Bone Marrow Rejuvenation Accelerates Re-Endothelialization and Attenuates Intimal Hyperplasia After Vascular Injury in Aging Mice

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Background: Aging-associated functional impairment of endothelial progenitor cells (EPCs) contributes to delayed re-endothelialization after vascular injury and exaggerated intimal hyperplasia (IH). This study tested if bone marrow (BM) rejuvenation accelerates post-injury re-endothelialization in aging mice.

Methods and Results: Using BM transplantation (BMTY→WT, youngGfp to youngWild (YTY), oldGfp to oldWild (OTO), youngGfp to oldWild (YTO), and oldGfp to youngWild (OTY) groups were created. After vascular injury, IH was significantly greater in the old group than the young group (P<0.001). BM rejuvenation (YTO) significantly accelerated re-endothelialization and attenuated IH. Compared with the OTO group, the YTY and YTO groups had earlier and greater EPC-derived re-endothelialization (P<0.001). The number of Sca-1*KDR* EPCs mobilized in the circulation induced by vascular injury was higher in young, YTO, and YTY mice than in old mice (P<0.05). Sca-1* BM cells from the young, YTO, and YTY groups had better migration and adhesion capacities than those from the old group (P<0.05). The increase in blood vascular endothelial growth factor (VEGF) levels after vascular injury was higher in young than in old mice. PI3K, Akt, and FAK pathways played a pivotal role in VEGF-associated EPC migration. Specifically, EPCs from young and YTO mice, compared with old mice, demonstrated stronger FAK phosphorylation after VEGF stimulation.

Conclusions: EPCs play a critical role in vascular repair in aging mice. BM rejuvenation accelerates re-endothelialization by improving EPC function. (Circ J. 2013; 77: 3045–3053)

Key Words: Aging; Bone marrow rejuvenation; Endothelial progenitor cells; Intimal hyperplasia; Re-endothelialization

A s a result of decreasing age-specific mortality and decreasing fertility in both developing and developed countries, gains in life expectancy have led to rapid aging of populations. Aging is an independent risk factor for developing coronary artery disease (CAD). Despite numerous cellular and molecular differences between young and aged populations, age-related endothelial dysfunction is a major factor contributing to delayed re-endothelialization and exaggerated intimal hyperplasia (IH) after vascular injury. Re-endothelialization is believed to be substantially controlled by adjacent endothelial cells (ECs) within the vessel wall; however, accumulating evidence indicates that circulating endothelial progenitor cells (EPCs) also play a pivotal role.

Bone marrow (BM) is the major reservoir for adult organ-specific stem and progenitor cells, including EPCs. A complex impairment of the BM microenvironment involving the endothelial compartment may be involved in a defective EPC-dependent tissue-repair capacity, such as mobilization and biological functions. This regulatory dysfunction of the BM may link the decrease in the number of circulating EPCs to aging, coronary risk factors, increased Framingham risk scores, and heart failure. Although extensive interventions have been made to improve the number and function of circulating EPCs, BM could actually be a novel primary target.

Aging-associated functional senescence of the BM may constitute a potential limitation to the ability of EPCs to sustain tissue repair. Young adult BM-derived EPCs were shown to restore aging-impaired cardiac angiogenic function. However, whether BM rejuvenation accelerates re-endothelialization in aged mice has not been tested. Similar concepts can be applied to conditions exacerbating BM dysfunction, such as diabetes...
and heart failure.

Our study hypothesized that increased mobilization of functioning EPCs repairs post-injury endothelial damage in young BM-reconstituted aging mice and eventually attenuates IH. Understanding the mechanisms responsible for the effects associated with BM rejuvenation may facilitate prevention or provide new therapeutic targets for vascular occlusive diseases in aging populations, especially in the era of induced pluripotent stem cells.

