The Correlation Between Platelet Activation and Liver Injury by Conditioning and Bone Marrow Transplantation


ABSTRACT

Hepatic veno-occlusive disease (HVOD) is a common severe complication after transplantation which is closely related to liver sinusoidal endothelial cell (LSEC) injury. Although platelet activation might play a key role in the progress of HVOD, the relationship between the P-selectin and HVOD is still unclear. In this study, the P-selectin, liver functions, and observed damage of LSEC after transplantation were detected. The P-selectin, alanine aminotransferase (ALT) and bilirubin were first detected in the patients. The ALT of patients reached the first peak of 144 ± 45.04 U/L on day 7 after busulfan + cyclophosphamide (BU/CY) pretreatment, and reached the second peak of 220.5 ± 40.58 U/L on day 7 after transplantation. Similarly, the concentration of bilirubin increased BU/CY pretreatment and reached peak on day 15 after transplantation at 38.8 ± 5.99 µmol/L. However, the level of P-selectin was significantly higher than normal levels for a long time and peaked on day 3 after BU/CY pretreatment and on day 12 after transplantation (P < .05). Then the P-selectin, liver function, liver index, hepatocytes, and LSEC were observed using a transplantation model of C57BL/6 (H-2b) donor to BALB/c (H-2d) recipient. The P-selectin, liver index, and liver function of total body irradiation (TBI) mice and bone marrow transplantation (BMT) mice were significantly increased, but the increase of TBI mice were more significant. On day 5, the ratio of TBI mice peripheral blood platelets which expressed P-selectin was higher and reached an earlier peak at 15.6 ± 2.63%, whereas that of BMT mice was 6.59 ± 1.17%, and peaked on day 15 at 8.36 ± 1.55% (P < .05). Moreover, the ALT and bilirubin levels of TBI mice were higher and reached earlier peaks on day 5 at 58.65 ± 1.43 U/L and 27.14 ± 1.32 µmol/L, respectively, whereas those of BMT mice peaked on day 5 at 50.22 ± 2.02 U/L and day 30 at 39.57 ± 1.55 µmol/L, respectively (P < .05). The LSEC damage and hepatocyte edema in BMT mice were most serious at day 15, and at day 30 injuries did not allow recovery. The liver score of TBI mice peaked on day 5, whereas that of BMT mice peaked on day 15. Moreover, the degree of damage and platelet activation positively correlated. This study implied that P-selectin could be used as a predictor of HVOD.
HVOD was the leading cause of death within 2 months of transplantation [1–3], mainly due to lobular central vein and sublobular vein injuries leading to lumina stenosis and occlusion, followed by abnormal liver function. At present, the pathogenesis is still unclear, but research has proven that the LSEC injury was the first step toward HVOD [4,5]. Injured endothelial cells not only release von Willibrand factor and thrombomodulin, but also some cytokines such as tumor necrosis factor-α, interleukin-6, and endothelin-1 and so on, and may further promote the release of P-selectin [6–9], and a large number of cytokines which cause hypercoagulability and inflammatory cell infiltration and also further increase endothelial cell injury, thus creating a vicious cycle toward HVOD. Currently researchers are studying how to break the cycle of liver injury using transplantation conditioning to control the occurrence of HVOD. To that end, we present a simple experiment on irradiated mice and mice that have had bone marrow transplantation using dynamic monitoring of related cytokines and studying the links to LSEC injury with a comprehensive analysis of the extent of endothelium injury using pathological and immunohistochemistry results.

MATERIALS AND METHODS

Animals

Specific pathogen-free C57BL/6 (H2Kb) and BABL/c (H2Kd) mice at 8 to 12 weeks of age were obtained from Yangzhou University. Mice were housed in sterilized cages at the Experimental Animal Center of Xuzhou Medical College. All experiments were performed according to the Institutional Animal Care and Use Committee (IACUC) guidelines.

