Leukemia Research 55 (2017) 49–54

Contents lists available at ScienceDirect

Leukemia Research

journal homepage: www.elsevier.com/locate/leukres

Simvastatin ameliorates graft-vs-host disease by regulating angiopoietin-1 and angiopoietin-2 in a murine model

Peng Zheng[a,1], Qiu-ling Wu[a,1], Bei-bei Li[a], Ping Chen[a], Di-min Nie[a], Ran Zhang[a], Jun Fang[a], Ling-hui Xia[a], Mei Hong[a,b,*]

[a] Institute of Hematology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China
[b] Collaborative Center of Hematology, Soochow University, Suzhou, China

ARTICLE INFO

Article history:
Received 30 November 2016
Accepted 9 January 2017
Available online 10 January 2017

Keywords:
Graft-versus-host disease
Angiopoietin
Simvastatin
Endothelial damage

ABSTRACT

Angiopoietins play an important role in vascular endothelial function. Endothelial damage is an important pathogenesis relating with acute graft-versus-host disease (GVHD) after allogeneic hematopoietic stem cell transplantation (allo-HSCT), protecting endothelial cells (ECs) from damage may be a potent prophylaxis and therapeutic strategy of acute GVHD (aGVHD). In this study, we explored changes in Angiopoietin-1 (Ang-1) and Ang-2 expression in a aGVHD mouse model and determined whether simvastatin prevents GVHD through regulating Ang-1 and Ang-2 expression. In vitro simvastatin administration increased Ang-1 production and release but conversely inhibited Ang-2 release from EA.hy926 ECs. Simvastatin improved the survival of aGVHD mice, attenuated the histopathological GVHD grades and plasma levels of Ang-2, and elevated the plasma levels of Ang-1 as well as the aortic endothelial levels of Ang-1 and Ang-2. In summary, simvastatin represents a novel approach to combat GVHD by increasing Ang-1 production while suppressing Ang-2 release to stabilize endothelial cells.

1. Introduction

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is the only potentially curative treatment for malignant hematological diseases. However, the major serious complication associated with HSCT is graft-versus-host disease (GVHD). Acute GVHD (aGVHD) comprises a substantial portion of early transplant-related morbidity and mortality [1]. However, the mechanisms involved in aGVHD are not completely clear.

In recent decades, endothelial cell damage is found to contribute to the initiation and development of several posttransplant complications, including GVHD [2]. During the early phase of aGVHD, endothelial cells (ECs) are destroyed by a preconditioning process or immunosuppressive drugs [3]. In an animal model of aGVHD, ECs were revealed to be destroyed and detached from the lamina elastic interna by electron microscopy. Furthermore, endothelium-dependent vasodilation was found significantly reduced in the mesenteric resistance arteries [4]. Thus, protecting ECs from damage may be a potent prophylaxis and therapeutic strategy of aGVHD.

Ang-1 and Ang-2 belong to the endothelial growth factor family and function as ligands to the shared receptor Tie2, which is mainly expressed on ECs [5–7]. Ang-1 is thought to stabilize the vessel endothelium and prevent permeability [8]. In contrast, Ang-2 destabilize quiescent vascular endothelia by counteracting Ang-1 function [9]. However, the precise regulatory mechanism of angiopoietins in GVHD patients remained elusive.

Conventional aGVHD therapies mainly focus on suppressing alloreactive T cells. Drugs include corticosteroids, calcineurin inhibitors and mammalian target of rapamycin (mTOR) inhibitors. Because of their multiple targets, many patients experienced adverse side effects including high risk of severe opportunistic infections and malignancy relapse after transplantation [10,11]. Consequently, novel therapies with low adverse effects are under investigation. Statin, a widely used lipid-lowering agent, was shown to block 3-hydroxy-methylglutaryl-coenzyme A (HMG-CoA) reductase activity [12]. Recently, statin drugs are thought to positively influence ECs [13,14]. Koichi et al. found that simvastatin improved endothelial-dependent vasodilation[15]. Moreover, some studies found the protective effect of simvastatin on ECs was related with the regulation of Ang-1 and Ang-2. Several studies found simvastatin significantly reduced intravitreal levels of Ang-2

* Corresponding author at: Institute of Hematology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, 1227#Jiefang Road, Wuhan, 430022, China.
E-mail address: meihongcncn@aliyun.com (M. Hong).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.leukres.2017.01.017
0145-2126/

in diabetic patients and rats [16–18]. Meanwhile, the positive regulation of Ang-1 by simvastatin was observed in rats after stroke [19,20]. Therefore, it can be predicted that simvastatin alleviates aGVHD by protecting EC function. However, researches about simvastatin in GVHD were absent so far.

Therefore, in this study, we explored changes in Ang-1 and Ang-2 expression in a well-established aGVHD mouse model and determine whether simvastatin prevents GVHD through regulating Ang-1 and Ang-2 expression.

2. Materials and methods

Ethics statement. All animal procedures performed were approved by the Committee on Animal Handling of Huazhong University of Science and Technology.