Methods

Animals
Male wild-type and enhanced green fluorescent protein-positive (eGFP+) C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME, USA) were bred and maintained in micro-isolation cages on a 12-h day/night photoperiod in the Laboratory of Animal Experiments at Chang Gung Memorial Hospital. Mice were used for studies at 2 (young) and 18 (old) months of age. The study protocol was reviewed and approved by the Institutional Animal Care and Use Committee. The experiments were conducted according to the guidelines for the Care and Use of Laboratory Animals (NIH publication no. 86-23, revised in 1985).

BM Transplantation (BMTx)
Young and old recipient C57BL/6 mice were lethally irradiated with a total dose of 9.0 Gy. Young and old eGFP transgenic mice (C57BL/6 background) were used as donors. After irradiation, the wild-type recipient mice received unfractionated BM cells (5×10⁶ cells) from eGFP mice by a tail vein injection (BMTxGfp → Wild mice), including mice with BMTx from young to old (YTO), from young to young (YTY), from old to young (OTY), and from old to old mice (OTO). At 8 weeks after the injection, these mice were used for a variety of in vitro and in vivo studies. Repopulation by eGFP+ BM cells was approximately 95% as measured by flow cytometry. In the BM cells harvested from the BMTxGfp → Wild mice, flow cytometry was performed to enumerate sca-1+ KDR+ EPCs. In the sca-1+ KDR+ population, 96.5±0.6% of the cells were eGFP+ (n=10).

Femoral Artery Wire-Injury (Vascular Injury) Model
A mouse femoral artery wire-injury model was adopted to induce mechanical endothelial injury. Transluminal mechanical
injury of the femoral artery was carried out under a dissecting microscope. Briefly, either the left or right femoral artery was exposed by blunt dissection. The accompanying femoral nerve was carefully separated, but the femoral vein was isolated from the artery. The femoral artery and vein were looped together proximally and distally with 6-0 silk sutures for temporary vascular control during the procedure. A small branch between the rectus femoris and vastus medialis muscles was isolated, and looped proximally and ligated distally with 6-0 silk sutures. The vein and connective tissues around the artery were carefully removed with microsurgical forceps. The exposed muscular branch artery was dilated by topically applying a drop of 1% lidocaine hydrochloride. A transverse arteriotomy was performed on this branch. A straight spring wire (0.014-inch in diameter, ASAHI Intecco Co, Japan) was carefully inserted into the femoral artery toward the iliac artery. The wire was left in place for 1 min to denude and dilate the artery. The wire was then removed, and a silk suture looped around the proximal portion of the muscular branch artery was secured. Blood flow in the femoral artery was restored by releasing the sutures placed proximally and distally. At different time points, the femoral arteries were excised, fixed in OCT compound (TissueTek, Tokyo, Japan), and used for immunofluorescence studies. In addition, flow cytometry was used to estimate the mobilization of EPCs 24 h after vascular injury.

**Flow Cytometry**

A fluorescence-activated cell sorting FACSCaliber flow cytometer (Becton Dickinson) was used to assess EPC mobilization from the BM. Basal levels of circulating EPCs were estimated in blood collected by a retro-orbital approach. After 2 weeks of recovery, whole blood was obtained by cardiac puncture 24 h after vascular injury. Antibodies including PE-conjugated anti-Sca-1 (clone E13-161.7; Pharmingen) and FITC-conjugated anti-KDR (Avas12α1; Pharmingen) were used. EPCs were considered to be from the mononuclear cell population and were positive for both Sca-1 and KDR.

**Migration Assay**

The migratory function of sca-1+ BM cells was evaluated by a modified Boyden chamber assay (Transwell, the volume of the upper well was 100 µl, and the chamber diameter was 6.5 mm; the volume of the lower chamber was 600 µl). Briefly, cells were starved in serum-free X-VIVO 15 medium at 37°C, 5% CO2 for 24 h. After starvation, 1×10^5 isolated sca-1+ BM cells in serum-free X-VIVO 15 medium were placed in the upper chamber with a polycarbonate membrane (3-µm pores). Mouse VEGF (50 ng/ml) in the medium was placed in the lower chamber. After incuba-
Nuclei were stained with Hoechst 33258 (Sigma). For semi-quantification of re-endothelialization, the length of the whole vascular surface and the length of the surface covered with cells expressing endothelial markers, with and without eGFP expression were all measured. The length of these measured sections was divided by the length of the whole vascular surface and presented as percentage.

