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Original Article

Simvastatin combined with aspirin increases the survival time of heart allograft by activating $CD4^+CD25^+$ Treg cells and enhancing vascular endothelial cell protection

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ABSTRACT

Objective: The objective was to investigate whether the combination of simvastatin and aspirin treatment prolongs the survival time of the heart allograft in rat and its underlying mechanism.
Methods: Heterotopic heart transplantation was performed using Wistar rats as donors and Sprague–Dawley (SD) rats as recipients. The SD rats were randomly divided into five groups (n=20/group): sham, HT (heart transplantation), HT+simvastatin (HT+S), HT+aspirin (HT+A), and HT+aspirin+simvastatin (HT+A+S). After transplantation, at 3, 7, 10, 15, 20, 30, and 40 days, the endothelial nitric oxide synthase (eNOS) expression was assessed by immunohistological staining; nitric oxide (NO) levels were analyzed by Griess assay; the activation of $CD4^+CD25^+$ T regulatory lymphocytes (Tregs) was analyzed by flow cytometry; and pathological changes in the graft heart were determined by histology.
Results: Combined treatment of hearts with simvastatin and aspirin significantly prolonged the mean survival time of heart allografts [8±1.2 days (n=18), 20±3.4 days (n=19), 21±2.8 days (n=19), and 39±5.3 days (n=19) for HT, HT+S, HT+A, and HT+A+S group, respectively; HT vs. HT+A+S, P<.001; HT vs. HT+S or HT+A, P<.05]. In addition,

1. Treatment together with simvastatin and aspirin resulted in less pathological changes (inflammatory cell infiltration, myocardial and vascular damage) in graft hearts and reduced vascular damage.
2. The eNOS expression and NO secretion were enhanced by the combined treatment of simvastatin and aspirin.
3. The circulating level of $CD4^+CD25^+$ Tregs in HT+A+S rats was significantly increased [2.2%±0.5%, 2.9%±0.8%, 4.3%±1.0%, 8.3%±1.7%, and 14.3%±3.7% for sham, HT, HT+S, HT+A, and HT+A+S, respectively; HT vs. HT+A (P<.05) or HT+A+S (P<.01)].

Conclusions: Simvastatin given in combination with aspirin delayed the development of pathological changes in the myocardium, reduced vascular damage and prolonged the survival time of cardiac allograft. The underlying mechanism is linked with $CD4^+CD25^+$-Treg-cell-induced immune tolerance and enhanced vascular endothelial cell protection.

1. Introduction

Cardiac transplantation is the preferred surgical therapy for patients with end-stage heart diseases. However, allograft rejection is the major challenge in the cardiac transplantation [1–4]. We found that there was less allograft rejection in transplanted organ if vasculature is intact. It was thought that the vascular damage in the transplanted organ may be the important initiating factor for the allograft rejection. It has been demonstrated that simvastatin can improve vascular endothelial function and that aspirin decreases the risk of cardiovascular diseases. The underlying mechanism remains elusive. In this study, we investigated the effect of combined treatment of simvastatin and aspirin in the survival time of heart allograft in rats and its underlying mechanisms.

2. Methods

2.1. Animals

Adult Wistar and Sprague–Dawley (SD) rats (weight, 200–300 g) were used as donors and recipients, respectively. All rats were purchased from the Medical Experimental Animal Center of Sun Yat-sen University and housed in the animal care facility at Sun Yat-sen University. All rats

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were kept under standard temperature, humidity, and time light conditions and fed standard chow and water ad libitum. All experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Sun Yat-sen University.

2.2. Experimental protocol

The rats were anesthetized by a single intraperitoneal injection of ketamine/xylazine (100:10 μg/kg). A midline incision was made on the abdominal wall. By using standard vascular microsurgical techniques, the recipient's abdominal aorta was anastomosed end-to-side to the donor's ascending aorta and the recipient's inferior vena cava was anastomosed to the donor's pulmonary artery. All rats were randomly divided into five groups ($n=20$/group): sham, HT (heart transplantation), HT+simvastatin (HT+S), HT+aspirin (HT+A), and HT+aspirin+simvastatin (HT+A+S). In the sham group, the SD rats underwent laparotomy only. In the HT group, hearts from Wistar rats (donors) were transplanted into SD rats (recipients) according to the procedure previously reported with slight modifications [5–7]. In the HT+S, HT+A, and HT+A+S groups, hearts from Wistar rats (donors) were transplanted into SD rats (recipients). Simvastatin (5 mg/kg/day), aspirin (9mg/kg/day), or the combination of the two drugs was given to the SD rats by gavage 1 day after operation. Graft survival was monitored by daily monitoring of palpable heartbeat. Graft rejection was defined as the cessation of heartbeat. At different time points (day 3, 7 10, 15 20, 30, and 40) after transplantation, endothelial nitric oxide synthase (eNOS) expression, nitric oxide (NO) levels, $CD4^+CD25^+$ Treg cells, and histopathological change in graft heart were analyzed.