Antibodies

Fluorescein isothiocyanate conjugated (FITC) anti-mouse CD62P (RB40.34), APC conjugated anti-mouse CD41 (MWReg30), FITC conjugated anti-mouse immunoglobulin G (IgG; AX5-1), PE conjugated anti-mouse CD61 (2C9.G2), and FITC conjugated anti-human CD41 (HIPS) mice were all purchased from BD Biosciences (San Jose, Calif, United States). PE conjugated anti-human CD62P (AK-4) and PE conjugated anti-human IgG (G18-145) were all purchased from eBioscience (San Diego, Calif, United States). Rat anti-mouse endothelial cell monoclonal antibody (MECA-32) and horseradish peroxidase conjugated rabbit anti-rat IgG monoclonal antibody were purchased from Biolegend (San Diego, Calif, United States). Platelet anticoagulant and platelet buffer were prepared by the laboratory.

Clinical Criteria of HVOD

Clinically, the diagnosis of stem cell transplantation–related HVOD is based on a triad of findings that include: painful hepatomegaly, hyperbilirubinemia (bilirubin ≥ 34.2 μmol/L), and unexplained weight gain ≥ 2% or 5% over baseline, any two of which could be used to diagnose HVOD.

Liver Index Calculation

In this study, 45 BABL/c mice were randomly divided into three groups: the control group; the total body irradiation (TBI) group, which were only given a single total 7.5 Gy 60Co source, dose rate 0.67 Gy/min; and the bone marrow transplantation (BMT) group, which were also given TBI, then were injected intravenously with bone marrow cells from C57BL/6 mice. On days 5, 10, 15, and 20 after TBI and BMT, mice were weighed, anesthetized to cut the belly open to obtain integrity liver samples, and weighed again using a Mettler AT261 electronic balance. Liver index = liver weight/body weight.

Observation of Histologic Pathology

To assess morphologic changes, 4% paraformaldehyde preserved tissues from day 5, 10, 15, 20, and 30 mice embedded in paraffin were cut into 4-μm paraffin sections, which were stained with hematoxylin and eosin. To observe hepatocyte changes respectively, LSECs were observed by immunohistochemical staining after de-waxing the 4-μm paraffin sections that were incubated by 3% H2O2. After blocking with 5% goat serum, sections were incubated with the first antibody (MECA-32) for 1~2 hours at 37°C. The secondary antibody was horseradish peroxidase conjugated rabbit anti-rat IgG monoclonal antibody. The sections were incubated for 30 minutes at 37°C, rinsed, colored with 3, 3′-diaminobenzidine, and counterstained with hematoxylin. The slices were sealed for further observation using a light microscope. Complete endothelial cells were dyed brown to determine the number of LSECs according to the color depth.

Activated Platelets Detection

P-selectin is a sign of platelet activation. In this study, we detected P-selectin expression in peripheral blood in response to platelet activation.

Transplantation patients activate platelets detection. Blood samples obtained from transplantation patients from 10 days before to 30 days after transplantation were incubated with FITC anti-human CD41 and PE conjugated anti-human CD62P for 20 minutes at 4°C, then diluted with platelet buffer. Stained cells were then analyzed for P-selectin (CD41+, CD62P+).

Mice activate platelets detection. Blood samples obtained from mice on days 0, 5, 10, 15, 20, and 30 after TBI or BMT and TBI were incubated with APC anti-mouse CD41, FITC anti-mouse CD62P (RB40.34) and PE conjugated anti-mouse CD61 for 20 minutes at 4°C, then diluted with platelet buffer. Stained cells were then analyzed for P-selectin (CD41+, CD61+, CD62P+).

Data and Statistical Analysis

Data were expressed as mean values ± standard deviation (SD), and comparison of two means were analyzed with unpaired Student t test. The correlation between P-selectin and liver injury score was expressed as a Pearson coefficient. P < .05 was considered statistically significant.

