Regulation of Endothelial Progenitor Cell Release by Wnt Signaling in Bone Marrow

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PURPOSE. Endothelial progenitor cells (EPC) have been shown to participate in ischemia-induced retinal neovascularization (NV). Overactivation of Wnt signaling has a pathogenic role in ischemia-induced retinal NV. The purpose of this study is to determine whether Wnt signaling regulates EPC release.

METHODS. Oxygen-induced retinopathy (OIR) was used as a model of retinal NV and Wnt pathway activation. The EPC, marked as c-Kit⁺/Tie-2⁺ cells in the peripheral blood and bone marrow, were quantified using flow cytometry following immunolabeling. The Wnt signaling activity was evaluated by measuring nonphosphorylated β -catenin levels and X-gal staining in the Wnt reporter mice (Bat-gal mice).

RESULTS. The c-Kit⁺/Tie-2⁺ cells were increased significantly in the peripheral blood and bone marrow of mice with OIR, compared to non-OIR mice. Overexpression of kallistatin, an endogenous inhibitor of the Wnt pathway, in kallistatin transgenic (kallistatin-TG) mice with OIR attenuated the increases of c-Kit⁺/Tie-2⁺ cells in the peripheral blood and bone marrow, compared to WT mice with OIR. When the Bat-gal mice were crossed with kallistatin-TG mice, kallistatin overexpression suppressed the OIR-induced increases of X-gal-positive cells in the retinas and bone marrow, suggesting inhibition of Wnt signaling in these tissues. Furthermore, intraperitoneal injection of LiCl, a Wnt signaling activator, increased c-Kit⁺/Tie-2⁺ cells in the peripheral blood of normal mice. Consistently, LiCl activated Wnt signaling in the retina and bone marrow cells in Bat-gal mice.

CONCLUSIONS. The Wnt signaling pathway has an important role in EPC release during retinal NV in OIR.

Keywords: EPC, bone marrow, peripheral blood, retina, Wnt

Endothelial progenitor cells (EPC) are a subpopulation of bone marrow cells that express some typical endothelial markers, and can differentiate into endothelial cells during vasculogenesis.¹ Under certain physiological and pathologic conditions, EPC are mobilized from the bone marrow, and participate in the process of vascular endothelium repair and neovascularization (NV).^{2,3} Growing evidence suggests that EPC participate in postnatal blood vessel formation under physiological and pathophysiological conditions, such as tissue repair following injury, cardiac or skeletal ischemia, wound healing, cancer, retinopathy of prematurity (ROP), and proliferative diabetic retinopathy.^{1,4}

To our knowledge, EPC have not been defined clearly, since specific, unique cell surface markers that permit the identification of EPC have not been established.⁵ However, recent studies have shown that cells expressing CD117 (c-Kit)/Tie-2 or CD34/VEGFR2/CD133 markers can have an important role in neoangiogenesis.^{6–8} Interestingly, modulation of EPC is believed to have therapeutic potential in conditions when NV is the desired result, such as in cardiovascular diseases, and in conditions when NV is detrimental, such as ischemia-induced retinal NV, in which inhibition of EPC release might attenuate or slow the progression of NV.⁹

The Wnts are a family of evolutionarily conserved, secreted signaling molecules that have significant roles in embryonic development,¹⁰ bone remodeling,¹¹ and normal blood cell formation.^{12,13} Further, the Wnt signaling pathway also mediates inflammatory responses, and modulates angiogenesis during development and under disease conditions.^{14–16} Our recent study showed that overactivation of Wnt signaling has pathogenic roles in ischemia-induced retinal NV and diabetic microvascular complications.¹⁷

A growing body of evidence suggests that Wnt signaling has a crucial role in the modulation of the bone marrow stromal microenvironment on the balance between hematopoietic stem cell self-renewal and differentiation.¹⁸ Recent evidence suggests that hematopoietic stem cells (HSC) respond to the canonical Wnt pathway in their normal microenvironment.¹³ In addition, constitutive activation of the canonical Wnt pathway results in the loss of HSC repopulation ability and the blockade of multilineage differentiation.¹⁹ Similarly, a recent study using β -catenin deletion with the Vav-Cre system showed that HSC self-renewal capacity was hampered significantly by the interruption of Wnt signaling.²⁰ Still, the role of Wnt signaling in modulation of the contribution of proangiogenic EPC to retinal NV is unclear.

