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Ginsenoside Rb1 Attenuates Homocysteine-Augmented Guidewire Injury-Induced Intimal Hyperplasia in Mice

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Abstract

Background.—Intimal hyperplasia (IH) is the primary cause for post-angioplasty restenosis. The purpose of this study is to investigate the effects of homocysteine (Hcy) and ginsenoside Rb1 (Rb1) on IH using a guidewire injury animal model.

Methods.—In 12-wk-old C57BL/6J mice, the left common carotid artery (CCA) was denuded with a guidewire and the right CCA was used as the uninjured control. They were treated with saline (NS), Hcy, Rb1, or Hcy + Rb1 for 4 wk prior to sacrifice. Animals were sacrificed at 4, 6, or 8 wk. Both CCAs were harvested and intimal-medium thickness (IMT) ratios were calculated. Local macrophage distribution was also studied.

Results.—Histology analyses demonstrated consistent internal elastic lamina disruption and focal IH in the injured CCA segments. The degree of IH correlated to the lengths of time following injury. Hcy treated group had significant increase in IMT compared with the NS group ($P < 0.05$), while Rb1 group was similar to the NS group. In addition, Hcy + Rb1 group showed significant improvement in IMT compared with Hcy group ($P < 0.01$). Furthermore, Hcy significantly increased local macrophage content as compared with either lesion alone or Rb1 treated animals.

Conclusions.—Our study showed that Hcy increased the degree of IH and macrophage content in the injured CCA and that Rb1 attenuated these adverse effects. These changes might be mediated through antioxidative effects of Rb1. Our data suggests a potential clinical application of ginseng in controlling Hcy-related vascular injuries.

Keywords

intimal hyperplasia; guidewire injury; homocysteine; ginsenoside Rb1; macrophage

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INTRODUCTION

Neointima formation and remodeling can lead to restenosis, which decreases the long-term clinical success of percutaneous coronary and peripheral interventions [1]. Restenosis may occur in 30% to 50% of percutaneous procedures within the first 6 mo of interventions [2]. Recent application of local therapies with sirolimus-eluting stent showed considerable promise for the prevention of neointimal proliferation, restenosis, and associated clinical events in coronary interventions [3]. However, clinical benefit of drug-eluting stents in peripheral interventions is still lacking, and the molecular mechanism of restenosis is poorly understood.

Many clinical studies have suggested an association between the plasma homocysteine (Hcy) concentration and arteriosclerosis. Researchers have shown that moderate hyperhomocysteinemia (HHcy, between 31 and 100 $\mu\text{mol/L}$) is linked to symptomatic peripheral vascular, cerebrovascular, and coronary heart disease [4]. It has been hypothesized that HHcy is a marker of oxidative and redox stress [5]. Our previous study showed that Hcy significantly impaired endothelium-dependent vasorelaxation and increased reactive oxygen species (ROS) production in porcine coronary arteries [6]. Still, further studies are warranted to better understand the molecular mechanism of Hcy-related or -induced vascular injury and potential treatment strategies.

Ginseng root has been used extensively in Chinese medicine and is becoming increasingly popular in the Western world for its alleged tonic effect and possible curative and restorative properties. There is increased clinical evidence of potential benefits of ginseng roots in cardiovascular diseases. Studies have shown that the ginseng root and ginsenosides, the major constituents of ginseng, have protective effects on atherosclerotic plaque formation due in part to their roles in free radical scavenging [6, 7]. The data from our previous study showed that ginsenoside Rb1 (Rb1) effectively blocks Hcy-induced impairment of endothelium vasomotor function in porcine coronary arteries. In addition, Rb1 successfully improved Hcy-induced reduction of endothelial nitric oxide synthase (eNOS) expression, and reduced Hcy-induced oxidative stress [6]. We propose that Rb1 can attenuate the detrimental effects of Hcy *in vivo* through its anti-oxidative effects.

Macrophages and macrophage-derived foam cells have been shown to significantly contribute to the neointimal lesion, which further lead to the local inflammatory response and neointimal growth [8]. Extensive infiltration of macrophages and T cells will also activate inflammation and promote plaque instability. However, the effect of Hcy or Rb1 on macrophage has not been studied previously. In this study, we investigated the molecular mechanisms of and the functional effects of Rb1 and Hcy on guidewire-induced vascular injury in C57BL/6 mice. We also studied the effects of Hcy and Rb1 on macrophage distribution. The data from our study may suggest a potential clinical application of ginseng in controlling Hcy-related vascular injuries.

MATERIALS AND METHODS

C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) at 12 wk old were allowed standard laboratory food and water *ad libitum*. All procedures were performed under protocols approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine and Stanford University. All protocols follow Federal and Institutional Guidelines for Animal Welfare.

