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Bone marrow-derived mononuclear cell seeded bioresorbable vascular graft improves acute graft patency by inhibiting thrombus formation via platelet adhesion☆

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ABSTRACT

Background: Acute thrombosis is a crucial cause of bioresorbable vascular graft (BVG) failure. Bone marrow-derived mononuclear cell (BM-MNC)-seeded BVGs demonstrated high graft patency, however, the effect of seeded BM-MNCs against thrombosis remains to be elucidated. Thus, we evaluated an antithrombotic effect of BM-MNC-seeding and utilized platelet-depletion mouse models to evaluate the contribution of platelets to acute thrombosis of BVGs.

Methods and results: BVGs were composed of poly(glycolic acid) mesh sealed with poly(L-lactideco-ε-caprolactone). BM-MNC-seeded BVGs and unseeded BVGs were implanted to wild type C57BL/6 mice ($n = 10$/group) as inferior vena cava interposition conduits. To evaluate platelet effect on acute thrombosis, c-Mpl$^{-/-}$ mice and Pf4-Cre^{+}; iDTR mice with decreased platelet number were also implanted with unseeded BVGs ($n = 10$/group). BVG patency was evaluated at 2, 4, and 8 weeks by ultrasound. BM-MNC-seeded BVGs demonstrated a significantly higher patency rate than unseeded BVGs during the acute phase (2-week, 90% vs 30%, $p = .020$), and patency rates of these grafts were sustained until week 8. Similar to BM-MNC-seeded BVGs, C-Mpl$^{-/-}$ and Pf4-Cre^{+}; iDTR mice also showed favorable graft patency (2-week, 90% and 80%, respectively) during the acute phase. However, the patency rate of Pf4-Cre^{+}; iDTR mice decreased gradually after DTR treatment as platelet number recovered to baseline. An in vitro study revealed BM-MNC-seeding significantly inhibited platelet adhesion to BVGs compared to unseeded BVGs, (1.75 ± 0.45 vs $8.69 \pm 0.68 \times 10^3$ platelets/mm^2, $p < .001$).

Conclusions: BM-MNC-seeding and the reduction in platelet number prevented BVG thrombosis and improved BVG patency, and those results might be caused by inhibiting platelet adhesion to the BVG.

1. Introduction

Vascular bypass and replacement surgeries are common and successful treatments for patients with cardiovascular disease. However, current synthetic grafts have limitations due to risk of thrombosis, stenosis, calcification, and the lack of growth capacity. Bioresorbable vascular grafts (BVGs) have emerged to address these limitations. In 2001, a first-in-human implantation of the pulmonary artery BVG seeded with endothelial cells cultured from the patient was reported [1]. Thereafter, a clinical trial implanting the BVGs seeded with bone marrow-derived mononuclear cells (BM-MNCs) into the venous circulation for extra total cavo-pulmonary connections has been performed in Japan and U.S., and favorable long-term results have been reported [2]. Although BM-MNC-seeding was applied to BVGs to promote vascular remodeling initially, it was also revealed that seeding BM-MNCs on BVGs improved the patency of the BVGs at both acute and chronic phases in mouse models [3–5]. However, the crucial mechanism for how BM-MNC-seeding prevents stenosis/occlusion of BVGs was still unclear.

☆ This author takes responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation.

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We have previously shown that BVGs transform into functional mature blood vessels via an inflammation-mediated process of vascular remodeling, and BVG stenosis is driven excessive inflammation by infiltration of host monocytes and macrophage into BVG [4,5]. Although BM-MNC-seeding was shown to prevent chronic BVG stenosis by attenuating this inflammatory process [6], the role of BM-MNC-seeding in acute BVG stenosis, which is thought to be caused by acute thrombosis, has not been established. The purpose of the present study was to evaluate the antithrombotic effect of BM-MNC seeded BVGs in a mouse model and in vitro. Furthermore, platelet depletion models were utilized to evaluate the effect of platelets in BVG thrombosis on the basis of our previous report that platelets play a crucial role in BVG stenosis/occlusion during the early phase [6].

2. Materials and methods

2.1. Bioresorbable vascular grafts

All BVGs were provided by Gunze Ltd. (Kyoto, Japan). The BVGs were composed of nonwoven poly(glycolic acid) fiber mesh coated with a 50:50 copolymer sealant solution of poly(L-lactide-co-ε-caprolactone) (PLCL) as previously described [7]. Each graft was 3.0 mm in length with an inner diameter of 0.85 mm.