2.3. Mononuclear cells isolation

A volume of 0.3–0.5 ml of peripheral blood was taken into a sterile heparinized syringe from the caudal vein of all rats. The peripheral blood mononuclear cells (PBMCs) were isolated by gradient density centrifugation as we described previously with slight modifications [8]. Briefly, blood was diluted in 2 ml phosphate-buffered saline (PBS) and then gently layered over equal volume of gradient medium (Histopaque-1083; Sigma, Fairfax, VA, USA) for centrifugation at 800*g* for 30 min at 4°C. The PBMCs in the interface layer were transferred to a new tube, washed with PBS, centrifuged at 400*g* for 5 min at 4°C, and used for flow cytometric analysis.

2.4. $CD4^+CD25^+$ Treg cells analysis

The $CD4^+CD25^+$ T regulatory lymphocytes (Tregs) were identified by double positive expression of membrane-specific markers CD4 and CD25. The percentage of $CD4^+CD25^+$ Tregs in the peripheral blood from all experiment groups was analyzed at different time points (day 3, 7 10, 15 20, 30, and 40). The PBMCs were collected as described above and resuspended with PBS and incubated with fluorescein-isothiocyanate-conjugated anti-rat CD4 and PE-conjugated anti-rat CD25 antibodies (Caltag, USA) for 30 min at 4°C in the dark. Isotype matched [immunoglobulin G (IgG)] nonspecific antibodies served as negative controls. The concentrations of antibodies were applied according to manufacture instructions. A total of at least 10,000 events were collected and analyzed by using Accuri C6 flow cytometer and CFlow Plus Analysis software (Ann Arbor, MI, USA). A live lymphocyte gate was created on dot plots using forward scatter and side scatter plots. The percentage of Tregs was determined within the settled gate by double positive staining of CD4 and CD25.

2.5. Immunohistochemical detection of eNOS

Cross or longitudinal sections of vessels were examined. Anti-eNOS antibody was purchased from Abcam (USA). The experiment was performed according to the instructions. Briefly, after dewaxing process, the slides were heated in a microwave oven for 15 min in 10-mm sodium citrate buffer (pH 6.0) and then incubated in the buffer at room temperature for 20 min to retrieve antigens. After washing, the slides were incubated in 0.3% H_2O_2 in 100% methanol for 30 min to inactivate endogenous biotin. After washing with PBS and blocking in 1.5% normal goat serum, the slides were then incubated with anti-eNOS monoclonal antibody (1:100) overnight at 4°C. The second day, the slides were sequentially incubated with biotinylated goat anti-mouse IgG antibody, horseradish-peroxidase-conjugated streptavidin, and development reagent. The standard of eNOS expression in vascular endothelial cells is as follows: brown staining in nucleus or nucleus and cytoplasm of the cell was regarded as positive; no nuclear staining in the cell or brown staining in the cytoplasm alone was regarded as negative. Ten 400× visual fields were randomly selected for the positive cells counting in each group at the corresponding time points.

2.6. Measurement of NO levels

NO secreted from vascular endothelial cells is rapidly oxidized to nitrite in serum; therefore, determination of nitrite concentrations was used as a measurement of NO production. A colorimetric Griess assay (Juli Biological Research Institute, Nanjing, China) was used. Briefly, 0.5–1 ml of peripheral blood was taken into a sterile heparinized syringe from the caudal vein of the SD rats. Two hundred microliter of serum and 90 mmol/ml nitrate reductase 100 μl, 0.28 mmol/LNADPH 100 μl, 35 mmol/L FAD 100μl, 0.1 mol/L phosphate buffer pH 7.5, and 200 μl at 25°C were incubated for 1 h and boiled for 3 min. An equal volume of Griess reagent [N-2% naphthylethylamine:0.2% sulfonamide (1:1) mixture] was incubated at 60°C for 10 min and analyzed by Bio-Rad 450.

2.7. Histological examination of heart and vasculature

The heart and vascular samples were collected from all experimental groups. The heart and vascular samples harvested from donor (sham) rats served as control. All tissue samples were fixed in 10% (pH: 7.1-7.3) buffered formalin solution overnight, embedded in paraffin, sectioned (5–6-μm thickness) under a microtome, and stained with hematoxylin and eosin (H&E) using the standard method. Hearts were evaluated for general histology and rejection according to the International Society for Heart and Lung Transplantation [9]. Briefly, heart and vascular sections were evaluated by a trained pathologist and assessed for changes during rejection defined as follows: 1R=mild, focal perivascular and/or interstitial infiltrate without myocyte damage; 2R=medium, multifocal infiltrate with myocyte damage; 3R=severe, diffuse polymorphous infiltrate with extensive myocyte damage and/or edema and/or hemorrhage and/or vasculitis. Vascular damage was determined by Image-Pro Plus 6.0 software (USA). All samples were analyzed under light microscopy in a blinded fashion.

2.8. Statistical analysis

Data were expressed as mean±S.E.M. Means for two groups were compared using Student's *t* test. Multiple comparisons were performed by one-way analysis of variance. Graft survival was plotted using the Kaplan–Meier method, and allograft survival rates were analyzed by using the log-rank test. *P* values <.05 were considered statistically significant.

3. Results

3.1. Treatment with combination of simvastatin and aspirin prolongs the survival time of heart allografts

The survival time of transplanted hearts in HT, HT+S, HT+A, and HT+A+S groups was 8±1.2 days ($n=18$), 20±3.4 days ($n=19$), 21±2.8 days ($n=19$), and 39±5.3 days ($n=19$), respectively. The data