Guidewire Injury

Mice were anesthetized for all surgeries by intraperitoneal injection of ketamine (80 mg/kg) and xylazine (5 mg/kg). Bilateral carotid arteries were exposed through a midline incision on the ventral side of the neck. Left common carotid artery (CCA), external carotid artery (ECA), and internal carotid artery (ICA) were isolated using blunt and sharp dissection techniques. Distal left ECA was then ligated with an 8-0 silk suture. Following control of the left proximal CCA and ICA with microclamps (S and T model B1 and B2; Fine Science Tools, Foster City, CA, respectively), an arteriotomy was made on the ECA proximal to the silk suture. A rigid 0.010 in. guide wire was introduced to the mid CCA. A total of 10 passes were made to ensure adequate denudation on the mid and distal CCA. Subsequently, proximal ECA was ligated with an 8-0 silk suture proximal to the arteriotomy. Last, the microclamps were released to re-establish in-line flow from CCA to ICA. During the procedure, the right carotid artery was isolated but not injured, and the right CCA was used as an uninjured control. After hemostasis was achieved, the skin was closed with a 4-0 nylon suture. Animals were sacrificed at 4, 6, or 8 wk after surgery. Both left and right CCA were harvested and processed for histomorphometry analysis. Once 8-wk post-procedure was determined to be the optimal time for study intimal hyperplasia (IH), the remainder of the study was performed using 8 wk as the end point. Subsequently, the animals were randomly divided into 4 treatment groups following guidewire injury. Groups were vehicle control (saline) alone, homocysteine (Hcy, 14.5 mM) alone, ginsenoside Rb1 (Rb1, 0.35 mM) alone, or combined Hcy (14.5 mM) and Rb1 (0.35 mM) (Hcy + Rb1). Each group consisted of at least 8 mice. Animals were sacrificed at 8 wk after surgery and both left and right CCA were harvested and processed, followed by analysis. A 28-d mini-osmotic pump (ALZET, model 2004; Cupertino, CA) was implanted in the peritoneum cavity for continuous treatment administration. All chemicals were purchased from Sigma (St. Louis, MO) unless otherwise specified.

Histomorphometry

At the designated harvesting time, animals were anesthetized and perfused with 0.9% NaCl by placement of a 22-gauge butterfly angiocatheter in the left ventricle. The mice were subsequently perfusion-fixed *in situ* by infusion with 10% buffered formalin (pH 7.0) for 20 min at a constant pressure of 100 mmHg. The CCA on both sides were dissected from surrounding tissue, harvested, and fixed in 10% buffered formalin for an additional 48 h before going through paraffin embedding.

Serial tissue sections (5 μ m) were obtained from both the left and right common carotid arteries, starting at the bifurcation. Three sections (50 μ m apart) at the site of maximal

luminal reduction were stained with hematoxylin and eosin. Areas within lumen, internal and external elastic laminae, were measured by planimetry of digitized images obtained by a bright-field microscope (Infinity 1; Meiji, Santa Clara, CA) with the use of ImageJ software (NIH, Bethesda, MD). To calculate initial-medium thickness (IMT) for each vessel cross-section, the linear distance between internal elastic lamina and external elastic lamina was measured independently in 4 places, each at 90° apart and averaged. The average of the 4 measurements at 4 quadrants was recorded and the ratio of intima-to-media was normalized using the contralateral unlesioned artery (normalized IMT).

Quantitative Immunohistochemistry of Macrophages

Sections (3 to 4 per mouse, 50 μ m apart) from neointimal lesions of mice ($n = 5$ per group) were immunostained for macrophages by using antibody against Mac-2 or isotype controls. Endogenous peroxidase activity was blocked by incubating the tissue sections in 0.3% H₂O₂. Specific binding was detected with a biotinylated-secondary antibody, avidin-biotin peroxidase complex, and 3,3'-diaminobenzidine substrate (Vector Labs, Burlingame, CA). Digital images were analyzed for total neointimal area. The percentage of lesion area occupied by staining of Mac-2 was quantitated through the use of ImageJ. Data for each group were expressed as percentage of the immunostained area per total neointimal area.

Statistical Analysis

All values were expressed as mean $\pm$ SEM. When only 2 groups (injured arteries and contralateral control arteries) were compared, differences were assessed by a paired Student's *t*-test. Multiple comparisons were first tested by analysis of variance. When the analysis of variance demonstrated significant differences, individual mean differences were analyzed by the Student-Newman-Keuls test. Statistical software MiniTab (State College, PA) was used in statistical analysis. For all statistical analyses, $P < 0.05$ was considered significant.

