation, and that this process regulates production of collagen in hearts with diastolic failure.22 In heart allografts, we found that collagen was deposited mainly in the interstitium, around the blood vessels and cardiac myocyte with fiber derangement. The results showed that the expression level of CTGF was elevated in heart grafts with more severe fibrotic lesions and chronic rejection. CTGF-mediated fibrosis does not appear to be heart allograft specific, as in kidney allografts that overexpress CTGF, which can induce epithelial–mesenchymal transformation to myofibroblasts (α-smooth muscle actin positive) and thus lead to allograft fibrogenesis.17 In our 2-week allografts, there was increased CTGF and fibrosis formation, and because myocardial fibrosis is considered as a marker for non-immune-mediated graft injury independently associated with an increased incidence of CAV in acute rejection,23 this suggests that we should treat cellular rejection more actively.

However, there was no detectable expression of CTGF in normal control rat hearts, which contrasts with the observations of Chen et al, who reported CTGF expression in cultured cardiomyocytes and fibroblasts.14 This outcome might be explained by the difference in the specimens: we used fresh rat ventricular walls, while Chen et al used cultured ventricular cells and fibroblasts.

There are several limitations to the present study. As a result of the non-availability of commercial reagents for ELISA detection of CTGF, we did not detect CTGF expression in the blood, which hindered the consideration of CTGF as a biomarker of chronic rejection. The limited number of rats used and the lack of a study on patients limit the power of the study. We also did not test the role of CTGF on myocardial vessel SMCs in vitro, thus we did not demonstrate a functional role of CTGF in CAV formation in this model. However, the functional role of CTGF has been made clear by neutralization or downmodulation of its activity in vitro using siRNA24 or by antisense oligonucleotides in an ex vivo setting.25 Further study should concentrate on this to make it clinically practical.

In summary, we demonstrated that CTGF was expressed in cardiomyocytes during CAV and overexpression of CTGF correlated with allograft fibrosis and CAV in a rat model of cardiac allograft chronic rejection. Further study into the functional role of CTGF in the development of CAV, and its potential as a surrogate marker of graft rejection status and outcome is required.
References