RESULTS

Transplantation Conditioning and BMT Could Damage Liver of Patients

To clarify the liver changes of transplantation patients before and after conditioning, we examined the P-selectin, alanine aminotransferase (ALT), and bilirubin changes of 10 cases transplantation patients; we found that the ALT reached the first peak on day 7 after busulfan and cyclophosphamide (BU/CY) pretreatment, the peak of 144 ± 45.04 U/L, after a slight decrease, on the day 7 after transplantation, reached the second peak, then decreased to the normal range gradually (Fig 1A). Bilirubin increased...
BU/CY pretreatment and reached peak on day 15 after transplantation at 38.8 ± 5.99 μmol/L, then decreased to the normal range on day 24, but began to increase again. The value was still higher than normal levels on day 30 after transplantation at 25.43 ± 3.12 μmol/L (Fig 1B). From the start of conditioning to day 20 after transplantation, P-selectin in the peripheral blood continued to be higher than normal levels, peaked on day 3 after BU/CY pretreatment, and on day 7 after transplantation, and the difference was statistically significant (P < .05). (C) The P-selectin changes of patients after conditioning and transplantation. P-selectin in the peripheral blood continued to be higher than normal levels, peaked on day 3 after conditioning, and on day 7 after transplantation, and the difference was statistically significant (P < .05). (D) Positive correlation was observed between ALT and P-selectin in the peripheral blood. ALT was a sensitive indicator of liver damage, and we observed a positive correlation between ALT and P-selectin in the peripheral blood. Therefore, we concluded a positive correlation between liver damage and P-selectin in the peripheral blood.

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To further define the pathological changes of the liver by conditioning and BMT, we gave the mice TBI, then allogeneic BMT, and observed pathological changes regularly. Conditioning and BMT Damage the LSECs and Injury Persists

LSEC injury was the first step toward HVOD; we observed the pathological changes of LSECs by immunohistochemical staining. On day 5 after conditioning we found that
Conditioning and BMT Promote Platelet Activation

P-selectin is an important indicator of platelet activation. To understand platelet activation, we detected the ratio of peripheral blood platelet expressed P-selectin at different times. On day 5 after TBI, the ratio of mouse peripheral blood platelet expressed P-selectin reached a peak of 15.6 ± 2.65%, and on day 10 it was lower than before, as normal mice 16.30 ± 2.76 times (P < .05), which suggested that conditioning promotes mouse platelet activation and forms a hypercoagulable state (Fig 3A). On days 5 and 10 after TBI and BMT, the ratio of peripheral blood platelet expressed P-selectin was lower than that of mice received TBI only, but higher than that of normal mice. The ratio peaked on day 15 at 8.36 ± 1.55%, as 9.61 ± 3.46 times of that of normal mice (P < .05). Thus, we concluded that TBI conditioning promoted platelet activation and after TBI together with BMT the ratio of mouse peripheral blood platelet expressed P-selectin continued to be higher than normal levels, which made it easy to form a hypercoagulable state (Fig 3A).

Conditioning and BMT Damages Hepatocytes and Injury Persists

TBI conditioning and BMT damaged LSECs promoted platelet activation and thrombosis, followed by hepatocyte injury, and caused liver index and liver function changes. The liver index value reflected the liver edema and was an indicator of liver function. We measured the liver index of mice after conditioning and found that liver index of TBI mice only peaked on day 5 at 6.25 ± 0.19%, as 1.128 ± 0.034 times of that of normal mice (P < .05). On day 10, the liver index was slightly lower than before, but still higher than that of normal mice (P < .05; Fig 3B). The liver index of mice that received TBI and BMT began to increase on day 2 after TBI and BMT, reached the first peak on day 15, as 1.348 ± 0.038 times of that of normal mice (P < .05), on day 20 the liver index was still higher than that of normal at approximately 1.159 ± 0.024 times that of normal mice (P < .05), and reached the second peak of the normal 1.370 ± 0.045 times on day 30, which suggested that liver edema of mice persisted within 30 days after transplantation; therefore, the liver damage continued (Fig 3B).

Further observation by microscopy showed that hepatocyte edema and nucleoli disappeared and enhanced eosinophilic cytoplasm could be found on day 5 after TBI only; however, degeneration and necrosis of hepatocytes could also be found. The edema and necrosis of hepatocytes on day 10 were slighter than before. We also found that on day 5 after TBI and BMT, the edema and necrosis of hepatocytes of mice were slighter than that of mice given TBI only, but more obvious than that of mice given TBI only on day 10. The injury, including hepatocyte edema and necrosis, were most obvious on day 15, and continued to exist within 30 days after transplantation (Fig 4).