FIGURE 1. Quantification of c-Kit⁺/Tie-2⁺ cells in OIR mice by FACS analysis. Blood and bone marrow cells were immunostained with antibodies for c-Kit and Tie-2, and analyzed by FACS. Representative FACS results of the peripheral blood (A–C) and bone marrow (D–F) are shown. (A, D) Mice at constant normoxia (NOR) at P16. (B, E) WT mice with OIR at P16. (C, F) Kallistatin-TG (KS-TG) mice with OIR at P16. (G, H) Quantification of c-Kit⁺/Tie-2⁺ cells in the peripheral blood (G) and bone marrow (H). All values are mean $\pm$ SD ($n = 8$ –12). * $P < 0.05$, ** $P < 0.01$.

In our study, we investigated the role of Wnt signaling in regulation of EPC release under ischemia using Wnt reporter mice (Bat-gal) with oxygen-induced retinopathy (OIR), transgenic mice overexpressing kallistatin, an endogenous inhibitor of Wnt signaling, and LiCl, an activator of Wnt signaling.

MATERIALS AND METHODS

Animal Models

Care and use of all animals in this study were in strict agreement with the guidelines in the Use of Animals in

Ophthalmic and Vision Research, and approved by the Institutional Animal Care and Use Committee at the University of Oklahoma. All of the animal experiments were performed in compliance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research.

The OIR model was generated as described previously.²¹ Briefly, animals were exposed to 75% oxygen from postnatal day (P7) to P12 with the nursing mother and then returned to room air at P12 to induce retinal NV. Kallistatin transgenic (kallistatin-TG) mice overexpressing kallistatin were generated as described previously.²² The Wnt signaling reporter mice,

FIGURE 2. Activation of Wnt signaling in the retina of OIR mice. (A) Retinal sections of Bat-gal mice under normoxia and with OIR, and Bat-gal × kallistatin-TG with OIR were stained with X-gal at P16, and representative sections are shown. Scale bar: 50 μ m. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. (B) NP- β -catenin levels in the retinas of normal Bat-gal mice and Bat-gal mice with OIR were measured at P12, P14, P16, P18, and P20 by Western blot analysis. (C) NP- β -catenin in the retinas of Bat-gal and Bat-gal × kallistatin-TG mice at P16 with OIR. The Western blotting results were semiquantified by densitometry, normalized by β -actin levels, averaged in three independent experiments, and expressed as percentage of the respective control. All values are mean $\pm$ SD ($n = 9$). * $P < 0.05$, ** $P < 0.01$.

Bat-gal, which express the β -galactosidase reporter gene under the control of a promoter containing TCF/ β -catenin-binding sites, were purchased from Jackson Laboratories (Bar Harbor, ME).

Lithium chloride (LiCl) was injected intraperitoneally (IP, 200 mg/kg) to 4-week-old Bat-gal mice once a day for seven days, with the same dose of NaCl as control. After the injections, the mice were humanely killed for X-gal staining or flow cytometry analysis.

Bone Marrow Cell Isolation

Bone marrow cells were collected from the leg bone of mice individually. After careful removal of the muscle around the bone, the bone was crushed in 2 mL PBS. The bone marrow was collected and then filtered through a 530-nm filter in a centrifugal tube.

Flow Cytometry Analysis

To quantify circulating and bone marrow EPC by fluorescence-activated cell sorter analysis (FACS), mononuclear cells were

isolated using RBC Lysis buffer (Biolegend, San Diego, CA) from either 200 μ L of peripheral blood or bone marrow. The cells were incubated for 30 minutes on ice with a FITC-conjugated anti-mouse CD117 (c-Kit) antibody (Biolegend) and PE-conjugated anti-mouse CD202b (Tie-2) antibody (Biolegend). The cells then were washed with PBS and fixed in 4% paraformaldehyde. Data were acquired using a FACS Calibur flow cytometer, and analyzed using Cell Quest software (BD Biosciences, Franklin Lakes, NJ) with a 530-nm filter. In each sample, 20,000 cells were analyzed, and data were analyzed by FlowJo software (Tree Star, Ashland, OR). To quantify EPCs, c-Kit⁺/Tie-2⁺ double-positive cells within the monocytic cell population were counted.

Western Blot Analysis

Western blot analysis was performed as described previously.²³ Antibodies for nonphosphorylated- β -catenin (NP- β -catenin) were purchased from Cell Signaling (Danvers, MA) and were used at 1:1000 dilution. The antibody for β -actin (Sigma-Aldrich, St. Louis, MO) was used at 1:5000 dilution.

