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Original Article

8-methoxypsoralen previously activated by ultraviolet A ameliorates acute graft-versus-host disease in a murine model of allogeneic hematopoietic cell transplantation

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Abstract: Background: Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is an important method in the treatment of malignant and benign hematologic diseases, such as leukemia and aplastic anemia. However, acute graft versus host disease (aGVHD) induced by the alloreactivity of donor T cells is still a major challenge. The effectiveness and safety of extracorporeal photopheresis (ECP) in patients with glucocorticoid-resistant or -dependent aGVHD have been shown. However, ECP therapy has some disadvantages, such as transient hypotension during apheresis, and it is time consuming and relatively costly. Methods: 8-methoxypsoralen (8-MOP) is the key component of ECP therapy. We hypothesized that the venous injection of 8-MOP previously activated by ultraviolet A could be used in the prophylaxis and treatment of aGVHD. Thus, we investigated the survival time, leukocyte recovery, clinical score and histopathology of aGVHD of the recipient mice in a murine allo-HSCT model treated with previously activated 8-MOP (PAM), unactivated 8-MOP (UM) or physiological saline (PS). Results: PAM significantly prolonged the survival time of recipient mice and alleviated the symptoms of aGVHD compared with UM and PS. The histopathology of the liver and intestine from the UM and PS groups showed remarkable damage compared to the PAM group. PAM also promoted the recovery of white blood cells (WBC). Conclusions: 8-methoxypsoralen previously activated by ultraviolet A ameliorated aGVHD in a murine allo-HSCT model.

Keywords: Allogeneic hematopoietic stem cell transplantation, acute graft-versus-host disease, extracorporeal photopheresis, 8-methoxypsoralen

Introduction

Acute graft versus host disease (aGVHD) remains the most common severe complication in patients treated with allogenic hematopoietic stem cell transplantation (allo-HSCT), affecting 20-80% of these patients [1, 2]. This disease is triggered by immunocompetent cells, such as T lymphocytes, derived from the donor. Currently, glucocorticoids are the major compounds widely used as the first line and mainstay therapy for aGVHD, which exhibit an effect in approximately 50% of patients [1]. However, the prognosis in patients resistant to glucocorticoids is very poor [3]. As immunosuppressive agents, glucocorticoids also increase the risk of lethal infections.

Approximately 30 years ago, Edelson et al introduced the use of extracorporeal photopheresis (ECP) to treat cutaneous T-cell lymphoma [4]. ECP was found to be effective in the treatment of some autoimmune diseases, such as scleroderma and systemic lupus erythematosus [5-7]. ECP also shows good clinical efficacy in the treatment of solid organ allograft rejection [8]. Briefly, in ECP, peripheral blood mononuclear cells (PBMNCs) are collected from the patient by apheresis and then irradiated by ultraviolet A (UVA) in the presence of the photoactive drug 8-methoxypsoralen (8-MOP) in vitro. The photoactivated PBMNCs are then reinfused into the patient [4]. In recent years, more and more clinical practice has demonstrated that ECP is an effective and safe therapy for the treatment of glucocorticoid resistance or dependent aGVHD. The response rate was shown to range from 61% to 82% according to the affected organ [9-12]. The study from Shaughnessy and coworkers also showed that ECP administered

prior to a standard myeloablative preparative regimen allo-HSCT could prevent aGVHD without affecting either engrafting or relapse rates [13]. Different from traditional immunosuppressive therapies, ECP rarely increases the rate of infection and relapse [14, 15] because it induces immunotolerance other than immunosuppression in patients [16].

Although the safety and tolerability of ECP has been proven, a small number of patients experienced hypotension while undergoing ECP, and some patients accepted deep vein catheterization because the peripheral vein was unfit for apheresis. The procedure for ECP therapy is time consuming, and the patient must lie on a sickbed and maintain a fixed position for 2-4 hours during apheresis. Patients suffering from severe aGVHD may not be able to endure such a procedure because of their poor performance status. If the amount of plasma collected by apheresis is not sufficient, a photoallergy may result from the photomodification of plasma proteins induced by UVA irradiation [17]. Additionally, the cost-efficacy of the ECP treatment should be considered in developing countries.