Conditioning and BMT Damages Liver Function and Injury Persists

ALT mainly in the cytoplasm of hepatocytes was considered to be the most sensitive indicator of liver damage. In this experiment, we found that ALT had been at a high level after TBI, reached a peak on day 5, which was 58.65 ± 1.43 U/L, about the normal 3.22 ± 0.078 times (P < .05),
although it was lower than the previous on day 10, still higher than normal level, about 1.90 ± 0.072 times of that of normal mice ($P < .05$; Fig 3C). Whereas after TBI and BMT, ALT levels continued to be high, and the peak also appeared on day 5, but was lower than that of mice given TBI only, about the normal 2.76 ± 0.11 times ($P < .05$). The liver index of BMT mice sustained higher than normal level, which suggested that liver edema of BMT mice persisted within 30 days after transplantation, therefore the liver damage continued to exist. (C) The levels of ALT in mice peripheral blood on days 0 (normal control), 5, 10, 15, 20, 30 after TBI only or TBI together with BMT. The ALT and bilirubin of BMT mice sustained higher than normal level, which suggested that liver damage persisted within 30 days after transplantation.

In this experiment, we found that bilirubin of mice given TBI only reached a peak on day 5 the same as ALT, which was 27.14 ± 1.32 µmol/L, about the normal 1.49 ± 0.072 times ($P < .05$), and was 27.14 ± 1.32 µmol/L on day 10, still higher than normal level ($P < .05$; Fig 3D). Compared with that of mice given TBI only, while after TBI and BMT, the bilirubin of mice reached the peak on day 15, which was 31.57 ± 0.93 µmol/L, about the normal 1.73 ± 0.051 times ($P < .05$), then began to decrease to a minimum on day 20, and later began to increase, then reached the second peak of 39.57 ± 1.55 µmol/L on day 30, which was significantly higher than normal level ($P < .05$; Fig 3D). The ALT and bilirubin of BMT mice sustained higher than normal levels, which suggested that liver damage persisted within 30 days after transplantation.
Liver Injury Score After Conditioning and Correlation With Activated Platelets

According to DeLeve et al [10], who use liver injury scoring criteria of HVOD to grade the liver after TBI conditioning, we concluded that the injured liver score of mice given TBI only reached the peak 11 points on day 5, and decreased to 9 points on day 10. Whereas the injured liver score of mice given TBI together with BMT reached the peak on day 15 of 9 points, then gradually decreased, to 5.5 points on day 20 (Fig 5A). We also observed a positive correlation between the injured liver score and P-selectin in the peripheral blood \( (r = 0.878, P = .022; \text{Fig 5B}). \) So, we conclude that the level of P-selectin in the peripheral blood could be used as a parameter to assess the extent of liver injury and as an indicator of HVOD, which suggested that the higher the score the greater the likelihood of HVOD.

DISCUSSION

HSCT is currently the primary means of treatment for hematological malignancies; however, when clearing the recipient bone marrow cells, the conditioning could also damage multiple recipient organs, including liver, intestine, and lung, in which LSECs, central vein, and lobular vein injury is particularly evident [11–13]. In recent years, with the bone marrow and stem cell transplantation widely used, HVOD has become the most common and severe in patients who have life-threatening complications.

HVOD generally occurs within 3 weeks after transplantation, the frequency and severity of which vary considerably from different conditioning, transplantation type, patient characteristics, and diagnostic criteria, generally ranging from 10% to 60%. Related retrospective studies [14] have shown that within 100 days after transplantation, the cause of death is 24% from HVOD and close to 100% for severe HVOD [15,16]. At present in the bone marrow and HSCT, to avoid major risk factors is still the primary method to prevent HVOD, such as for patients who have acute hepatitis and could be delayed transplant period, reducing the pretreatment dose of cytotoxic drugs, extending the BU/CY combination of interval time, fractionated TBI and reducing the dose rate which reduce the conditioning regimen for liver toxicity and selecting a fully matched donor of HLA typing and so on. During conditioning and after transplantation, diuretics and drugs of hepatoprotection and anti-platelet aggregation are still the main drug to prevent the HVOD, such as low molecular heparin, recombinant tissue type plasminogen activator (t-PA) and defibrotide, but the remission rates are all less than 50% [17]. The available clinical research data further show that the above prevention means are not reducing the incidence of HVOD after transplantation and finding effective means of prevention and treatment of HVOD is imperative.

In this study we found that after administering TBI, both liver index and liver function (ALT and bilirubin) levels of the mice tended to increase, suggesting that TBI conditioning had great influence on liver index and liver function. However, after administering TBI and BMT, liver index and liver function of the mice were still higher than the normal control within 30 days after transplantation, suggesting that the liver injury from TBI and BMT could be last a long time and the risk of HVOD be greatly increasing, which may explain the BMT is the major cause of HVOD at present.

