Cordyceps sinensis Extracts Attenuate Aortic Transplant Arteriosclerosis in Rats

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Background. Transplant arteriosclerosis is a hallmark of chronic rejection and is still the major limiting factor affecting the success of long-term organ transplants. Development of transplant arteriosclerosis is refractory to conventional immunosuppressive drugs, and adequate therapy is not yet available. The aim of this study was to determine the role of Cordyceps sinensis extracts in reducing the formation of transplant arteriosclerosis in a rat aortic transplant model.

Methods. Lewis rat aortic allografts were transplanted into Brown-Norway recipient rats. Recipients received 0.5, 1, 2, and 5 mg/kg of Cordyceps sinensis extracts (or control saline) daily via intragastric injection for 60 d. Grafts were harvested 60 d post-transplantation and intimal thickness determined microscopically following hematoxylin and eosin (H and E) staining and abdominal aorta protein profiles determined by Western blot analysis. Cellular localization was assessed by histology and immunohistochemistry and the serum analyzed for tumor necrosis factor alpha (TNF-α) and intercellular adhesion molecule-1 (ICAM-1) by enzyme-linked immunosorbent assay (ELISA).

Results. C. sinensis administration resulted in a significant reduction in neointimal formation (neointimal thickness 8.27 ± 1.95 μm [0.5 mg/kg], 3.69 ± 1.43 μm [1 mg/kg], 3.69 ± 1.43 μm [1 mg/kg], 3.69 ± 1.43 μm [1 mg/kg] versus 11.42 ± 2.67 μm [control]) and in the proliferative activity of vascular smooth muscle cells. In addition, localized expression of TNF-α and ICAM-1 in transplant aortas was characterized by immuno-

INTRODUCTION

Although advances in immunosuppressive therapies have dramatically increased clinical organ transplantation success rates, transplant arteriosclerosis presentation has become a major obstacle in long-term allograft survival, a major cause of late allograft dysfunction and late death in some patients [1]. Therefore, developing new therapies aimed at attenuating transplant arteriosclerosis is critical. Cordyceps sinensis (the Chinese caterpillar fungus) is a highly valued medicinal fungus used in Chinese herbal medicine that has been used in tonics to increase longevity, endurance, and vitality, and its medicinal value has gained worldwide attention in recent years [2, 3]. C. sinensis has been shown to have multiple pharmacologic activities, including antiproliferative activities on a variety of tumor cells [4]. Recently, it has also been shown that C. sinensis inhibited the migration and proliferation of VSMC (vascular smooth muscle) and neointima formation [5]. Although these data suggest that C. sinensis possess

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properties that may affect vascular diseases [5, 6], a defined mechanism for C. sinensis in mediating transplant arteriosclerosis success has not been established. The present study was designed to determine the effects of C. sinensis on the success of transplant arteriosclerosis in a rat model, as well to define potential molecular mechanisms.

MATERIALS AND METHODS

Animals

Inbred male BN (Brown-Norway) and Lewis rats weighing between 250 and 300 g were purchased from Beijing Vital River Laboratory. Rats had access to standard rat chow and water ad libitum and were maintained following conditions established by the Guide for the Care and Use of Laboratory Animals.

Cordyceps sinensis Extracts Preparation

C. sinensis was obtained from the Zhongmei Huadong Company (Hangzhou, China). C. sinensis and asexual phase Histutella sinensis isolates were cultured in hydrolysable nutrient media at 15°C for 45 d to generate sufficient mycelium for subsequent experiments. As mycelia are not water-soluble, they were filtered to obtain a pure mycelium stock that was dried and then crushed to generate mycelium micropowder; 150 g micropowder was added to 100 mL of 80% medicinal alcohol, soaked for 2 d, back flow collected twice, and then filtered to obtain the alcohol extract, which was then vacuum-concentrated (completed the ethanol extraction step). Remaining dregs were mixed in 100 mL water and boiled for 2 h. After the samples cooled, they were again boiled for 2 h to obtain the aqueous extract that was then vacuum-concentrated and then heated (completed the aqueous extraction process). Remaining dregs were treated with trypsin and then microwave-treated to inactivate the trypsin. The trypsin mixture was then centrifuged at 300 × g to remove particulate matter and obtain the zymohydrolysis extract. Finally, the three extracts were combined, homogenized, water bath-sterilized at 60°C and reconstituted at 5 g/mL in supplemented 0.75% Tween and stored at −80°C until use.

