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Combined use of rapamycin and leflunomide in prevention of acute cardiac allografts rejection in rats

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ABSTRACT

This study aimed to evaluate the role of combined use of rapamycin and leflunomide(Lef) on the prevention of acute allograft rejection in rats. After cardiac transplantations, rats were randomly divided into untreated group, rapamycin group, Lef group and rapamycin + Lef group. The drugs were given by gavage from day 0 to day 9 after transplantations. Graft survival time was observed. Some grafts were harvested for histopathological investigation on day 10 after transplantations. The levels of CD_4^+ and CD_8^+ T lymphocytes and the concentrations of interleukin 2(IL-2) and interferon (IFN)γ in peripheral blood were examined on day 10 after transplantations. At the same time, the body weight, the hepatic function, renal function and the haemoglobin of the recipients were also examined. The graft survival time of untreated group was 7.14 ± 1.07 days. Rapamycin group was 11.14 ± 1.35 days. Lef group was 11.29 ± 1.80 days. While in rapamycin + Lef group, the graft survival time was prolonged to 13.86 ± 1.57 days($P<0.05$). Histological changes of the allografts in rapamycin + Lef group were much milder than either of the two single drug groups. The absolute number and the percentage of CD_4^+ T lymphocytes in peripheral blood in rapamycin + Lef group were lower than those of rapamycin or Lef group on day 10 after transplantations($P<0.05$), while the percentage of CD_8^+ T lymphocytes in rapamycin + Lef group was higher than that of rapamycin or Lef group($P<0.05$). The absolute number of CD_8^+ T lymphocytes was not significantly different among rapamycin group, Lef group and rapamycin + Lef group. The levels of IL-2 and IFN-γ in rapamycin + Lef group were significantly lower than that of rapamycin group or Lef group($P<0.05$). The body weight, the hepatic function, renal function and the haemoglobin were not significantly different among rapamycin group, Lef group and rapamycin + Lef group ($P>0.05$). Combined use of rapamycin and Lef had better effect on the prevention of acute cardiac allografts rejection in rats than monotherapy.

1. Introduction

Although a variety of new immunosuppressants has become available or are being investigated for clinical use, none of them is perfect because of their side effects. To achieve maximum therapeutic effect with minimum toxicity, two or more immunosuppressants are most often used in combination. So there is growing interest in developing new strategies to use various combinations of immunosuppressive drugs with differing mechanisms of action to prevent allograft rejection in organ transplant recipients. In addition, tumor onset is a known risk factor after organ transplantation. Strategies to minimize the risk of developing malignancy in the transplant population are needed. Since studies had shown that calcineurin inhibitors had been linked with post-transplant malignancies, while mammalian target of rapamycin (mTOR) inhibitors had shown antineoplastic activities [1] and clinical studies had demonstrated a lower incidence of new malignancies after renal transplantation in recipients receiving immunosuppression with mTOR inhibitors compared with calcineurin inhibitors [2], therapeutic protocols involving mTOR inhibitors may protect an allograft from immunological rejection, while at the same time solving the problem of cancer in this high-risk population. Rapamycin (sirolimus), a mTOR inhibitor, as an immunosuppressive and antitumor agent had been demonstrated in both animal experiments and clinical use. Although rapamycin has these advantages, it has dose-dependent adverse effects, including hyperlipemia [3], myelosuppression [4] and overimmunosuppression, which may reduce their overall benefits for long-term survival. Moreover, rapamycin alone sometimes could not achieve the ideal immunosuppressive effect [5]. To minimize rapamycin-induced toxicity or get the better effects, it may be necessary to use reduced doses rapamycin in combination with other immunosuppressive regimens. Leflunomide (Lef) as an isoxazole derivative is an immunosuppressant widely used in autoimmune disorders. Previous study had shown that Lef was synergistic in its activity with cyclosporine

Abbreviations: Lef, leflunomide; mTOR, mammalian target of rapamycin; CsA, cyclosporine; AST, aspartate aminotransferase; ALT, alanine aminotransferase; Crea, creatinine.

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(CsA) without increasing the side effects [6], or Lef and calcineurin-inhibitor could be used together [7]. Compared with CsA, mTOR inhibitors have advantages due to their antitumor activities, slight effects on kidney function [8–10] and their benefits among HIV positive patients [11,12], so combined use of rapamycin and Lef may have better prospects. In addition, because rapamycin is associated with significant financial costs while Lef is inexpensive, rapamycin-sparing regimens may substantially save patients' costs. Although rapamycin and Lef are potent immunosuppressive agents in their own right, it is not clear the immunosuppressive role of rapamycin and leflunomide is additive or antagonistic. In order to evaluate the immunosuppressive effect of combined use of rapamycin and Lef, we used the two drugs in combination after cardiac transplantations in rats.

2. Objective

This study was conducted to evaluate the immunosuppressive effect of combined use of rapamycin and Lef on prevention of acute cardiac allografts rejection in rats.

