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Evaluation of the effects of preconditioning regimens on hepatic veno-occlusive disease in mice after hematopoietic stem cell transplantation

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ABSTRACT

Pre-conditioning regimens before hematopoietic stem cell transplantation (HSCT), such as total body irradiation (TBI) or busulfan/cyclophosphamide (BU/CY), are associated with hepatic veno-occlusive disease (HVOD). However, the mechanism of these regimens on hepatic veno-occlusive disease remains unclear. The aim of this study is to evaluate the effect of TBI or BU/CY on HVOD in mice after HSCT. Mice received TBI or BU/CY followed by HSCT. Analysis of liver pathology and function, and platelet aggregation were performed. Both these regimens caused damage to liver sinusoid endothelial cells, leading to loss of normal structural integrity of liver sinusoid, abnormal liver function, fibrin deposition, inflammatory cells infiltration and platelet aggregation. No differences of liver function in these regimens were observed. Increased hepatic lipid droplets, mitochondrial swelling and higher incidence of HVOD were observed in BU/CY. In conclusion, both TBI and BU/CY caused damage to liver sinusoid endothelial cells and occurrence of HVOD with higher incidence for BU/CY. Meanwhile, inflammation and platelet activation was also observed, suggesting targeting them maybe beneficial in the prophylaxis of HVOD.

1. Introduction

Hepatic veno-occlusive disease (HVOD), known as sinusoidal obstruction syndrome (SOS), remains a significant early complication leading to higher incidence of mortality and mobility in patients after hematopoietic stem cell transplantation (HSCT). This syndrome is characterized by clinical features such as ascites, hepatomegaly, jaundice, weight gain (Bearman, 1995; Richardson and Guinan, 1999) and usually occurs within 30 days after transplantation (Richardson et al., 2012). The incidence of HVOD after HSCT is reported to be highly variable, from 10 to 60% due to the diagnosis based on the clinical criteria (Wadleigh et al., 2003), with a higher rate in patients after allogeneic than autologous HSCT. The severity of this syndrome ranges from a mild reversible disease to a severe syndrome, which is associated with multi-organ failure (MOF), characterized by pulmonary edema, renal failure and encephalopathy (Carreras et al., 2011; Coppell et al., 2010).

The pathophysiology of HVOD is very complicated, with multifactors involved. There is an increasing body of evidence demonstrating that the main cause of HVOD is to be initiated by the damage to sinusoidal endothelial cells in zone 3 of the hepatic acinus resulting from the application of preparative conditioning regimens (Baglin, 1994; el Mouelhi and Kauffman, 1986; Guglielmelli et al., 2012). Fibrin deposition, clot formation, extravasation of red blood cells, leukocytes into the space of Disse (Bearman, 1995; Coppell et al., 2003), resulting from activated sinusoidal endothelial cells due to injury, have been reported to be contributed to the narrowing of the sinusoids, reduction of hepatic venous outflow, leading to central venular-occlusion, hepatic enlargement with capsular distension, as well as portal venous flow reversal, ultimately resulting in HVOD.

Previous studies revealed a lot of risk factors contributing to HVOD with the most important being the preparative conditioning regimen itself (Carreras et al., 1998; Rozman et al., 1996; Wadleigh et al., 2003). Total body irradiation (TBI) or busulfan (BU) combined with cyclophosphamide (CY) (BU/CY) is the main conditioning regimen for HSCT in patients with myeloid malignancies. However, the major complication associated with HSCT is the occurrence of HVOD, resulting from the employment of TBI or BU, with a higher incidence rate for BU. Given the critical roles in the development of HVOD, it would be valuable to investigate the exact mechanism of these conditioning regimes on the

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pathogenesis of HVOD following HSCT. However, most of cohort studies are focused on the conditioning regimes' effect on the overall survival, relapse or transplant-related mortality (TRM) and there is little information regarding the effect of TBI and BU/CY on the development of HVOD. In this study, we aimed to evaluate and compare the effects of TBI or BU/CY on the pathogenesis of HVOD in mice before and after HSCT, in terms of the incidence rate, liver pathology, function, liver sinusoidal endothelial cells (LSECs), peripheral white blood cells, reticulocytes as well as activated platelets.

2. Materials and methods

2.1. Materials

FITC-conjugated anti-mouse P-selectin antibody (CD62P), IgG antibody and PE-conjugated anti-mouse CD61 antibody were purchased from BD Biosciences (San Jose, USA). APC-conjugated anti-mouse CD41 antibody was from Ebioscience (San Diego, USA). Rat anti-mouse endothelial cell monoclonal antibody (MECA-32) and HRP-conjugated rabbit anti-mouse IgG were purchased from Biolegend (San Diego, USA). 3, 3′-diaminobenzidine was from Sigma (CA, USA).

2.2. Animals and treatment

Male C57BL/6 (H-2K^b) (donor) and female BALB/c (H-2K^d) (recipient) mice, aged 10–12 weeks and weighed 20–25 g, were purchased from SLAC LABORATORY ANIMAL, Shanghai, China. The mice were housed in sterilized cages at the Experimental Animal Center of Xuzhou Medical College. All animal care and experimental procedures followed ethical standards of animal use and were approved by the Jiangsu Society for Animal Welfare, China.