FIGURE 3. Activation of Wnt signaling in the bone marrow of OIR mice. Bone marrow cells from Bat-gal mice under normoxia (control), Bat-gal mice with OIR, and Bat-gal $\times$ KS mice with OIR were stained with X-gal at ages indicated. (A) Representative images of bone marrow cells after X-gal staining at P16. (B) Quantification of X-gal-positive cells in the bone marrow of Bat-gal mice with OIR or under normoxia at P12, P14, P16, P18, and P20 and expressed as percentage of total cells. (C) Comparison of X-gal-positive cells in the bone marrow of Bat-gal mice and Bat-gal $\times$ kallistatin-TG mice with OIR at P16. All values are mean $\pm$ SD ($n = 10$). * $P < 0.05$, *** $P < 0.001$.

X-Gal Staining

The eyeballs from the Bat-gal reporter mice were fixed for 30 minutes in 1 $\times$ fixation buffer (Sigma-Aldrich), incubated at 37°C overnight in X-gal staining solution (Sigma-Aldrich), and sectioned according to manufacturer's instruction.

Bone marrow cells were spun down by centrifugation at 500g for 5 minutes, resuspended and fixed in 1 $\times$ fixation buffer for 30 minutes. The cells then were incubated at 37°C overnight in the X-gal staining solution. To quantify X-gal-positive cells, X-gal-positive cells were counted under microscope in 10 random areas of each sample and averaged within each group.

Statistical Analysis

Student's *t*-test was used for statistical analysis between two groups. One-way ANOVA was used for comparison of multiple groups. Statistical significance was accepted when the *P* value was less than 0.05.

RESULTS

Increased Circulating c-Kit⁺/Tie-2⁺ Cells in the OIR Model

The OIR is a commonly-used model of ischemia-induced retinal NV.^{21,24} Our previous studies showed that aberrant activation of the Wnt signaling pathway in the retina has an important pathogenic role in retinal NV and inflammation in OIR.¹⁷ To

induce the increase of circulating endothelial cells (including EPC and mature endothelial cells), C57 mice were exposed to 75% oxygen from P7 to P12, and then returned to room air to induce retinal NV. The c-Kit⁺/Tie-2⁺ double-positive cells in the peripheral blood and bone marrow were quantified by FACS. Age-matched mice maintained in constant room air were used as non-OIR controls. Compared to non-OIR mice, numbers of circulating c-Kit⁺/Tie-2⁺ cells in the blood and bone marrow were significantly increased in OIR mice at P16, correlating with the most aggressive stage of retinal NV²¹ (Figs. 1A, 1B, 1D, 1E, 1G, 1H).

Kallistatin is an endogenous inhibitor of Wnt signaling and angiogenic inhibitor.²² To determine whether kallistatin overexpression affects EPC release, OIR was induced in kallistatin-TG mice overexpressing kallistatin. The c-Kit⁺/Tie-2⁺ cells from the peripheral blood and bone marrow were quantified by FACS at P16. Kallistatin-TG mice with OIR showed significantly decreased numbers of circulating c-Kit⁺/Tie-2⁺ cells at P16 in the peripheral blood and bone marrow, compared to WT mice with OIR at the same time point (Figs. 1C, 1F–H). These results suggested that inhibition of the Wnt signaling pathway by kallistatin suppressed the generation and release of EPC, which may be a mechanism responsible for the antiangiogenic effect of kallistatin.

Wnt Signaling Was Activated in the Retina With OIR

To confirm further the effect of OIR on Wnt signaling in the retina, we measured the transcriptional activity of β -catenin in the retina. We crossed kallistatin-TG mice with Wnt reporter Bat-gal mice. The OIR was induced in the Bat-gal mice and Bat-

FIGURE 4. The FACS analysis of circulating c-Kit⁺/Tie-2⁺ cells after LiCl injections. Blood and bone marrow cells were isolated from mice after the LiCl treatment, immunostained with antibodies for c-Kit and Tie-2, and analyzed by FACS. Representative FACS results of peripheral blood (A, B) and bone marrow (C, D) are shown. (A, C) Controls with NaCl injections. (B, D) mice injected with LiCl. (E, F) Quantification of c-Kit⁺/Tie-2⁺ cells showed that c-Kit⁺/Tie-2⁺ cells were increased by LiCl in the blood (E), but not in the bone marrow (F). All values are mean $\pm$ SD ($n = 10$). * $P < 0.05$.

gal $\times$ kallistatin-TG mice. The Bat-gal mice without OIR were used as control. At P16, the retinas were stained with X-gal, to evaluate the activity of β -galactosidase reporter driven by β -catenin. The OIR Bat-gal mice showed more intense X-gal staining in the retina, compared to non-OIR Bat-gal mice, further confirming the OIR-induced activation of Wnt signaling in the retina. Under the same conditions, Bat-gal $\times$ kallistatin-TG mice with OIR showed reduced X-gal staining in the retina, compared to age-matched WT Bat-gal mice with OIR (Fig. 2A),

confirming that kallistatin overexpression attenuated the OIR-induced activation of Wnt signaling in the retina.