To seed BM-MNCs onto BVGs, syngeneic C57BL/6 mice were euthanized and bone marrow was collected from femurs and tibias with 5.0 ml of RPMI 1640 (Thermo Fisher scientific, Waltham, MA) and 1% penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO). After filtration through a 70 μm cell strainer, the bone marrow was layered on Histopaque solution (1083, Sigma-Aldrich) in a 1:1 ratio (*v*/v). BM-MNCs were isolated using a density-gradient centrifugation method. A total of 1.0×10^6 cells suspended in 5 μl RPMI 1640 were seeded onto the scaffold for 10 min and then incubated overnight at 37 °C 5% CO_2 [8]. Cell concentrations were measured with Trypan blue exclusion using a Countess automated cell counter (Invitrogen, Carlsbad, CA).

2.2. Animal preparation

All animals received humane care in compliance with the National Institutes of Health Guideline for the Care and Use of Laboratory Animals. The Institutional Animal Care and Use Committee at Nationwide Children's Hospital approved the use of animal and all procedures described in this study. C57BL/6, C57BL/6-Tg(Pf4-cre)Q3Rsko/J (*Pf4-cre*), and C57BL/6-Gt(ROSA)26Sor$^{<tm1(HBEGF)Awai>}$/J (*iDTR*) mice were obtained from The Jackson Laboratories (Bar Harbor, ME). Homozygous c-Mpl–deficient (*c-mpl*$^{-/-}$) mice on a C57BL/6 background were obtained from Genentech (South San Francisco, CA). *Pf4-cre* mice were mated with the *iDTR* line to generate *Pf4-cre*; *iDTR* mice in our facilities. To induce megakaryocyte ablation in the *Pf4-cre*; *iDTR* model, diphtheria toxin (DT) (Sigma-Aldrich) was injected intraperitoneally every other day at a dose of 250 ng for two weeks after graft implantation.

2.3. Platelet number

To survey platelet number in C57BL/6, *c-Mpl*$^{-/-}$, and *Pf4-Cre*$^{+}$; *iDTR* mice, platelet numbers were counted using an ABX Micros 60 hematology analyzer (Horiba, Edison, NJ). Blood was collected from the retro orbital sinus of mice anesthetized with 1.5% *v*/v inhaled isoflurane by a capillary tube.

2.4. In vitro assessment of platelet attachment on BVGs

To measure platelet attachment on grafts, an LDH assay was performed as described before [9]. 0.6–1.2 ml of whole blood was obtained from a C57BL/6 mouse by intracardiac puncture with a 25 gauge needled affixed to 3 ml syringe preloaded with 100 μl of citrate-dextrose solution (C3821, Sigma-Aldrich). Platelet rich plasma (PRP) was prepared by centrifugation (120 g for 8 min ×2), and platelet number was counted by an ABX Micros 60 hematology analyzer. PRP was diluted with PBS to obtain a concentration of 0.2×10^6 cells/μl. BM-MNC-seeded BVGs and unseeded BVGs were incubated in PRP for 60 min at 37 °C, 5% CO_2 (n = 5, respectively). In addition, other BM-MNC-seeded BVGs were incubated into PBS for 60 min at 37 °C, 5% CO_2 (n = 5) as controls. Non-adherent platelets were removed by washing scaffolds with 1.0 ml of PBS. To lyse attached platelets, BVGs were soaked in 2% Triton X-100 (93,427, Sigma-Aldrich) for 60 min at room temperature, then an LDH assay was performed according to the manufacturer's instructions (MK401, Takara Bio, Kusatsu, Japan). Absorbance was measured at 490 nm using a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA), and a standard curve was applied to obtain the total number of platelets attached to each graft. To exclude the effect of BM-MNC number into BVGs, platelet numbers attached on BM-MNC-seeded BVGs were measured as the difference of absorbance between BM-MNC-seeded BVGs incubated into PRP and BM-MNC-seeded BVGs incubated into PBS, and are compared with those attached on unseeded BVGs.