BALB/c mice were randomly divided into five groups with n = 20 in each group, control, TBI, TBI + HSCT, BU/CY and BU/CY + HSCT. Normal mice without any treatments were used as control. HSCT mice model with TBI preconditioning treatment was established as previously described (Zeng et al., 2012). For BU/CY treatment, BU (20 mg/kg/day for 4 days) and CY (100 mg/kg/day for 2 days) was intraperitoneally injected into mice from the 7th to 4th days and mice from 3rd to 2nd days respectively before HSCT. The dose of BU and CY was based on previously studies (Al-Hashmi et al., 2012; Sadeghi et al., 2008). All the mice were fed with sterilized water containing gentamicin (320,000 U/L) and ceftriaxone (0.25 g/l) 1 week before tail injection of 5×10^6 bone marrow mononuclear cells containing 1.14 ± 0.26% hematopoietic stem cells isolated from C57BL/6 mice as previously described (Zeng et al., 2013). Samples for liver analysis and other purposes were collected 7 days (d7) after treatment in TBI group, on the day of HSCT (d0) in BU/CY and 15 days post-HSCT (d15) in TBI + HSCT and BU/CY + HSCT group.

2.3. Histological pathology and immunohistochemical staining of LSECs

The histological sections were stained by hematoxylin–eosin. The liver was fixed with formaldehyde solution, dehydrated, waxed, and sliced into 4 μm thickness by RM2126 microtome. After H&E staining, pathologic changes were evaluated by a light microscope.

LSECs were identified by immunoperoxidase stain. The slices were incubated with 3% H_2O_2 at room temperature and blocked with 5–10% goat serum. Then the slices were incubated with pan-endothelial cell monoclonal antibody (MECA-32) at 37 °C for 1–2 h followed by incubation with secondary antibody which was horseradish peroxidase-conjugated rabbit anti-rat antibody. Color was developed with 3, 3′-diaminobenzidine.

2.4. Evaluation of bone marrow chimerism and liver index

Bone marrow chimerism was evaluated as previously described (Zeng et al., 2013). Liver index was calculated as liver weight/body weight.

2.5. Liver tissue ultrastructure analysis

The ultrastructure of liver tissues was evaluated by high resolution transmission electron microscopic (HRTEM). The livers were fixed with 3% glutaraldehyde, post-fixed with osmium tetroxide for 2 h at 4 °C, dehydrated with graded ethanol and acetone, and embedded in Epon. Thin sections were cut on a LKB-IV microtome and examined with an H-600 electron microscope.

2.6. Measurement of platelet activation

20.5 μl whole blood obtained from eyeballs was placed into tube A (containing 2 μl platelet anticoagulant, 2 μl APC-CD41 monoclonal antibody, 2 μl PE-CD61 monoclonal antibody and negative control antibody) and tube B (containing 2 μl platelet anticoagulant, 2 μl APC-CD41 monoclonal antibody, 2 μl PE-CD61 monoclonal antibody and 2 μl of FITC-CD62P monoclonal antibody). After mixing, the tubes were incubated for 20 min in the dark followed by the addition of 500 μl platelet buffer and analyzed by FACS Calibur (BD Biosciences, USA).

2.7. Statistical analysis

Date is represented as Mean ± SD. By using SPSS16.0 software, two-tailed student's unpaired *t*-test was used to compare the parameters in TBI or BU/CY group (with or without following HSCT) with that in control group as well as for the comparison between TBI and BU/CY treatment (with or without following HSCT). $P < 0.05$ was considered to be statistically different.

3. Results

3.1. Phenotype

On d7 after treatment in TBI group, weight loss, hunchback and curl hairs were observed. On d15 after HSCT in TBI + HSCT and BU/CY + HSCT, listlessness, reduced food intake, hunchback and curl hairs were seen. However, abdomen distention was observed in BU/CY + HSCT group.

3.2. Liver pathology and function in TBI and BU/CY group

The normal liver lobule is a hexagonal mass of tissue primarily composed of plates of hepatocytes radiating from the region of the central vein toward the periphery. On d7 post-TBI treatment, H&E staining (Fig. 1A) showed loss of structural integrity of liver lobule, edema and coagulative necrosis of hepatocytes, as well as partly obstruction of liver sinusoid, swelling of LSECs and infiltration of inflammatory cells. On d0 in the BU/CY treatment group, H&E staining revealed edema and severe necrosis of hepatocytes, narrowing and obstruction of central vein and liver sinusoid and a large number of macrophages infiltration. Immunohistochemical staining (Fig. 1B) with MECA-32 antibody demonstrated abnormal structure of liver lobule, loss of integrity of sinusoid wall and detachment of LSECs in TBI group and necrosis of liver lobule, dilatation of liver sinusoid, damage and detachment of LSECs and interruption of the continuity of sinusoid wall in BU/CY group.

After pre-HSCT conditioning regimen treatment, plasma was isolated from anti-coagulated blood which was drawn from mice in TBI and BU/CY group to evaluate the effect of regimens on the liver function. The level of alanine transaminase (ALT) was 88.65 ± 3.43 U/L ($P < 0.05$) in mice treated with TBI and 124.43 ± 12.56 U/L ($P < 0.05$) treated with BU/CY, which was significantly increased compared to control group (48.75 ± 4.02 U/L) (Table 1). However, there was no difference between TBI and BU/CY group. The similar results were obtained regarding the level of aspartate transaminase (AST), alkaline phosphatase (ALP) and total bilirubin (TBIL) in control, TBI and BU/CY