Previous studies showed that the peaks of VEGF overexpression and vascular permeability in the retina occurred at P16 in the OIR model.²⁵ In this study, we also measured NP- β -catenin levels in the retina using Western blot analysis at P12, P14, P16, P18, and P20, in mice with OIR and control mice under normoxia. In the OIR mice, retinal NP- β -catenin levels were significantly increased, with a peak approximate-

FIGURE 5. The LiCl-induced activation of Wnt signaling in the retina. **(A)** Representative retinal sections of Bat-gal mice were stained with X-gal after the LiCl injections or NaCl injections. *Scale bar:* 50 μ m. **(B)** The NP- β -catenin in the retinas was measured by Western blot analysis after the mice were treated with LiCl, and semiquantified by densitometry, normalized by β -actin levels, averaged in three independent experiments, and expressed as percentage of the respective control. All values are mean $\pm$ SD ($n = 6$). * $P < 0.05$.

ly 2-fold higher than that in the non-OIR control at P16 (Fig. 2B).

Under the same condition, Bat-gal mice with OIR and Bat-gal $\times$ kallistatin-TG mice with OIR were humanely killed at P16 to compare the NP- β -catenin levels. Retinal NP- β -catenin levels were significantly lower in the Bat-gal $\times$ kallistatin-TG mice with OIR (Fig. 2C), compared to age-matched Wt Bat-gal mice with OIR. Taken together, these results suggested that the Wnt signaling pathway is activated in the retina in the OIR model, which is attenuated by overexpression of kallistatin.

Wnt Signaling Was Activated in Bone Marrow With OIR

Previous evidence demonstrated that EPC from the bone marrow participated in ischemia-induced retinal NV.⁹ To determine whether Wnt signaling also was activated in bone marrow cells, we stained bone marrow cells from Bat-gal mice with OIR or without OIR using X-gal. The Bat-gal mice with OIR showed increased numbers of X-gal staining positive cells (blue), compared to Bat-gal mice under normoxia condition. In contrast, Bat-gal $\times$ kallistatin-TG mice with OIR showed reduced numbers of X-gal-positive cells in the bone marrow, compared to age-matched Bat-gal mice with OIR (Fig. 3A). Quantification of X-gal-positive cells showed significantly increased X-gal-positive bone marrow cells in OIR mice between P14 and P18 compared to the age-match non-OIR mice. The peak of X-gal-positive bone marrow cells was observed at P16 (Fig. 3B), correlating with the peak of EPC number in the circulation and the peak of retinal NV. Under the

same condition, Bat-gal $\times$ kallistatin-TG mice with OIR at P16 showed significantly decreased X-gal-positive cells in the bone marrow, compared to age-matched WT Bat-gal mice with OIR (Fig. 3C). These results are consistent with the findings in the retinal NV in the OIR model, suggesting that Wnt activation in the bone marrow may contribute to EPC generation and release in ischemia-induced retinal NV.

Activation of Wnt Signaling by LiCl Increased the Number of Circulating c-Kit⁺/Tie-2⁺ Cells

To confirm further that the Wnt signaling pathway has a regulatory effect on circulating endothelial cells release, we injected LiCl (200 mg/kg, IP), an activator of Wnt signaling via inhibiting GSK-3 β , once a day for 7 days to the 4-week-old C57 mice. Age-matched mice were injected with NaCl at the same dose as controls. The c-Kit⁺/Tie-2⁺ cells in the peripheral blood and bone marrow were quantified by FACS. The LiCl injections significantly increased c-Kit⁺/Tie-2⁺ cells in the peripheral blood of normal mice, compared to the NaCl control (Figs. 4A, 4B, 4E). This result further supported that Wnt signaling activation has an important role in the EPC release. However, numbers of c-Kit⁺/Tie-2⁺ cells in the bone marrow were not significantly different between the NaCl injection and LiCl injection groups (Figs. 4C, 4D, 4F).

LiCl Induced Wnt Signaling Activation in the Retina

To determine whether Wnt signaling also was activated by LiCl in the retina, we stained the retina from the LiCl-injected Bat-

FIGURE 6. The LiCl-induced activation of Wnt signaling in the bone marrow. (A) Representative images of X-gal staining of bone marrow cells from mice after the LiCl injections or NaCl injections. Arrows indicate X-gal-positive cells (blue). (B) Quantification of X-gal-positive cells. All values are mean $\pm$ SD ($n = 8$). *** $P < 0.001$.

gal mice with X-gal. The LiCl injection group showed more intense X-gal staining in the retina, compared to the control group with NaCl injections (Fig. 5A). This result indicated that LiCl injections induced Wnt signaling activation in the retina. Western blot analysis showed that retinal NP- β -catenin levels also were increased in the LiCl injection group compared to the NaCl control (Fig. 5B). These findings demonstrated that LiCl reached the retina and induced Wnt activation in the retina.