For the deficiency of ECP mentioned above, modifications of the ECP procedure, which could provide a more comfortable treatment experience with less financial burden for the patient, should be developed. In view of these factors, we investigated whether the venous injection of 8-MOP, which was previously activated by UVA in vitro, could function in the prophylaxis and treatment of aGVHD in a murine allo-HSCT model.

Material and methods

PAM preparation

8-MOP (Sigma, USA) was diluted in sterile water for injection (20 μg/ml) and was irradiated by UV-A (365 nm, 2 J/cm^2) for 30 min before intravenous injection via the tail vein of mice.

Mice

In this study, female BALB/c recipient mice and male C57BL/6 donor mice were 8-12 weeks of age. All mice were purchased from Vital River (Beijing, China). The mice were given sterile water and standard chow *ad libitum* and housed in a germ-free environment. The experiments were conducted according to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publications No. 96-01).

Murine model of acute GVHD

Allogeneic hematopoietic stem cell transplantation was performed as previously described [18]. Briefly, lethal total body irradiation (TBI) with a dose of 9.5 Gy was applied as a conditioning regimen. BALB/c mice received 2×10^7 bone marrow cells and 0.5×10^7 spleen cells from C57BL/6 mice 4 hours after TBI. After transplantation, the recipient mice were kept in cages for one day to rest.

Grouping of allo-HSCT mice and administration of PAM

Thirty-nine recipient mice were divided into three groups (n=14 in PAM group, n=13 in UM group, n=12 in PS group). From day 1 to day 14 post-transplantation, mice were treated with PAM (6 μg/day/each), UM (6 μg/day/each) or PS (0.3 ml/day/each), respectively, by tail vein injection.

Survival time of recipient mice and clinical score of aGVHD

After transplantation, mice were housed in an experimental animal room and closely observed for 30 days. To evaluate the therapeutic effect of PAM on aGVHD, the survival time of recipient mice and the clinical score of aGVHD were monitored daily. As described elsewhere [19], aGVHD was scored according to weight loss, posture, activity, fur changes and skin integrity.

Histopathology of target organs of aGVHD

For the histopathological examinations of target organs of aGVHD, 3 mice from each group were selected randomly and sacrificed on day 14 after transplantation. The liver and intestine were harvested and fixed in 4% formalin for 24 hours, transferred to 70% ethanol, dehydrated, paraffin-embedded, sectioned and stained with hematoxylin and eosin for histopathologic evaluation by a pathologist who was blinded to the experimental procedures.

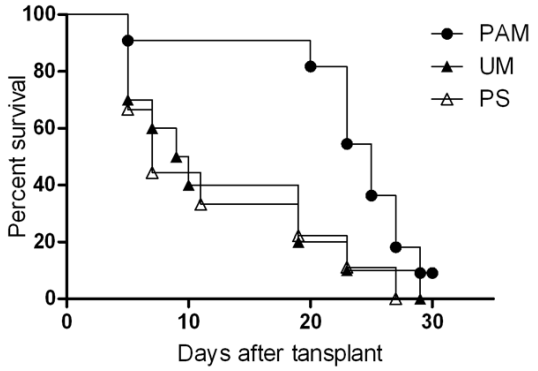

Figure 1. Survival curves according to Kaplan-Meier analysis of mice in the treated groups and control group. After transplantation, mice were injected with previously activated 8-MOP (PAM), unactivated 8-MOP (UM) or physiologic saline (PS) through the tail vein from day 1 to day 14 post-transplantation and survival was checked daily for 30 days. Survival, $P<0.05$ for PAM group versus UM group and versus PS group. Log-rank test $P=0.019$ for PAM group versus UM group and 0.011 for PAM group versus PS group.

