Thrombosis Research 136 (2015) 1245–1251

Contents lists available at ScienceDirect

Thrombosis Research

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

Full Length Article

Prevention of occlusive arterial thrombus formation by a single loading dose of prasugrel suppresses neointimal hyperplasia in mice

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ARTICLE INFO

Article history:
Received 25 June 2015
Received in revised form 5 October 2015
Accepted 5 October 2015
Available online 9 October 2015

Keywords:
Thrombosis
Platelet aggregation
Neointimal hyperplasia
Prasugrel
Thienopyridine
$P2Y_{12}$ receptor

ABSTRACT

The present study examined the effects of prasugrel in a mouse model of thrombosis-induced neointimal hyperplasia. Following carotid artery injury by application of ferric chloride solution, thrombus formation was assessed on Day 1 and neointimal thickening was assessed on Day 21. Single administrations of prasugrel at 0.3–3 mg/kg (p.o.) resulted in a dose-related and sustained inhibition of ADP-induced platelet aggregation through 24 h. Single and multiple (1 and 3 weeks) administration of prasugrel (3 mg/kg loading and 1 mg/kg/day maintenance doses) resulted in a marked inhibition of neointimal thickening in the injured artery. In the dose–response study, a single administration of prasugrel at 0.3–3 mg/kg (p.o.) dose-relatedly inhibited thrombus formation and neointimal thickening on Days 1 and 21, respectively. The degree of neointimal hyperplasia in the injured artery correlated significantly with the thrombus indices, time to occlusion and patency rate. To explore possible mechanisms of inhibition of neointimal hyperplasia by prasugrel, mRNA expression levels of inflammatory and fibrosis markers were determined in injured arteries. Prasugrel treatment resulted in reduced MCP-1, ICAM-1 and TGF-β mRNA levels on Day 2 (24 h after the injury) and Day 8 (1 week after the injury) in the target arteries. In conclusion, we found that a single oral loading dose of prasugrel markedly prevented neointimal hyperplasia by inhibiting platelet activation and thrombus formation and was associated with inhibition of the expression of inflammatory and fibrosis markers, including MCP-1, ICAM-1 and TGF-β, in the injured arteries.

1. Introduction

Percutaneous coronary intervention (PCI) is widely used to treat patients with obstructive atherothrombosis. However, neointimal proliferation after stent implantation remains a concern [1]. A major limitation of PCI is its association with a high risk of lumenal restenosis during the first 6 months after intervention [2,3]. Restenosis is caused by intervention-associated vascular injury and is mediated by arterial remodelling and neointimal hyperplasia comprising primarily of smooth muscle cells (SMCs) and inflammatory leukocytes [4,5]. Notably, de-endothelialization promotes platelet adhesion, activation, release of growth factors and infiltration of inflammatory cells, which initiate/promote vascular SMC migration and proliferation by releasing proinflammatory mediators, such as monocyte chemotactic protein-1 (MCP-1), interleukin 6 and prostaglandins [6,7].

Histopathological studies in humans have demonstrated that after coronary stent placement, thrombus formation and acute inflammation are followed by vascular SMC proliferation, resulting in neointimal thickening and restenosis [8,9]. Thus, platelets may play a key role in not only early thrombus generation but also in later neointimal formation secondary to arterial injury. Arterial insult occurs at the time of PCI or as a consequence of vessel wall inflammation secondary to atherosclerosis. Accordingly, various antiplatelet agents, including aspirin and clopidogrel, have been shown to inhibit neointimal formation in various experimental models [10–13]. However, despite promising experimental observations, clinical investigations aimed at demonstrating a significant diminution of restenosis using antiplatelet agents (e.g. aspirin or clopidogrel) have failed or provided confusing results [1]. However, more effective antiplatelet agents such as ticagrelor and prasugrel have not been widely studied in these scenarios.

In a mouse model of thrombosis-induced hyperplasia, Patil et al. [14] provided evidence that inhibition of the $P2Y_{12}$ ADP receptor by ticagrelor [15–17] reduced the pathological vessel wall response to injury. However, it remains unclear whether this effect was solely driven by $P2Y_{12}$ inhibition or secondary to adenosine accumulation, resulting from ticagrelor mediated inhibition of adenosine transporters that scavenge adenosine [1,18,19]. Prasugrel is the third generation thienopyridine antiplatelet prodrug which selectively inhibits $P2Y_{12}$ [20,21], and preliminary data have found no adenosine-related effects of prasugrel (data not shown). Prasugrel provides a more consistent

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http://dx.doi.org/10.1016/j.thromres.2015.10.010
0049-3848/

and greater antiplatelet effect than clopidogrel [22]. However, to our knowledge, the effect of prasugrel on neointimal hyperplasia has not yet been reported.

In the present study, the effects of prasugrel were assessed in a mouse model of ferric chloride-induced hyperplasia. In addition, the effects of prasugrel on inflammatory and fibrotic mRNA expression in the injured artery were evaluated to clarify possible mechanisms involved in the attenuation of intimal hyperplasia secondary to $P2Y_{12}$ inhibition.

2. Materials and methods

2.1. Materials

Adenosine-5′-diphosphate (ADP) (MCM ADP) was purchased from LMS Co., Ltd. (Tokyo, Japan). Prasugrel hydrochloride (prasugrel) was provided by Ube Industries Ltd. (Ube, Yamaguchi, Japan). Ferric chloride ($FeCl_3$) was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Gum arabic and trisodium citrate were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). RNAlater was purchased from Qiagen (Venlo, Netherlands).