Experimental Design

Aortic transplants were performed in rats using Lewis donors and BN recipients. Transplant procedures were performed using a modified technique described by Hillebrands et al. [7]. Briefly, a segment of abdominal aorta located between the left renal artery and the bifurcation (approximately 15 mm long) was harvested and transferred to recipient animals. Recipient aortas were clamped and then transected with sharp microvascular scissors. A proximal end-to-end anastomosis was performed using 6-0 nylon sutures. Aortic grafts were subsequently repositioned and the anastomosis procedure completed using single interrupted sutures. After the transplantation procedure, animals were randomly divided into five groups: group 1, untreated controls (n = 10); group 2, C. sinensis treated (0.5 mg/kg/d, equivalent of a human daily half-dose, n = 10); group 3, C. sinensis treated (1 mg/kg/d, equivalent of a human daily dose, n = 10); group 4, C. sinensis treated (2 mg/kg/d, equivalent of a human daily dose, n = 10); group 5, C. sinensis treated (5 mg/kg/d, equivalent of a human daily dose, n = 10). C. sinensis was administered intragastrically using a 16 G stainless steel gavage needle.

Arterial Harvest and Morphometric Analyses

Day 60 post-transplantation, aortic grafts and serum samples were harvested. Aortas were formaldehyde fixed, aortic segments embedded in paraffin, and 5 μm sections stained with hematoxylin and eosin (H and E) as a means of assessing morphology, cytolgy, and neointimal thickness in transplanted aortas. Quantitative morphometric analyses of aortic grafts were carried out using a bio-image analysis system (Bio-Profile JangSu JEDA Science-Technology, Nangjing, China). Five non-overlapping fields per section were examined using light microscopy and the thickness between endothelial and smooth muscle cells measured, and the results averaged.

Measurements of Serum TNF-α and ICAM-1 Levels by ELISA

Serum TNF-α and ICAM-1 levels were measured after transplantation as measures of systemic inflammation following treatment with or without C. sinensis. Arterial blood was collected into calcium-containing tubes from the abdominal aorta using an 18-gauge intravenous cannula at the time aortas were harvested. TNF-α and ICAM-1 levels were then measured in duplicate using a commercial ELISA kit (Raybiotech, Inc., Norcross, GA, USA).

Characterization of Local Tissue Inflammation by Western Blot Analysis

To determine whether C. sinensis treatment suppressed both systemic inflammation and local tissue inflammation, Western blots were used to determine tissue expression levels of TNF-α and ICAM-1 in the harvested transplanted artery. Vessel tissues were homogenized in lysis buffer and protein concentrations determined using a Micro BCA Protein Assay kit (Pierce, Rockford, IL, USA). Twenty micrograms of protein per specimen were separated on a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), blotted onto nitrocellulose membranes and probed with antibodies specific for TNF-α (Santa Cruz Biotechnology, Santa Cruz, CA) or ICAM-1 (Santa Cruz Biotechnology). Anti-β-actin antibodies (Santa Cruz Biotechnology) were used to detect actin as a loading control.

Assessment of Local Tissue Inflammation by Immunohistochemistry

Serial sections (5 μm thick) were obtained from formalin-fixed, paraffin-embedded rat abdominal aortic graft specimens from each recipient. To enhance immuno-staining signals, antigens were pre-retrieved in citrate buffer by heating in a microwave twice for 5 min. Sections were then treated with 3% hydrogen peroxide to block endogenous peroxidase activity. Immunohistochemical staining was then performed using the avidin-biotin peroxidase complex method and the Liquid DAB Substrate Kit (Zymed Laboratories Inc., South San Francisco, CA, USA) that resulted in a brown staining representing the presence of TNF-α or ICAM-1 expression, respectively. Sections were stained for TNF-α and ICAM-1 using a primary monoclonal anti-mouse TNF-α antibody (Santa Cruz Biotechnology) at 8 μg/mL or an anti-ICAM-1 antibody (Santa Cruz Biotechnology) at 10 μg/mL. Finally, sections were counterstained with hematoxylin. Proteins were then quantified by scanning densitometry using a bio-image analysis system (Bio-Profile).

Proliferative Activity

To determine whether suppressed inflammation was accompanied by reduced VSMC proliferation, we performed immunohistochemical staining designed to detect the presence of proliferating cell nuclear antigen (PCNA) (Bioworld Technology, Louis Park, MN, USA). VSMC proliferation after transplant was quantified by counting the
percentage of PCNA-positive cells/total nucleated cells in five different fields per vessel section (n = 10 per group).