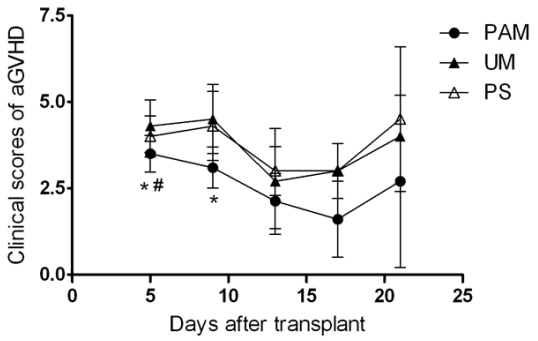

Figure 2. Clinical scores of aGVHD in recipient mice treated with previously activated 8-MOP (PAM), unactivated 8-MOP (UM) and physiological saline (PS). Recipient mice received treatment daily, starting from day 1 to day 14 post-transplantation. PAM significantly attenuated the clinical score of aGVHD on day 5 and day 9 after transplantation compared with the group treated with UM and PS (day 5: 3.5±0.5 for PAM versus 4.3±0.8 for UM, $^{*}P<0.05$ and versus 4.1±0.6 for PS, $^{\#}P<0.05$) (day 9: 3.1±0.6 for PAM versus 4.5±1 for UM, $^{*}P<0.05$ and versus 4.3±1 for PS, $^{\#}P<0.05$).

Recovery of leukocytes in peripheral blood

To evaluate the impact of PAM on engraftment after transplantation, the leukocytes in peripheral blood were counted regularly from the tail veins of recipient mice in each group on day 0, 7 and 14 after transplantation, as described previously [20].

Statistical analysis

Data were analyzed with IBM SPSS Statistics 19. Difference between groups in survival rates were compared by using the log-rank test, and survival curves were calculated using Kaplan-Meier analysis. For all other results, the two-sample Student's T test was used. Data are expressed as the mean ± SD and $P<0.05$ was considered significant.

Results

PAM treatment could prolong the survival time of recipient mice

The survival rates of recipient mice after transplantation can be influenced by the severity of the aGVHD. Treatment with PAM from day 1 to day 14 post-transplantation could rescue mice from lethal aGVHD compared with UM and PS. At the 15th day post-transplantation, the survival rates of recipient mice treated with PAM, UM and PS were 90.9%, 40% and 33.3%, respectively (**Figure 1**, $P<0.05$). These data showed that PAM could significantly prolong the survival time of allo-HSCT mice (**Figure 1**).

PAM treatment could alleviate the severity of aGVHD of recipient mice

The clinical score of aGVHD consisted of 5 components, including weight loss, posture, activity, fur changes and skin integrity. Each component was scored as 0 or 1 or 2 according to the severity of symptoms: 0 for normal, 1 for slightly or moderately abnormal and 2 for severely abnormal. The clinical score of aGVHD was the sum of the 5 components. As shown in **Figure 2**, PAM significantly attenuated the clinical score of aGVHD day 5 and day 9 after transplantation compared with UM ($P<0.05$) and PS ($P<0.05$). We proved that PAM treatment could alleviate the severity of aGVHD of recipient mice.

PAM treatment could promote the leukocyte reconstitution of recipient mice

The speed of hematopoietic reconstitution was assessed by the peripheral blood leukocyte counts (**Figure 3**). On day 7 post-transplantation, leukocyte number in recipient mice treated with PAM [$(0.062\pm0.032)\times10^9$/L] was significantly higher than that in recipient mice treated