RESULTS

Guidewire Injury Induced IH

After 4, 6, or 8 wk survival period, guidewire-injured animals were sacrificed and paraffin-embedded sections of right (uninjured) and left (wire-injured) carotid arteries were processed for neointimal lesion analysis. In the 4 and 6 wk survival group, we noticed an increase in IMT when normalized with the right CCA ($110.3 \pm 4.6\%$, $127.5 \pm 12.8\%$, respectively). However, neither group reached statistical significance ($P > 0.05$). On the other hand, there was a significant increase in the normalized IMT ratio ($153.5 \pm 11.6\%$, $P < 0.01$) in the 8 wk survival group compared with the non-injured side (Fig. 1). Thus, we decided to use 8 wk as the primary end point for the following experiments.

Effects of Hcy and Rb1 on IH

After establishing the guidewire injury model in C57BL/6J mice, we further investigate the effects of Hcy and Rb1 on the IH. An osmotic pump filled with Hcy (14.5 mM) or Rb1 (0.35 mM) or both was implanted intraperitoneally after the carotid injury and continuously released chemicals for 28 d. The doses of Hcy or Rb1 were determined based on our

previous studies [6, 9, 10]. After an 8-wk survival period, there was a significant increase in IMT ratio in the Hcy group after being normalized with the uninjured side ($232.0\% \pm 24.7\%$) compared with the control (NS) group ($153.5\% \pm 11.6\%$, $P < 0.01$). Interestingly, Hcy + Rb1 group had significantly less normalized IMT ratio ($108.7\% \pm 3.9\%$) than both the control and Hcy group ($P < 0.01$). Furthermore, Rb1 group had the lowest IMT ratio ($98.2\% \pm 7.9\%$) among all 4 groups (Fig. 2A and B).

Effects of Hcy and Rb1 on Local Distribution of Macrophage

We investigated macrophage changes in the lesion area using Mac-2 antibody 8 wk after injury. The percentage of lesion area occupied by staining of Mac-2 was quantified and presented as macrophage content. In lesion only groups, the macrophage content was $10.5\% \pm 1.2\%$. When animal received Hcy treatment, the macrophage content increased to $17.8\% \pm 1.7\%$. Rb1 co-treatment with Hcy (Hcy + Rb1) showed $12.8\% \pm 1.5\%$, which was lower than Hcy-treatment group but not statistically significant ($P = 0.09$). Rb1 treated animals showed a significant decrease in macrophage content compared with the control ($P < 0.05$) and Hcy groups ($P < 0.01$) (Fig. 3A). The staining of Mac-2 can be seen in both intima and media (Fig. 3B).

DISCUSSION

Post-angioplasty restenosis poses a significant challenge to the long-term efficacy of percutaneous surgical interventions. In this study, we demonstrated that guidewire-induced carotid artery injury was a reproducible *in vivo* model for IH. While injury led to IH and local macrophage accumulation in C57BL/6J mice, Hcy significantly augmented these detrimental effects and Rb1 effectively abolished both the injury alone and Hcy-augmented changes. This study underscores the potential role of ginseng and its constituents, Rb1, in treating IH.

Restenosis as the consequence of neointimal formation and remodeling impairs the long-term clinical success of peripheral arterial interventions. In recent years, multiple murine models of vascular restenosis have been proposed to investigate this challenging dilemma in cardiovascular interventions [11, 12–14]. Among them, guidewire injury model closely mimics clinical scenarios by maintaining in-line flow following guide wire injury. Therefore, it was adapted and modified for our study. Additionally, genetically modified mice, including apolipoprotein E deficient mice (ApoE^{−/−}) allowed us to create an intimal hyperplastic lesion that resembles human lesion. The specific strain of C57BL/6J mice was chosen due in part to its close genetic relationship to ApoE^{−/−}, which will be utilized for our future investigations. To establish a consistent and reliable *in vivo* model using a guidewire injury technique, we utilized C57BL/6J and harvested CCA at different time points. We demonstrated that IH started as early as 4 wk after wire injury. However, only at 8 wk a statistically significant difference was shown. This finding is consistent with other studies [15]. Furthermore, we confirmed that our modified guidewire injury technique of smaller rigid wire (0.010 in.) with multiple passes (10 passes) could create a reliable and reproducible restenotic lesion in mice.

Ginsenoside Rb1 has been shown to reduce superoxide anion production after HIV protease inhibitor Ritonavir treatment in porcine coronary artery rings [9]. It is also reported to activate the eNOS system and increase nitric oxide production [6], reduce the local concentration of thromboxane A2 [16], as well as activate tetraethylammonium-sensitive K⁺ channels [17]. Previously, we have reported that Hcy can directly induce endothelial damage in porcine coronary arteries through impairment of eNOS system and enhancing oxidative stress, while Rb1 effectively blocking Hcy-induced changes [6]. However, the effect of Rb1 on IH is unknown, especially related to vascular injuries. In this murine model, we demonstrated that continuous infusion of Hcy significantly augmented guidewire injury-induced IH. Rb1 treatment reversed intimal changes caused by guidewire-induced and Hcy-augmented injuries. This study confirmed the benefit effect of ginsenoside Rb1 on IH in C57BL/6J mice. The study also highlights the potential benefits of Rb1 in patients with hyperhomocysteinemia. Further investigation on ApoE^{-/-} mice will provide additionally valuable information. Based on the previous data, maintaining/restoring eNOS functions and reducing oxidative stress could be the underlying mechanisms for Rb1. Further investigations on eNOS or ROS system in this animal model as well as cell culture studies are warranted.