Statistical Analysis

Results were assessed using a one-way ANOVA for comparisons between groups. Differences were assessed using the Bonferroni post-test, with P < 0.05 considered indicative of significant differences. Data are expressed as the mean ± standard error of the mean (SEM).

RESULTS

Effect of C. sinensis on Neointimal Thickness

When recipient rats were treated daily with 0.5 mg/kg C. sinensis, neointimal thickness was significantly decreased compared to untreated controls (8.27 ± 1.95 μm versus 11.42 ± 2.67 μm, P < 0.05; Fig. 1). Daily treatment with 1 mg/kg, 2 mg/kg or 5 mg/kg C. sinensis also significantly reduced neointimal thickness compared to thickness observed in untreated controls (neointimal thickness of 3.69 ± 1.43 μm [1 mg/kg], 3.78 ± 1.65 μm [2 mg/kg], 3.66 ± 1.58 μm [5 mg/kg] versus 11.42 ± 2.67 μm [control]; Fig. 1). Neointimal thickness in groups that received daily treatments of 1 mg/kg C. sinensis were significantly decreased compared to animals treated with 0.5 mg/kg C. sinensis (P < 0.05). However, there were no additional beneficial effects observed in animals treated with 2 mg/kg or 5 mg/kg C. sinensis compared with rats treated with 1 mg/kg C. sinensis (P > 0.05).

Effects of C. sinensis on Systemic Inflammation

The levels of serum ICAM-1 and TNF-α in serum were significantly decreased in the 0.5 mg/kg C. sinensis treatment group compared with untreated controls (P < 0.05). Moreover, rats treated with 1, 2, or 5 mg/kg C. sinensis showed significantly decreased ICAM-1 and TNF-α serum levels compared with controls or animals treated with 0.5 mg/kg C. sinensis. However, there were no significant differences between the 1, 2, and 5 mg/kg C. sinensis treatment groups (P > 0.05, Table 1).

Effect of C. sinensis Treatment on Local Inflammation

We next examined the effect of C. sinensis on local inflammation defined by the expression of ICAM-1 and TNF-α. ICAM-1 staining was observed on endothelial cell surfaces and TNF-α staining was observed in the cytoplasm of endothelial cells as well as on neointima tissues. Immunohistochemical staining demonstrated that animals treated with 0.5 mg/kg/d C. sinensis had significantly reduced ICAM-1 (Fig. 2) and TNF-α (Fig. 3) expression levels in transplanted aortas compared with untreated controls (P < 0.05). Rats treated with 1, 2, and 5 mg/kg C. sinensis presented with

FIG. 1. Effect of C. sinensis on neointimal thickness. Aortas were harvested from recipient rats that were either (A) untreated, magnification 100×, (B) treated with 0.5 mg/kg C. sinensis, magnification 100×, (C) treated with 1 mg/kg C. sinensis, magnification 100×, (D) transplanted aorta from untreated rats, magnification 40×, (E) treated with 0.5 mg/kg C. sinensis, magnification 40×, (F) treated with 1 mg/kg C. sinensis, magnification 40×. Blue segments indicate the thickness measurements between the endothelium and smooth muscle. Aortas were harvested 60 d post-transplant and stained with H and E. (Color version of figure is available online.)
a significantly decreased ICAM-1 (Fig. 2) and TNF-α (Fig. 3) expression scores in transplanted aortas compared with controls or 0.5 mg/kg C. sinensis-treated animals. However, there were no significant differences between the 1, 2, and 5 mg/kg C. sinensis treatment groups (P > 0.05) (Table 2).

Western blot analyses of transplanted aorta extracts also revealed that the expression of ICAM-1 and TNF-α in the untreated controls was significantly increased (Fig. 4). The administration of C. sinensis at various concentrations significantly attenuated both ICAM-1 and TNF-α expression following C. sinensis treatment (Fig. 5).

Effect of C. sinensis on the Proliferative Activity of VSMCs

A significant decrease in the number of PCNA-positive VSMCs was demonstrated in the medial layers of arteries harvested from C. sinensis-treated rats versus controls (Fig. 4). The percentage of PCNA-positive cells in vessel walls was 32.7% ± 6.1%, 10.6% ± 1.3%, 11.2% ± 1.6%, 10.8% ± 1.4% in the 0.5, 1, 2, and 5 mg/kg/d C. sinensis-treatment groups, respectively, and 46.8% ± 7.9% in the untreated group (P < 0.05).