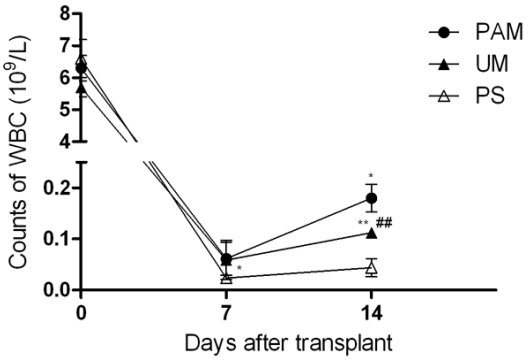

Figure 3. The changes of leukocyte counts in mice treated with previously activated 8-MOP (PAM), unactivated 8-MOP (UM) or physiological saline (PS) after transplantation. The leukocyte counts on day 7 in the PAM group were higher than in the PS group [$(0.062\pm0.032)\times10^9$/L versus $(0.024\pm0.008)\times10^9$/L, $^*P<0.05$]. On day 14, PAM recovered WBC more quickly than the other two groups treated with UM and PS [$(0.18\pm0.027)\times10^9$/L for PAM versus $(0.113\pm0.006)\times10^9$/L for UM, $^*P<0.05$ and versus $(0.044\pm0.018)\times10^9$/L for PS, $^{**}P<0.01$)]. The WBC count in recipient mice treated with UM was also significantly higher than that in the PS group [$(0.113\pm0.006)\times10^9$/L for UM versus $(0.044\pm0.018)\times10^9$/L for PS, $^{\#\#}P<0.01$].

with PS [$(0.024\pm0.008)\times10^9$/L, $P<0.05$]. On day 14 post-transplantation, white blood cells (WBC) in PAM recovered more quickly than that in the other two groups [$(0.18\pm0.027)\times10^9$/L in PAM versus $(0.113\pm0.006)\times10^9$/L in UM, $P<0.05$, and versus $(0.044\pm0.018)\times10^9$/L in PS, $P<0.01$]. The WBC count in recipient mice treated with UM was also significantly higher than that in the PS group [$(0.113\pm0.006)\times10^9$/L in UM versus $(0.044\pm0.018)\times10^9$/L in PS, $P<0.01$].

PAM could alleviate the injury of the target organs of aGVHD

The small intestine and liver are the two organs most frequently attacked by aGVHD. To compare the severity of aGVHD between groups, we also evaluated the histopathological changes in each group from the small intestine and liver tissues at day 14 post-transplantation. Representative images of H&E staining of the small intestine from physiological saline and the unactivated 8-MOP treatment group demonstrated greater infiltration of inflammatory cells in the lamina propria (**Figure 4**). Images of the liver tissue from physiological saline and unactivated 8-MOP treatment group also showed increased inflammatory infiltrates in the portal area than images of liver tissue from the previously activated 8-MOP treatment group (**Figure 4**).

Discussion

Our study revealed that intravenous injection of previously activated 8-MOP (PAM) could rescue experimental allo-HSCT mice from lethal aGVHD. In other words, PAM could increase the survival rate of recipient mice, alleviate injury in the target organs of aGVHD and promote hematopoietic reconstitution.

Acute GVHD is a severe complication of allo-HSCT, which is an important cause of transplant-related mortality and is also an important risk factor for the future development of chronic GVHD [21]. The pathophysiological process of aGVHD can be divided into three phases: phase 1 is the tissue damage and cytokine release caused by chemotherapy, infection or total body irradiation prior to allo-HSCT; in phase 2, donor T lymphocytes are activated and IFN-γ, IL-2 and TNF-α are released under the help of antigen-presenting cells derived from recipients; and phase 3 consists of the proliferation of donor T lymphocytes and the expansion of various cytotoxin or inflammatory molecules, which assault various host tissues [22]. Glucocorticoids are currently the first line therapy for aGVHD, and approximately 50% of all patients respond to such therapy [21]. There is currently no standard therapy used widely as the salvage treatment for the steroid-refractory or -dependent aGVHD. Many studies designed to treat these patients focus on the inhibition of alloreactive donor T lymphocytes, cytokine release or cytokine receptors [23].