2.1. Mice and BMT

Male C57BL/6 (H-2b) and male BALB/c (H-2d) mice were purchased from Hua Fukang Bioscience (Beijing, China). Mice were kept in a pathogen-free environment and sacrificed between 8 and 12 weeks of age. The BMT experiments were performed as previously described [21]. Briefly, BALB/c mice were intravenously injected with 1×10^7 bone marrow cells (BM cells) with or without 2×10^7 spleen cells from C57BL/6 mice as T cell (Tc) source after 8 Gy of lethal irradiation. Simvastatin powder (Selleck, USA) was dissolved in 0.5% methylcellulose. Mice received simvastatin (10 mg/kg) by oral gavage before the irradiation and administered daily for 7 days based on a previous study [22]. The animals in the control group received the same vehicle containing no simvastatin. All experiments were conducted twice with 10–15 mice per group. The mice's survival and weight were monitored daily. The degree of clinical GVHD was evaluated every 4 days by a previously described scoring system that included five clinical parameters [23]. Peripheral blood samples were collected at 0, +4, +8, +12, and +16 days after transplantation. The plasma was then processed by centrifugation at 3000*g* for 10 min after blood collection.

2.2. Histological analysis

Liver and small intestine tissues collected on day 16 were fixed with 4% formalin, embedded in paraffin, sectioned, slide-mounted, and stained with hematoxylin and eosin. Two blinded observers analyzed the histopathological signs of aGVHD under a light microscope (Olympus, Tokyo, Japan). GVHD severity was assessed according to a previously published histopathology scoring system [24].

2.3. Immunohistochemistry

The aortas were dissected on day 16 after transplantation. The paraffin sections were incubated with rabbit anti-Ang-1 and rabbit anti-Ang-2 (Abcam, Cambridge, UK) antibodies. The staining was observed under a light microscope at a magnification of 400 ×, and all fields of the aorta fragments were analyzed using a densitometric method by Image-Pro Plus software 6.0 as described previously [25].

2.4. Cell culture and simvastatin treatment

The HUVEC line EA.hy926 was purchased from the American Type Culture Collection (ATCC, Manassas, VA) and cultured in DMEM supplemented with 10% FBS. The cells were treated with simvastatin (1 μmol/l) for 12 h prior to stimulation with TNF-α (100 ng/ml, PeproTech, USA) for 24 h. Simvastatin was activated and the dose used was based on previous studies [26,27]. Then, the cell supernatant was collected by centrifugation at 1000 *g* for 10 min and then 3000 *g* for 5 min to remove cells and large debris, respectively. The untreated and treated cells were subject to various functional assays.

2.5. Measurement of ang-1 and ang-2 in cell culture supernatants and mouse plasma

Cell culture supernatants and mouse plasma were collected as described above. Measurement of Ang-1 and Ang-2 levels in the supernatant and plasma was performed by commercially available ELISA kits (Boster, Wuhan, China) according to the manufacturer's instructions.

2.6. Quantitative RT-PCR

Total RNA was extracted from EA.hy926 cells using RNAiso Plus (Takara Biotechnology, Dalian, China) according to the manufacturer's protocol. Quantitative PCR was performed with the SYBR Green real-time PCR method. All PCR reactions were performed at least in triplicate on an ABI 7500 FAST System (Applied Biosystems, Foster City, CA). The primer sets were as follows: human Ang-1 (forward: CTTTCCTCGCTGCCATTC and reverse: AGTTGCCATCGTGTTCTG), Ang-2 (forward: AAGCAGCATCAGCCAACCA and reverse: CCACCAGCCTCCTGTTAGC) and GAPDH (forward: CCACCCATGGCAAATTCCATGGCA and reverse: TCTAGACGGCAGGTCAGGTCCACC). The results for each sample were normalized to the respective GAPDH value.

2.7. Western blot analysis

After receiving the treatments, the cells were lysed. Protein (30 ug) was separated on 10% sodium dodecyl sulfate-polyacrylamide gels and transferred to polyvinylidene fluoride membranes. The blots were probed with primary antibodies against Ang-1 (Abcam, 1:10000), Ang-2 (Abcam, 1:1000) and GAPDH (Boster) overnight at 4 °C. Protein expression levels were measured by band densitometry using Image Lab Software (Bio-Rad Laboratories, Hercules, CA).

2.8. Statistical analysis

Data are presented as the mean ± SD. Differences in animal survival (Kaplan-Meier survival curves) were analyzed by log-rank test. For comparisons between experimental groups, a two-tailed Student's *t*-test was used. A probability value less than 0.05 was considered to be statistically significant.

3. Results

3.1. Simvastatin administration reduced acute GVHD

Simvastatin treatment significantly improved survival compared with that of the vehicle-treated group ($p < 0.05$) (Fig. 1a). On day 22 after transplantation, all vehicle-treated mice had died, whereas 50% of all simvastatin-treated mice were still alive. The survival rate of mice that had received BM only was 100% until the end point. All mice receiving bone marrow cells and spleen cells showed gradual weight loss (Fig. 1b). The body weight decrease was lower in the group treated with simvastatin than in the vehicle-treated group. Meanwhile, a lower clinical GVHD score was observed in the simvastatin-treated group. Mice that had received BM only did not show any signs of aGVHD (Fig. 1c).

In the aGVHD group that did not receive simvastatin treatment, liver histopathology showed a significantly increased pooled score