DISCUSSION

Although currently used immunosuppressive regimens are highly effective in preventing acute rejection, there are no adequate therapies available to prevent or treat chronic transplant disease. The most common histopathologic hallmark of chronic transplant disease is transplant arteriosclerosis [8, 9]. Immunosuppressive treatments based on calcineurin inhibition (cyclosporine, tacrolimus), as well as newer second messenger inhibitors such as the mammalian target of rapamycin

<table>
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<th>Table 1</th>
<th>Serum levels of ICAM-1 and TNF-αa</th>
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<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>51.96 ± 15.53</td>
</tr>
<tr>
<td>TNF-α</td>
<td>119.43 ± 35.36</td>
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*P < 0.05 compared with the control group.
**P < 0.05 compared with the 0.5 mg/kg C. sinensis group.
Data are expressed as the mean ± SE of 10 animals per group (pg/mL).

![FIG. 2. Immunohistochemistry analysis for ICAM-1 expression. Aortas were harvested from recipient rats and stained for the presence of ICAM-1-positive cells 60 d post-transplantation. (A) Transplanted aortas form the control group, magnification 100×, (B) transplanted aorta from rats treated with 0.5 mg/kg C. sinensis, magnification 100×, (C) transplanted aorta from rats treated with 1 mg/kg C. sinensis, magnification 100×, (D) transplanted aortas from untreated rats, magnification 200×, (E) transplanted aortas from rats treated with 0.5 mg/kg C. sinensis, magnification 200×, (F) transplanted aortas from rats treated with 1 mg/kg C. sinensis group, magnification 200×. The arrows indicate ICAM-1-positive cells. Boxes represent tissue samples shown in panels (D–F). (Color version of figure is available online.)](image-url)
(mTOR) inhibitors (sirolimus, everolimus) have not been successful at preventing transplant arteriosclerosis [10]. More recent adjuvant immunosuppressive therapies, such as mycophenolate mofetil (MMF) may provide some limited benefits, but the clinical data to date are inconclusive, generally demonstrating a limited impact over 5 years [11–13]. The development of transplant arteriosclerosis in allografts is a multifactorial process, with macrophages, T-cells, proinflammatory cytokines, adhesion molecules, growth factors, and alloantibodies associated with both the initiation and progression of this chronic inflammatory process [14, 15].

TNF-α is a pro-inflammatory cytokine, produced mainly by macrophages. TNF-α is released at the site of inflammation and up-regulates the expression of both adhesion molecules and major histocompatibility complex (MHC) molecules, activates endothelial cells, induces vasodilation and increases vascular permeability [16]. TNF-α is expressed during neointimal formation and coronary artery plaques [17, 18] but not in normal vessels, and circulating levels of TNF-α were shown to be elevated in patients with unstable angina and myocardial infarction [19]. TNF-α also has been shown to have direct negative effects on the myocardium [16, 20]. Furthermore, up-regulation of TNF-α was observed in chronic allograft nephropathy [21] and may have promoted the development of transplant vasculopathy [7, 22]. Persistent intragraft expression of TNF-α has also been suggested as a mediator of the cardiac allograft hypertrophy and fibrosis development in clinical transplantation [23].

**TABLE 2**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>0.5 mg/kg</th>
<th>1 mg/kg</th>
<th>2 mg/kg</th>
<th>5 mg/kg</th>
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<tr>
<td>ICAM-1</td>
<td>44.60 ± 8.23</td>
<td>36.45 ± 6.37</td>
<td>16.28 ± 3.39***</td>
<td>17.44 ± 3.61***</td>
<td>17.58 ± 3.25***</td>
</tr>
<tr>
<td>TNF-α</td>
<td>93.72 ± 13.60</td>
<td>79.22 ± 10.08</td>
<td>46.77 ± 6.65***</td>
<td>45.43 ± 5.98***</td>
<td>46.32 ± 6.03***</td>
</tr>
<tr>
<td>PCNA†</td>
<td>46.8 ± 7.9</td>
<td>32.18 ± 4.39</td>
<td>10.6 ± 1.3***</td>
<td>11.2 ± 1.6***</td>
<td>10.8 ± 1.4***</td>
</tr>
</tbody>
</table>

*P < 0.05 compared with control group.
**P < 0.05 compared with the 0.5 mg/kg C. sinensis group.
†Data are expressed as the mean ± SE of 10 animals per group.
‡Data expressed as % PCNA positive cells.
Intercellular adhesion molecules are also important in transplant arteriosclerosis because they mediate recipient inflammatory cell attachment to and migration into donor vessel walls [24]. In addition to their roles in leukocyte trafficking, adhesion molecules may also regulate the migration of smooth muscle cells within the vessel wall [25]. It has been shown that TNF-α mediated the activation of endothelial cells and induced up-regulation of adhesion molecules (including ICAM-1) that played critical roles in the development of transplant arteriosclerosis [22]. Deletion of the ICAM-1 gene in aortic graft recipients or blocking ICAM-1