ECP is now available at more than 200 centers worldwide to treat cutaneous T-cell lymphoma, scleroderma, lupus erythematosus, solid organ transplantation, Crohn's disease, atopic dermatitis, pemphigus and acute or chronic GVHD [24, 25]. According to a phase 2 study conducted by Greinix et al, in the response of steroid-refractory or -dependent aGVHD to ECP treatment, the CR rates were 82% for skin involvement and 61% each for gastrointestinal and liver involvement, and the median time to reach the best response was 1.3 months [11]. The safety and tolerability of ECP are considered to be good and the side effects are mild and

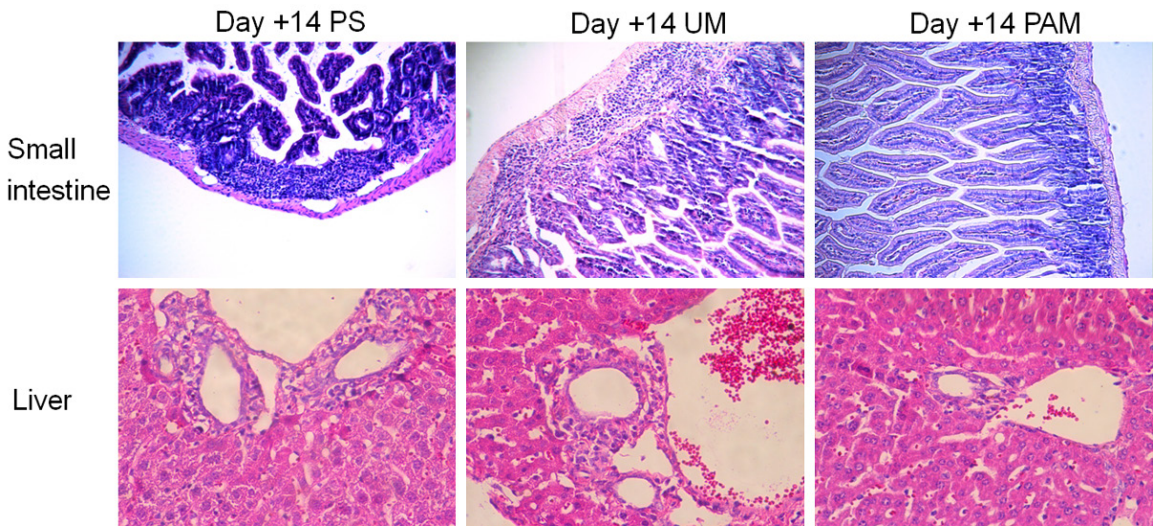

Figure 4. Histopathological changes in the small intestine (original magnification ×100) and liver (original magnification ×200). At day 14 after transplantation, the histopathological changes in the small intestine and liver were evaluated. Representative images of H&E staining of the small intestine and liver from unactivated 8-MOP (UM) and physiological saline (PS) treatment group demonstrating more leukocyte infiltration than images from the previously activated 8-MOP (PAM) treatment group.

include transient hypotension and a mild decrease in hemoglobin and/or platelets during treatment. The use of heparin or acid citrate dextrose in the process of apheresis may increase the risk of bleeding, especially in patients with thrombocytopenia or coagulation disorders. Patients with a history of previous heparin-induced thrombocytopenia or unsatisfactory cardiovascular function are not suitable for treatment with ECP [24]. For patients with a low body weight or without appropriate venous access, a special apheresis device and deep vein catheterization should be used. Patients with poor performance status are less able to tolerate apheresis because they must maintain a fixed position for several hours during the process. To improve the treatment experience, as a substitute for ECP cells, Budde et al collected leukocytes from donor mice through apheresis, mixed them with 8-MOP and irradiated them with UV-A. These cells were then infused into the recipient mice post-transplantation. Unfortunately, this modification was not as effective as ECP cells derived from recipient mice [26].

The present study showed that the direct injection of the 8-MOP previously activated by UV-A in vitro through the tail vein could diminish the severity of aGVHD and rescue recipient mice from lethal aGVHD. The main advantage of this modification is the avoidance of apheresis, that is, there is no need for a cell separation device; therefore, the side effects mentioned above may not develop. Additionally, this therapy could be applied easily according to the needs for treatment without restrictions due to the poor physical condition of patients who are not able to tolerate apheresis.

In conclusion, we provide experimental evidence that PAM can be used as an effective agent to prevent and treat aGVHD in a murine allo-HSCT model. The mechanisms of action of this therapy need to be further studied.

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Disclosure of conflict of interest

None.

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