Measurement of Plasma VEGF Levels
Whole blood, with EDTA as an anticoagulant, was collected from mice of each group before and 24 h after the vascular injury was created. Blood was centrifuged to isolate plasma and stored at −80°C for measurement. Plasma concentrations of VEGF were measured by a mouse VEGF enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems). At the end of the assay, the optical density was measured by an ELISA reader at 450 nm (DYNEX MRX-1).

Western Blot Analysis
BM sca-1+ cells were isolated from each group of mice and subjected to VEGF (50 ng/ml) stimulation. Cells were harvested at the indicated time points and lysed with RIPA lysis buffer containing freshly added protease inhibitor cocktail (Sigma-Aldrich, St. Louis) and 500 µmol/L phenylmethylsulfonylfluoride. Total protein (30 µg) was subjected to electrophoresis on SDS-
BM Rejuvenation in Aged Mice

Re-Endothelialization in Mice After BMTx

Re-endothelialization was quantified 12 and 28 days after wire injury in all BMTx groups. On day 12 (Figure 2), BM rejuvenation in old mice (YTO group) significantly increased the speed of re-endothelialization, compared with the OTO group. Transplantation of BM from old mice to young mice (OTY) resulted in a slightly decreased speed of re-endothelialization compared with the YTY group. Although re-endothelialization was the best in the YTY group, the number of eGFP+CD31+ EPCs in the YTO group was the highest compared with the other groups. However, a very small number of eGFP+vWF+ ECs were noted in the OTO and OTY groups.

EPC Migration in Vitro

In the BM, numbers of Sca-1+KDR+ EPCs did not significantly differ among groups (Figure 4A). The migratory ability of BM had significantly more IH. BM rejuvenation performed in the YTO group significantly attenuated the extent of IH compared with the OTO and old groups. Transplantation of BM from old mice to young mice (OTY) did not increase the thickness of IH, compared with that of the young and YTY groups.

Figure 4. Migratory and adhesion capacities. (A) Number of Sca-1+KDR+ cells in the bone marrow of different groups (n=6–10 in each group). (B) In vitro migratory capacity of Sca-1+ cells in response to vascular endothelial growth factor (VEGF) and serum (n=6 in each group). Data are shown as the fold change compared with the old mice group without VEGF or serum stimulation (presented as arbitrary unit). (C) Adhesive capacity of Sca-1+ cells to uncoated and fibronectin-coated dishes (n=6 in each group). YTO, YTY, OTY, and OTO respectively indicate bone marrow transplantation from young to old, from young to young, from old to young, and from old to old mice. *P<0.05 compared with “Old”; †P<0.05 compared with “Young”.

Statistical Analysis

Quantitative data are expressed as mean±SEM. Statistical analysis was performed using the unpaired Student’s t-test or analysis of variance (ANOVA) followed by Scheffé’s multiple-comparison post-hoc test. Data were analyzed using SPSS software (vers. 14; SPSS, Chicago, IL, USA). A probability value of <0.05 was considered to indicate statistical significance.
Sca-1+ BM EPCs was estimated in all 6 groups (Figure 4B). Compared with the old group, sca-1+ BM EPCs in the young, YTO, and YTY groups had better migratory capacity in response to VEGF as a chemoattractant. There were no significant differences among the old, OTY, and OTO groups. In the response to serum as a chemoattractant, the findings were similar to the experiments utilizing VEGF. However, in the YTO and YTY groups, the migratory capacity of their EPCs was remarkably better than those of the old and even the young groups.