From hematoxylin and eosin staining and immunohisto-staining of liver, hepatocyte edema, necrosis, LSEC loss and thrombosis could be found on day 5 after TBI only, and the liver injury was slighter than before on day 10, but still obvious. After TBI together with BMT, hepatocyte edema and necrosis, loss of EC of central vein and sublobular vein and LSEC were also be found. The liver injury of edema and necrosis and so on became obvious than before on day 15, on day 20 the hepatocyte edema and necrosis were slighter than before, but no significant improvement of LSEC could be found, and the liver injury, including hepatocyte edema, necrosis and EC loss had not yet returned to normal on day 30, on the contrary, further increase of inflammatory cell infiltration could be found. The changes of liver from hematoxylin and eosin staining and immunohisto-staining were basically the same as that of liver index and function, which suggested that TBI conditioning had serious damage on the liver, especially the LSEC, the injury of which could be last a long time after transplantation, leading to high risk of occurrence of HVOD.
According to DeLeve et al’s [10] liver injury scoring criteria of HVOD to grade the liver after TBI and BMT, the results showed that the peak of liver injury score of mice given TBI only and given TBI together with BMT, appeared on the day 5 and 15 respectively. Therefore, the time of the most serious liver damage of mice given TBI together with BMT delayed compared with that of mice given TBI only, but the peak of both had no significant difference (Fig 5A). P-selectin store in α-granules of platelet, releasing when platelets are activated and there is increased blood hypercoagulability. In our study, we found that the P-selectin of mice given TBI only peaked on day 5, whereas the P-selectin of mice given TBI together with BMT had two peak, appearing on the day 5, 15 respectively, and the levels of P-selectin continued to higher than normal control levels, which suggested that TBI conditioning and BMT both cause platelet activation. However, by detecting the levels of transplantation patients, we found high-dose chemotherapy could also cause platelet activation (Fig 1C). After correlation analysis of the results, surprisingly we observed a positive correlation between the injured liver score and P-selectin in the peripheral blood (Fig 5B), which explained the injury of hepatocyte and liver sinusoidal endothelial cells and platelet activation have positive correlation, and we conclude that P-selectin can be used as an indicator of HVOD, which have some clinical significance.

Currently, pathogenesis of HVOD is still not clear, but research has confirmed that LSEC injury is the first step toward HVOD [4,5] and is also involved in the development of the whole process of HVOD. Some studies also have proved that LSEC injury and platelet activation are closely linked; however, inhibition of platelet activation to reduce the hypercoagulability can reduce the incidence of HVOD, research data indicates that the current large number of anticoagulant and anti-platelet drugs widely used does not significantly reduce the incidence of HVOD. It appears that anticoagulation and anti-platelet drugs application did not break the vicious cycle of HVOD after transplantation, and the effects that only rely on anticoagulation and anti-platelet drugs to control HVOD are limited, so finding better ways to combat HVOD after transplantation is imperative.

Now a growing number of scholars mention the endothelial progenitor cells (EPC), which are considered to be precursor of the EC, usually settled in the bone marrow cavity, and in some cases, such as local vascular injury, ischemia, trauma and certain drugs stimulating and so on, EPC could be mobilized from the bone marrow cavity to peripheral blood to differentiate into EC, directly involve in the occurrence of new blood vessels and also produce angiogenic cytokines to promote vascular remodeling [18–20].

In liver cirrhosis, liver damage, and other models, some scholars gave EPC infusion, which could improve the function of the injured liver [21–23]. Ueno et al [24] further confirmed using a rat model of cirrhosis, after giving EPC transplantation, rat liver hepatocyte growth factor, transforming growth factor-alpha, epidermal growth factor, vascular endothelial growth factor, and other cytokines increased significantly, and proliferation and differentiation of hepatocyte occurred, which suggesting that hepatic fibrosis be inhibited and the injured liver start to regenerate. Whether EPC transplantation can reduce liver injury and promote the regeneration of LSEC, and whether BMT united EPC infusion can early repair the liver damage by the TBI conditioning or BMT to decrease the incidence of HVOD need we next to confirm.

REFERENCES