![Image of immunohistochemistry analysis for PCNA](image)

**FIG. 4.** Immunohistochemistry analysis for PCNA. Aortas were harvested from recipient rats and stained for the presence of PCNA-positive cells 60 d post-transplantation. (A) Transplanted aortas from the control group, magnification 200×, (B) transplanted aortas rats treated with 0.5 mg/kg C. sinensis, magnification 200×, (C) transplanted aortas from rats treated with 1 mg/kg C. sinensis, magnification 200×, (D) transplanted aortas from untreated rats, magnification 400×, (E) transplanted aortas from rats treated with 0.5 mg/kg C. sinensis, magnification 400×, (F) transplanted aortas from rats treated with 1 mg/kg C. sinensis, magnification 400×. The arrows indicate PCNA-positive cells. Boxes represent tissue samples shown in panels (D–F). (Color version of figure is available online.)

![Image of Western blot analysis of ICAM-1 and TNF-α expression](image)

**FIG. 5.** Western blot analysis of ICAM-1 and TNF-α expression. Aortas from the respective treatment groups were subjected to Western blot analysis to assess the expression levels of ICAM-1 and TNF-α. The same blots was stripped and reprobed with anti-actin antibodies to confirm equal protein loading.
in conjunction with lymphocyte function-associated antigen-1 strongly reduced neointimal proliferation [26, 27].

*C. sinensis* is an insect-borne fungus in the genus *Cordyceps* that has been used as part of herbal remedies for the prevention of various diseases [1, 2]. *C. sinensis* has been shown to have multiple pharmacologic activities; the most notable is its antiproliferative activity on a variety of tumor cells. It has been used as an antitumor herb in Chinese medicine and as an adjuvant of chemotherapies and radiotherapies used in the treatment of various cancers. While immunomodulation has been generally considered as the most possible mechanism for the beneficial effects of *C. sinensis* in the context of cancer therapy, some recent studies have shown that *C. sinensis* components mediated anti-inflammatory processes characterized by decreased TNF-α levels [28]. Previous studies have demonstrated that *C. sinensis* could prolong the survival of allogeneic heart grafts [29] and, in combination with CsA, had synergistic effects in blocking allogeneic graft rejection. In addition, *C. sinensis* in combination with reduced CsA doses decreased proteinuria and retarded chronic allograft nephropathy (CAN) progression [30, 31]. Xu et al. demonstrated that *C. sinensis* possessed certain therapeutic effects on CAN, i.e., it diminished injury to the glomerulus and tubular interstitium [32]. Moreover, *C. sinensis* significantly reduced CD8+ T-cell activity, suppressed acute rejection, and ablated allograft vasculopathy [33].

This study demonstrated that *C. sinensis* extracts attenuated ICAM-1 and TNF-α expression levels in transplanted aortas in addition to reducing serum levels of both ICAM-1 and TNF-α. This *C. sinensis*-mediated reduction in ICAM-1 and TNF-α expression in both transplant tissues and serum contributed to the attenuation of neointimal formation. Moreover, it has recently been demonstrated that cordycepin inhibited the migration and proliferation of VSMC and vascular neointima formation [31]. In this study, *C. sinensis* extracts inhibited the proliferative activity of vascular smooth muscle cells by decreasing PCNA expression, and effectively reduced the development of transplant arteriosclerosis, in addition to conferring protective effects on allograft vasculopathy. This model may explain why reductions in ICAM-1 and TNF-α and inhibition of PCNA expression during transplant arteriosclerosis in *C. sinensis*-treated rats reduced inflammation and proliferative activity leading to reduced neointima formation.

In summary, this study demonstrated that *C. sinensis* treatment attenuated neointima formation by inhibiting local and systemic inflammation as a result of interactions that affected various targets known to be involved in the pathophysiology of transplant arteriosclerosis and prevented neointimal VSMC proliferation.

Data presented in this study supported other work that demonstrated a role for *C. sinensis* in preventing renal transplant rejection and retarding CAN progression [30, 31]. These data suggest that *C. sinensis*-derived formulations may provide novel treatment options for preventing graft rejection.

**ACKNOWLEDGMENTS**

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**REFERENCES**