Macrophages are abundantly presented in spontaneous atherosclerotic plaques and neointimal lesions after vascular interventions. Exposure of cultured endothelial cells to Hcy has shown to increase ROS levels and amplify expression of chemokines and adhesion molecules [5]. These changes may facilitate the local accumulation of monocytes/macrophages. Additionally, endothelial cells exposed to oxidative stress is shown to produce macrophage colony-stimulating factor (M-CSF), which promotes transformation of monocytes to lipid-laden form cell [5]. In our study, a 69% increase of Mac-2 staining was observed in vessels treated with Hcy, 8 wk after guidewire injury. Rb1 treatment significantly decreased macrophage content in both lesion only and Hcy treatment groups. These data suggest that macrophages may be involved in IH induced by guidewire injury, and that Rb1 may potentially be used to minimize these detrimental changes through its anti-oxidative effects. Further investigations are warranted to explore the possibility sources of macrophages (local or blood-borne) as well as their detailed role on vascular intervention-related IH and restenosis.

In summary, this study showed that guidewire injury is a reliable murine model to study injury-induced IH. Hcy increases the local macrophage accumulation and IMT ratio in a guidewire injured vessel, while Rb1 effectively blocked these changes. Admittedly, all these observations were made on healthy carotid arteries in C57BL/6J mice. Nonetheless, this study validates our modified guidewire injury technique in a murine model and establishes the foundation for further investigation in more atherosclerosis prone ApoE^{-/-} mice, a daughter strain of C57BL/6J. Importantly, this study underscores the potential therapeutic benefits of ginseng and Rb1 in preventing IH follow percutaneous peripheral vascular interventions.

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FIG. 1.

Effects of guidewire injury on IH in C57BL/6J mice. Animals that received wire injury survived for 4, 6, or 8 wk. Serial tissue sections ($5\ \mu\text{m}$) were obtained from both left and right common carotid arteries. To calculate the neointimal and medial thickness for each vessel cross-section, the linear distance between internal elastic lamina and external elastic lamina was measured and ratio of intima-to-media was normalized using the contralateral unlesioned artery. In the 4 and 6 wk survival group, the normalized intima/medium thickness ratios (IMT) were not statistically significant ($P > 0.05$). However, in the 8 wk survival group, there was a significant increase of the IMT ratio ($P < 0.01$) compared with the non-lesioned arteries.

FIG. 2.

Effects of Rb1 and Hcy on guidewire induced IH in C57BL/6J mice. Animals that received wire injury were treated for 4 wk with normal saline, Hcy, Rb1, or both (Hcy + Rb1). The total survival period was 8 wk. (A) In animals treated with Hcy, there was a significant increase of the IMT ratio ($132.0\% \pm 24.7\%$) compared with lesion-only animals ($P < 0.01$). When injured mice were treated with both Hcy and Rb1, there was no increase in IMT ratio compared with non-lesioned animals ($P > 0.05$). Rb1 treatment alone showed no difference in IMT compared with non-lesioned animals. (B) Representative hematoxylin and eosin staining of changes of intimal thickness in animals receiving guidewire injury only (lesion) and/or Hcy/Rb1 treatment. Uninjured right common carotid artery was shown as control (normal). Bar = 10 μ M. (Color version of figure is available online.)

FIG. 3.

Effects of Rb1 and Hcy on guidewire induced local macrophage accumulation in C57BL/6J mice. Animals that received wire injury were treated for 4 wk with normal saline, Hcy, Rb1, or both (Hcy + Rb1). The total survival period was 8 wk. (A) The percentage of lesion area occupied by staining of Mac-2 was quantified and presented as macrophage content. In animals that received Hcy treatment, the macrophage content increased significantly compared with lesion only group ($P < 0.05$). Rb1 co-treatment with Hcy showed a lower macrophage content than Hcy-treatment group but not statistically significant ($P = 0.09$).

However, Rb1 alone showed a significant decrease of macrophage content compared with lesion alone ($P < 0.05$) and lesion plus Hcy groups ($P < 0.01$). (B) Representative Mac-2 immuno-histochemistry staining in animals receiving guidewire injury and or Hcy/Rb1 treatment. The staining of Mac-2 can be seen in both intima and media. Bar = 10 μ M. (Color version of figure is available online.)