2.5. Graft implantation

BM-MNC-seeded BVGs and unseeded BVGs were implanted into wild type C57BL/6 mice as inferior vena cava interposition conduits (n = 10, respectively). In addition, c-Mpl$^{-/-}$ and Pf4-Cre^{+}; iDTR mice were also implanted with unseeded BVGs using standard microsurgical technique by two micro surgeons (T.Y. and Y.L.) (n = 10, respectively) [8,10]. In brief, mice were anesthetized with intraperitoneal injections of ketamine (100 mg/kg), xylazine (10 mg/kg), and ketoprofen (5 mg/kg), a midline laparotomy incision was made, and the abdominal aorta was exposed. After transection of the inferior vena cava, a 3-mm graft was introduced with end-to-end anastomosis performed at both the proximal and distal ends using 10–0 monofilament nylon sutures in simple interrupted stitches. During implantation, grafts were flushed with a heparin solution (1000 U/ml, Fresenius Kabi USA, Lake Zurich, IL) to inhibit graft thrombosis. Following anastomosis, the surgical clamps were removed, and the abdomen was closed with a running 5-0 Prolene suture. Postoperatively, mice did not receive anticoagulation or antiplatelet therapy.

2.6. Ultrasound

Serial ultrasonography (Vevo Visualsonics 770; Visualsonics, Toronto, Ontario, Canada) was used to serially monitor grafts after implantation at 2, 4, and 8 weeks to determine patency. Before ultrasonography, mice were anesthetized with 1.5% *v*/v inhaled isoflurane. In this study, BVGs was defined as patent when venous flow was detected within the BVG, which was assessed by both color Doppler and pulse wave Doppler.

2.7. Histology and immunohistochemistry

All mice were sacrificed at 8 weeks. Explanted grafts were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned 5 μm thick. Sections were stained histologically with Carstair's Method (Rowley Biochemical Inc. Danvers, MA) to distinguish fibrin and platelets, von Willebrand factor antibody (Dako A0082, 1:1000, Agilent, Santa Clara, CA) for platelets, and Masson's Trichrome for collagen fibers. For immunofluorescence staining, endothelial cells were stained using rabbit anti-CD31 primary antibody (1:50, ab28364, Abcam, Cambridge, UK) and smooth muscle cells were stained using mouse anti-α-SMA primary antibody (1:500, Agilent). Secondary fluorochrome conjugated antibodies were Alexa Flour 488 anti-rabbit immunoglobulin G secondary antibody (1:300, Invitrogen, Carlsbad, CA) and Alexa Fluor 647 anti-mouse immunoglobulin G secondary antibody (1:300, Invitrogen), respectively. Images were obtained under a fluorescence microscope (Zeiss AXIO Observer Z1, Oberkochen, Germany).

2.8. Statistical analysis

Numeric values are listed as mean ± standard deviation. The number of experiments is shown in each case. For comparisons among multiple groups, data of continuous variables with normal distribution were evaluated by one-way ANOVA followed by Tukey–Kramer. A nonparametric Kruskal-Wallis test was used in instances when data had a non-normal distribution. A post hoc Mann–Whitney test was performed to detect significant differences between groups with Bonferroni-Holm correction for multiple comparisons, when the Kruskal-Wallis test was significant. Fisher's exact probability test was used for dichotomous variables of multiple groups. P values of <0.05 indicate statistical significance. All statistical analyses were performed using GraphPad Prism 7 (GraphPad Software, La Jolla, CA).

3. Results

3.1. Platelet numbers in platelet depletion models

To assess the effect of platelet number on graft patency, two mouse models were prepared (c-Mpl$^{-/-}$ and Pf4-Cre^{+}; iDTR). C-Mpl is the receptor for thrombopoietin, which is the main megakaryocyte (MK) growth factor. In c-Mpl knockout mice (*c-Mpl*$^{-/-}$ mice), it has been well described that platelet numbers are reduced >85% relative to littermate controls [11]. In this study, platelet numbers were compared between c-Mpl$^{-/-}$ mice and C57BL/6 mice before graft implantation (day 0) and at explantation (day 56). Platelet number in c-Mpl$^{-/-}$ mice was significantly lower than in C57BL/6 mice at both time points (day 0; 147.4 ± 35.8 vs $503.8 \pm 111.0 \times 10^3/mm^3$, $p = .012$, and day 56; 139.2 ± 15.0 vs 497.2 ± 21.3, $p < .001$) (Fig. 1A), indicating chronic depletion of platelets in this model. In contrast, in Pf4-Cre^{+}; iDTR mice, platelets and MKs can be conditionally depleted by a Cre-recombinase-medicated cell ablation system. Upon diphtheria toxin (DT) exposure, platelet and MKs that are platelet factor 4 (Pf4)-positive cell populations, are susceptible to DT-induced apoptosis, leading to their systemic depletion. We administered DT intraperitoneally to Pf4-Cre^{+}; iDTR mice every other day for 2 weeks, and we observed the change of