3. Materials and methods

3.1. Materials

Male adult Lewis rats weighing 200–250 g were used as recipients and male DA rats weighing 150–200 g were used as donors. The animals were purchased from animal central of the second affiliated hospital of Harbin Medical University. The animals adapted themselves to circumstance for at least one week prior to transplantations. They were treated as recommended in the Guide for the Care and Use of Laboratory Animals issued by the China Association of Laboratory Animal Care. Flowcytometry (FACSort) purchased from BD Company (USA). The anti-CD_3^+, CD_4^+ and CD_8^+ monoclonal antibodies were purchased from Serotec Ltd.(UK). Rapamycin was purchased from Wyeth Pharmaceuticals Company (USA), and Lef was from Changzheng-Cinkate (China) Pharmaceuticals Co. Ltd.

3.2. Surgical procedure

The non-suture cuff technique of heterotopic cervical cardiac transplantation model was used [13]. Briefly, the donor heart was flushed with cold perfusion, Perfadax® (Pharmacia & Upjohn, Uppsala, Sweden) and for cervical cardiac transplantation, the aortic root of donor being anastomosed with the common carotid artery of recipient and the pulmonary artery of donor with the jugular vein of recipient. All veins of the graft were ligated. The graft was placed under the neck skin. The total ischemia time was no more than 15 minutes. Graft survival time was evaluated by palpation through the neck skin once daily. Graft rejection was defined as cessation of palpable cardiac graft beats.

3.3. Immunosuppressive regimens

After transplantations, the recipients were divided into four groups randomly and treated with following regimens. In untreated group, physiological saline was given ($n=14$). In rapamycin group, rapamycin at dose of 0.5 mg/kg/day was given ($n=14$). In Lef group, Lef at dose of 0.6 mg/kg/day was given ($n=14$) and in rapamycin + Lef group, rapamycin at dose of 0.5 mg/kg/day and Lef at 0.6 mg/kg/day were combined ($n=14$). Drugs were administered by gastric gavage from day 0 to day 9.

3.4. Histology

Seven grafts per group were harvested on day 10 after transplantations. Graft sections were stained with hematoxylin and eosin. Two independent, blinded pathologists reviewed the slides to rate the extent of acute rejection. The grading criterion were based on the standardization of nomenclature in the diagnosis of heart and lung rejection formulated by the Heart Rejection Study Group of the International Society for Heart Transplantation in 1990 [14] and the revision of the 1990 working formulation for the standardization of nomenclature in the diagnosis of heart rejection by the International Society for Heart and Lung Transplantation in December 2004 [15].

3.5. Flow cytometry analysis

The proportion of CD_4^+ and CD_8^+ T cells of total lymphocytes and absolute number of CD_4^+ and CD_8^+ T cells in peripheral blood were examined by flow cytometry on day 10 after transplantations. Peripheral blood was collected from the tail vein. 50 μl blood was incubated with the optimal concentration of fluorochrome-labeled monoclonal antibody for 30 min at 4 °C in the dark. Cells were washed 2 times and resuspended by Flow Cytometer buffer. All cells were analyzed on a Flow Cytometer using Cell Quest software.

3.6. Quantification of interleukin 2 and interferon (IFN) γ

On day 10 after transplantations, peripheral blood was collected from the tail vein. Concentrations of interleukin 2 and IFN γ were measured by means of ELISA (enzyme-linked immunosorbent assay) with Quantikine Rat immunoassay kits according to the manufacturer's instructions.

3.7. Blood drug levels and drug toxicity to liver and kidney

Blood concentration of rapamycin was measured on day 10 after transplantations by high performance liquid chromatography/mass spectrometry (HPLC/MS). And the concentration of A771726, leflunomide's active metabolite, in serum was examined by HPLC. In order to observe the toxicity of the two drugs on liver and the kidney, the livers and the kidneys of seven recipients per group were also harvested on day 10 after transplantation for histological examination. At the same time, the most important parameters of the recipient's liver and renal function including aspartate aminotransferase (AST),

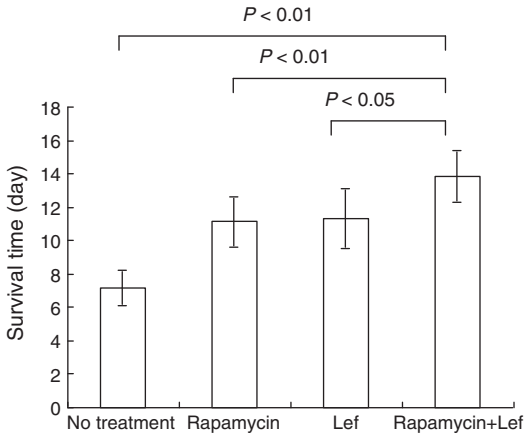

Fig. 1. Combining rapamycin and Leflunomide further improved the cardiac allograft survival. In untreated group, graft median survival time was 7.14 ± 1.07 days in heterotopic heart transplantation in rats. Rapamycin and Lef used alone prolonged the median survival time to 11.14 ± 1.35 days and 11.29 ± 1.80 days respectively. While in rapamycin + Lef group, the median survival time was further prolonged to 13.86 ± 1.57 days (compared with Rapamycin group, $P<0.01$, compared with Lef group, $P<0.05$).