2.2. Animals

C57BL/6J mice were purchased from Charles River Laboratories Japan, Inc. (Yokohama, Kanagawa, Japan). Upon arrival, the health status of each mouse was assessed and healthy mice were placed in housing with once daily examination during the quarantine/acclimation period. All animal studies were approved by the Institutional Animal Care and Use Committee (Nissei Bilis Co., Ltd. and Daiichi Sankyo Co., Ltd.).

2.3. Drug administration

Prasugrel was suspended in 5% gum arabic solution. The doses and dosing schedules for each agent are shown in each Figure and Table. The suspension of prasugrel or vehicle (for sham, vehicle and control groups) was orally administered once or once a day for 7 or 21 days in a volume of 10 mL/kg. Prasugrel doses used for the platelet function studies were based on the results of previous platelet aggregation and thrombosis studies [23,24].

2.4. Platelet aggregation

Platelet aggregation was measured 1, 4, 12 and 24 h after the first administration of study drug. The mice were anaesthetized before blood collection with isoflurane inhalation anaesthesia (induction: 3%–5%, maintenance: 1%–3%). Citrated blood (0.8 mL) was collected from the abdominal vein and centrifuged to obtain platelet-rich plasma (PRP) (80 × g, 15 min, at room temperature) and platelet-poor plasma (PPP) (2000 × g, 10 min, at room temperature). The platelet count was determined and PRP was diluted with PPP to a count of 3×10^5/μL. Following this adjustment, 120 μL of PRP was stirred for 1.5 min at 37 °C and ADP (final concentration: 20 μM) was added to induce platelet aggregation. The concentration for ADP was selected based on preliminary in vitro experiments. Platelet aggregation was measured using a 12-channel automated platelet aggregometer (PRP313M, IMI Co., Ltd) for 10 min with maximum platelet aggregation being automatically recorded.

2.5. Ferric chloride-induced thrombosis

Mice were anaesthetized with isoflurane and the right carotid artery exposed. A soft-cuff blood flow probe (DBF05S, Crystal Biotech America) was placed on the right carotid artery and blood flow was continuously monitored with a pulsed Doppler flowmeter (CBI-8000, Crystal Biotech America). Subsequently, filter paper (Qualitative filter paper No. 2; Advantec Toyo Kaisha, Ltd.) trimmed to 1 × 2 mm and saturated with 10% $FeCl_3$ solution was placed on the arterial surface for 3 min. In the sham group, carotid arteries were treated with distilled water in place of $FeCl_3$. The filter paper was removed and the time to occlusion (TTO; sec), defined as the time to arrest of blood flow for more than 1 min, was measured. Blood flow was monitored for 60 min after placement of the filter paper. If no arrest of blood flow for greater than 1 min was observed during the 60 min, TTO was defined as 60 min and monitoring was terminated. The patency rate (PR) was also calculated for an individual mouse as PR = ((60 − total occlusion time (min)) / 60) × 100. After blood flow monitoring, the incision was sutured and normal animal care was continued.

2.6. Neointimal hyperplasia measurement

On Day 21, mice were anaesthetized with pentobarbital sodium (50 mg/kg, i.p.) and exsanguinated with perfusion of saline via the left ventricle and an incision in the abdominal artery. Perfusion fixation was performed with a neutrally buffered 10% formalin solution for approximately 5–10 min, and injured carotid arteries were harvested and immersed in neutrally buffered 10% formalin. Pathology specimens were isolated from 3 areas of the carotid arteries: the approximate midsection of $FeCl_3$-injured segment, 100 μm distal from the midsection and 200 μm distal from the midsection. Two paraffin sections (total of 6 sections) were prepared, and 2 staining procedures were performed (HE: haematoxylin–eosin staining, EVG: Elastica van Gieson staining) for each segment. For EVG-stained specimens, the lumenal area (A), the area surrounded by internal elastic lamina (B) and the area surrounded by external elastic lamina (C) were determined using image analysis software (WinROOF 2013, MITANI CORPORATION). Using these values, the intimal area (B-A), medial area (C-B) and the intima/media (I/M) ratio were calculated, and values from the 3 segments were averaged.

2.7. mRNA measurement

Injured and control arteries on Days 2 and 8 were isolated and then immediately submerged in RNAlater. These time points were chosen since in pilot studies intima thickening was not observed until at least Day 8. All samples were incubated overnight at 4 °C to stabilize the RNA and the samples were subsequently stored frozen (−20 °C or below). Total RNA was isolated using an RNeasy micro kit (Qiagen), according to the manufacturer's protocol. Reverse transcription reactions were conducted using High Capacity RNA-to-cDNA kits (Life Technologies Corp.), according to the manufacturer's protocol. Real-time PCR assays were conducted using ViiA™ 7 Real-Time PCR System and TaqMan® Gene Expression Master Mix (both Life Technologies Corp.). Commercially available primers and probes contained in TaqMan® gene expression assays (Life Technologies Corp.) were used. Target gene mRNA expression was normalized to that of the cyclophilin B internal control for each animal. The markers investigated are listed in Tables 2 and 3.

2.8. Statistical analysis

Results for platelet aggregation, TTO, PR, the intimal area, medial area, I/M ratio and relative mRNA expression levels are given as mean ± or + standard errors. Dunnett's multiple comparison test was conducted for platelet aggregation at each time point using the vehicle group as control. For the intimal area, medial area and I/M ratio in the duration of action study, Student's t-tests were performed, using the data of the vehicle group as control. In the prasugrel dose–response study, Dunnett's multiple comparison tests were performed for the intimal area, medial area, I/M ratio, PR and TTO, using the data of the vehicle group as control. Correlations between TTO or PR and I/M ratios were assessed using Spearman's rank order. Differences in mRNA expression between the control, sham and prasugrel groups were assessed using Student's t-test. In all analyses, differences were considered significant