LiCl Induced Wnt Signaling in the Bone Marrow

To further confirm Wnt signaling is activated in bone marrow cells after the LiCl injections, we used X-gal staining of bone marrow cells from the Bat-gal mice injected with LiCl. There were significant increases in X-gal-positive cells in the LiCl treatment group, compared to the NaCl control group (Fig. 6). These results suggested that the LiCl injections also induced Wnt signaling activation in bone marrow cells.

DISCUSSION

Recent evidence indicates that vasculogenesis also contributes to ischemia-induced NV, such as retinal NV.^{26–29} The Wnt pathway modulates angiogenesis during development and under disease conditions.^{14–16} However, the role of Wnt signaling in the regulation of EPC release has not been investigated previously to our knowledge. Our study demonstrated for the first time to our knowledge that c-Kit⁺/Tie-2⁺ double-positive cells are increased in the peripheral blood and bone marrow in the OIR model, correlating with Wnt signaling activation in the bone marrow and retina. Further, overexpression of an endogenous Wnt inhibitor inhibited Wnt signaling in the bone marrow and led to suppressed EPC release in the OIR model. In contrast, activation of Wnt signaling alone without OIR also increased EPC release. Taken together, these results established that Wnt signaling activation promotes EPC release in the OIR model, representing a mechanism by which Wnt signaling contributes to ischemia-induced retinal NV.

The OIR is a commonly-used model of ischemia-induced retinal NV, as it has a well-characterized course of retinal NV.^{21,30} It is used as a model of proliferative diabetic retinopathy and ROP. Our previous study showed that the Wnt signaling pathway is overactivated in the retina in the OIR model, and peaks at P16, correlating with that of NV.²⁵ The present study demonstrated that the activation of the Wnt signaling pathway in bone marrow cells also peaks at P16 in the OIR model, correlating with the peak in the NP- β -catenin accumulation in the retina and with the most aggressive stage of the retinal NV in the OIR model. This result suggested that the Wnt signaling activation in the bone marrow may contribute to EPC release, leading to the spike in circulating endothelial cells and EPC, which contributes to retinal NV in the OIR model.

To establish the regulatory role of Wnt signaling in EPC release in OIR, we used transgenic mice overexpressing human kallistatin, which is an endogenous inhibitor of the Wnt pathway.²² Our recent study showed that kallistatin overexpression reduced the progression of retinal NV in the OIR model.²² Kallistatin-TG mice showed significant decreases in numbers of c-Kit⁺/Tie-2⁺ cells in the peripheral blood and bone marrow in OIR. We also have examined the Wnt signaling activity in vivo using Bat-gal mice, which express the β -galactosidase reporter gene under the control of Wnt signaling, and are widely used for evaluation of Wnt pathway activation in vivo. X-gal staining showed that overexpression of kallistatin resulted in fewer X-gal-positive bone marrow cells induced by OIR. This result is consistent with previous observation that the systemic injections of PEDF-34, a fragment of another endogenous inhibitor of Wnt signaling, significantly reduced the progression of retinal NV and attenuated the spike of circulating endothelial cells at P16 of the OIR model.⁹ These results further supported that Wnt signaling has a key role in modulating EPC release from the bone marrow.

To further confirm that Wnt signaling contributes to the EPC release, we injected LiCl, a Wnt pathway activator via inhibition of GSK-3 β , into the Wnt reporter Bat-gal mice. The LiCl induced Wnt pathway activation in the retina and bone marrow cells, compared to NaCl. Consistently, LiCl injection also increased c-Kit⁺/Tie-2⁺ double-positive cells in the peripheral blood. This result showed that systemic activation of Wnt signaling alone is sufficient to promote EPC release. Since lithium is a clinically-used drug, the effect on EPC release may be relevant in patients with neovascular diseases.

In summary, to our knowledge, our study is the first to demonstrate that Wnt signaling has a key role in the regulation of EPC release. However, the mechanism for the Wnt pathway activation in the bone marrow in the OIR model remains to be elucidated. Our study further supported that bone marrow abnormality also contributes to retinal NV, and, thus, Wnt signaling in the bone marrow is a potential drug target for the treatment of retinal NV.

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