### Adhesion Capacity

The adhesion capacity of Sca-1+ BM cells harvested from different groups for the uncoated and fibronectin-coated dishes was tested (Figure 4C). On uncoated dishes, compared with the old group, BM rejuvenation (the YTO group) significantly increased the adhesion capacity of Sca-1+ BM cells up to a level similar to that of the young group. The findings were similar on fibronectin-coated dishes, although the adhesion capacity increased on average in all groups.

### EPC Mobilization in Vivo

At baseline, before vascular injury was created, the numbers of circulating Sca-1+KDR+ EPCs did not significantly differ among all groups (Figure 5A). In response to stimulation by vascular injury, the increases in circulating Sca-1+KDR+ EPCs were significantly greater in the young, YTO, and YTY groups compared with the old group. The number of Sca-1+KDR+ EPCs inside the BM may be related to the extent of EPC mobilization. However, as shown in Figure 4A, there were no significant differences among groups.

### Blood Levels of VEGF

Before the induction of vascular injury, no significant differences were noted in blood VEGF levels among groups (Figure 5B). However, in response to the stress of vascular injury, increases in blood VEGF levels were significantly higher in the young and YTY groups compared with the old and YTO groups, respectively.

### Mechanisms Related to FAK and Akt Activation

Because the main effect of BM rejuvenation appears to manifest through an increased migratory ability of Sca-1+ KDR+ EPCs, migration-related cellular signals were investigated for Sca-1+ BM cells in young mice (Figure 6). In response to VEGF, significant phosphorylation was noted at 15 min for PI3K, Akt, FAK, and JNK, but not for ERK1/2, and P38. Subsequently, inhibitors were used to investigate the role of PI3K, Akt, and FAK in VEGF-induced cell migration in the old, YTO, and young mice. LY294002 (PI3K inhibitor) partially attenuated the migration capacity (Figure 7A). PF562271 (FAK inhibitor) (Figure 7B) and Triricibine (Akt inhibitor) (Figure 7A) completely blocked the cell migration. In addition, the effect of BM rejuvenation was also abolished. Akt and FAK phosphorylation were also quantified in old, YTO, and young groups 15 min after VEGF stimulation (Figure 7C). There was no difference in Akt phosphorylation among the 3 groups; however, the phosphorylation of FAK was upregulated in the YTO group compared with the old group.

### Discussion

Delayed re-endothelialization is one of the mechanistic links in aggravated IH. Impaired biological function of the endothelium and EPCs contributes to deficient endothelial recovery in aged mice. BM rejuvenation improves the migratory, mobilization, and adhesive functions of EPCs. Our study supports the hypothesis that BM-derived EPCs play a critical role in vascular injury in aging mice, but not in young mice. BM rejuvenation was able to accelerate the speed of re-endothelialization after wire-induced vascular injury by mechanisms associated with EPCs.

### Mechanism of IH in the Elderly

Aging-associated atherosclerosis and IH involve complex mechanisms. Smooth muscle proliferation plays a key role in path-
ways are obligatory for this biological function of sca-1+ EPC. It has been shown that VEGF induces tyrosine phosphorylation of FAK and the focal adhesion-associated protein, paxillin, to promote recruitment of FAK to new focal adhesions in mature ECs. It has been shown that VEGF mediates P38, JNK, and ERK1/2 in Sca-1+ bone marrow EPCs in response to VEGF (50 ng/ml) were quantified by Western blotting analysis (n=3).

**Effects Associated With BM Rejuvenation**

Increasing age has been shown to be associated with reduced levels of circulating EPCs in patients with CAD. Previous studies suggested that this was related to the reduced migratory and adhesion capacities of EPCs in elderly patients with CAD. Our present data showed that BM rejuvenation improved the in vitro EPC migratory and adhesion functions, and in vivo mobilization function in response to vascular injury. These beneficial effects were further supported by increased numbers of EPCs on the injured vascular wall at an early time point after wire injury and remarkably improved re-endothelialization by EPCs at a late time point in the mouse model. The associated mechanisms involving molecular signals related to VEGF-induced sca-1+ BM EPC migration are still unknown. Our data demonstrated that PI3K-Akt, and FAK pathways are obligatory for this biological function of sca-1+ EPC.

It has been shown that VEGF induces tyrosine phosphorylation of FAK and the focal adhesion-associated protein, paxillin, to promote recruitment of FAK to new focal adhesions in mature ECs. Both Src-dependent FAK signaling and Akt-dependent phosphorylation of endothelial nitric oxide synthase at Ser-1177 play an important role in VEGF-induced EC migration. Furthermore, our data suggest that enhanced FAK activation may be a critical mechanism associated with the functional improvement of EPCs after BM rejuvenation. However, it cannot be excluded that the aging intracellular cytoskeleton also contributes to the impaired migratory capacity of EPCs in old mice based on the similar activation levels of Akt among the 3 groups.

Vascular injury has been shown to enhance circulating EPC levels by VEGF-mediated mobilization of BM-derived EPCs. VEGF levels decrease with age, leading to limited mobilization and survival/differentiation of EPCs. However, some studies have shown that cytokines are not likely to play a direct role in BM progenitor cell mobilization. In our present animal studies, baseline VEGF levels did not significantly decrease in old and BM-rejuvenated old mice. However, in response to vascular injury, increases in the VEGF level were greater in young mice and hosts compared with old mice and hosts. This finding is consistent with a clinical report that the increase in VEGF level after coronary artery bypass grafting is depressed in older patients. Furthermore, progenitor cells from rejuvenated BM also responded better to VEGF-mediated migration. As shown in our study, this improvement appears to be derived from improved function of the KDR rather than to an increased number of KDR on EPCs. Changes in blood VEGF levels and the response of EPCs to VEGF both represent major mechanisms related to the effects of BM rejuvenation.
Role of BM in Young Mice
The role of EPCs in the spontaneous atherosclerosis process is still being debated. However, our present data support the pivotal role of EPCs in vascular repair in aging mice. In addition to the higher blood VEGF levels in young mice and better mobilization response of EPCs to vascular injury, healthy ECs in young animals also substantially contribute to rapid re-endothelialization, together with EPCs. The notion that old mice have inadequate endothelial function was supported by our finding that the recovery of endothelium on injured vascular surfaces mainly depended on EPCs in YTO mice. Moreover, the finding that EPCs from reconstituted BM have a better migratory capacity in response to serum than VEGF suggests certain important factors other than VEGF in the serum also play an important role in the chemotraction of EPCs.

An interesting finding from our study was that progenitor cells from YTY mice had certain biological functions, such as an even stronger migratory capacity than those from young mice. Although our study did not explore the associated reasons, the mechanisms may include the following: (1) during the process of transplanted cells homing back to the BM, the microenvironment in the BM may regulate the selection of progenitor cells with a strong migratory capacity; (2) transplanted progenitors migrate back to the BM but do not adhere to the BM microenvironment as firmly as those that remained in the BM niche from birth; or (3) transplanted progenitors are still not quiescent 2 months after BM reconstitution. This phenomenon is noted for the first time and remains to be elucidated.

Clinical Applications
From the findings in this study, BM rejuvenation potentially provides hope for tackling cardiovascular diseases associated with irreversible deficiencies related to aging. We cannot exclude the possibility that a similar therapeutic concept may be
applied to other chronic vascular diseases associated with multiple risk factors. Although it is still not feasible to replace BM as a regular antiatherosclerotic therapy, advanced progress in hematology does not exclude the possibility of achieving this goal in the future, especially in the era of induced pluripotent stem cells. The alternative is to functionally modify autologous progenitors before returning them to the body.

Conclusions

In both developing and developed countries, increased life expectancy has led to a rapid aging of the populations. BM rejuvenation provides a potential concept for targeting pathological vascular remodeling in the elderly after a variety of vascular interventions.

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Disclosures

The authors state that they have no conflicts of interest.

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