Erythropoietin Stimulates Endothelial Progenitor Cells to Induce Endothelialization in an Aneurysm Neck After Coil Embolization by Modulating Vascular Endothelial Growth Factor

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Key Words. Cerebral aneurysm • Endothelialization • Endothelial progenitor cells • Erythropoietin

ABSTRACT

This study explored a new approach to enhance aneurysm (AN) neck endothelialization via erythropoietin (EPO)-induced endothelial progenitor cell (EPC) stimulation. Results suggest that EPO enhanced the endothelialization of a coiled embolization AN neck by stimulating EPCs via vascular endothelial growth factor modulation. Thus, the promotion of endothelialization with EPO provides an additional therapeutic opportunity during vascular inner layer repair. The results provide important information on the unique role EPO plays during vascularization induction provides an additional therapeutic opportunity during vascular inner layer repair.

SIGNIFICANCE

Erythropoietin (EPO) is involved in erythropoiesis and related conditions and is reported to enhance stem-cell mobilization from bone marrow while elevating stem-cell viability and function. In this study, EPO was also found to stimulate endothelial progenitor cells to induce the endothelialization of a coiled embolic aneurysm neck via vascular endothelial growth factor modulation. Endothelialization induction provides an additional therapeutic opportunity during vascular inner layer repair and remodeling. The results provide important information on the unique role EPO plays during vascular repair and remodeling.

INTRODUCTION

Compared with surgical management, endovascular coil embolization is an attractive approach for the treatment of unruptured, saccular cerebral aneurysms (ANs) because it is minimally invasive and efficient [1]. However, endovascular coil embolization has a high rate of recurrence (6.1%–33.6%) and rebleeding (0.11%–1.6%) [2–6]. Because the lack of endothelialization plays a crucial role in AN recurrence, the promotion of endothelialization in the AN neck may help prevent both recurrence and rupture.

Endothelial progenitor cells (EPCs) were first identified from human peripheral blood and are derived from the bone marrow. Circulating EPCs promote endothelialization after coiled embolization treatments, and EPC therapy has been tested in many vascular diseases [7, 8]. Studies have shown a correlation between a lack of EPCs and the incidence of cerebral ANs and have also demonstrated that bone marrow-derived EPCs...
are involved in the process of AN repair [9]. However, the contribution of increased circulating EPCs after coiled embolization contributing to AN neck endothelialization remains unverified.

Erythropoietin (EPO) is known for its function in erythropoiesis and has been reported to enhance the mobilization of stem cells from bone marrow and strengthen their viability and function. EPO has shown therapeutic effects in the treatment of myocardial infarcts, cerebral ANs, brain ischemia, and traumatic injuries [10–12] and may also promote angiogenesis to improve microcirculation and neovascularization and protect neural tissue. EPO exhibits a strong capacity to promote EPC differentiation and maturation toward endothelial cell lineages. Increasingly, studies have strongly indicated that EPO can reduce the formation and progression of cerebral ANs by promoting EPC mobilization and targeting them to sites of vascular injury [13–15]. These findings support the hypothesis that EPO may be used to protect against the recurrence of cerebral AN by stimulating EPCs and promoting endothelialization.

We showed that EPCs enhanced AN neck endothelialization after coil therapy and that the administration of EPO increased the number and function of EPCs. Because of the potency of EPO in promoting EPCs, we explored the possibility of using EPO to promote AN neck EPC-induced endothelialization in a coiled embolization AN model.

**MATERIALS AND METHODS**

All animal procedures were carried out according to a protocol that was approved by the Institutional Animal Care and Use Committee (IACUC), and the experimental protocol was reviewed and approved by the Ethics Committee of Huashan Hospital affiliated with Fudan University in Shanghai, China. Adult Sprague-Dawley rats (150–200 g) were used in the experiments (Slac Laboratory Animal, Shanghai, China, [http://english.sibs.cs.cn/rs/fs/shanghailaboratorianimalcenters]). The coiled AN model was prepared as previously reported [16]. The AN-EPO group was administered 1.5 μg/kg Recombinant Rat EPO (R&D Systems, Minneapolis, MN, [https://www.rndsystems.com]) injected subcutaneously, and the AN group was given an equal amount of saline subcutaneously. The mock surgery (MS) group was given inhalation anesthesia and was treated with saline similarly to the AN group but without surgery. On days 10, 20, and 30, peripheral blood was collected to examine circulating EPCs via flow cytometry analyses. The viability of EPO-treated cells was tested via a Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan, [http://www.dojindo.com]) double staining and live and dead cells were distinguished using propidium iodide (PI) (BD Pharminogen, San Diego, CA, [http://www.bdbiosciences.com]) staining. The secreted VEGF, TNF-α, and IL-6 from cultured cells were analyzed by a MILLIPLEX MAP. Gene expression was evaluated by quantitative polymerase chain reaction (qPCR). The primer sequences are shown in supplemental online Table 1. Detailed methods are described in the supplemental online data.

**Statistical Analysis**

The statistical analysis was performed using IBM SPSS Statistics (Armonk, NY, [http://www.ibm.com]), and graphs were generated by GraphPad Prism (GraphPad, La Jolla, CA, [http://www.graphpad.com/company]). Two-way ANOVA tests were used to analyze the percent of circulating EPCs identified by flow cytometry. Independent sample t tests were used to determine the aneurysm repair score, von Willebrand factor (vWF)+ cell count, KDR+ cell count, and circulating cytokines [17]. One-way ANOVA tests were used to analyze the peripheral blood, the optical density values obtained from the cell viability test, the migration cell count, the levels of cytokines secreted from the cultured cells, and the gene expression levels obtained by qPCR. Differences with p < .05 were considered significant.

**RESULTS**

**Experimental Design, Histological Assessment, and Scanning Electron Microscopy Observations**

After the coil embolism aneurysm model was initiated, 90 rats survived until sacrifice. All coil embolism aneurysm specimens were acquired for subsequent use.

H&E staining under low magnification demonstrated that a more integrated aneurysm neck was formed, and more spindle-like slender cells were observed in the aneurysm necks of the AN-EPO-treated rats. In contrast, in the AN rats, fewer vascular endothelial cells and only sparse fibrous cells were observed. The aneurysm repair score was significantly higher in the AN-EPO-treated rats compared with the AN rats, p < .05 (Fig. 1B).

Scanning electron microscopy (SEM) examination revealed the level of endothelialization in the aneurysm neck and found better endothelial coverage in the AN-EPO-treated rats compared with the AN rats. This layer primarily consisted of simple squamous epithelial cells at the bottom of the aneurysm neck. Overall, rats treated without EPO demonstrated a similar sealing effect compared with the EPO-treated rats. However, endothelial cells were rarely observed in the AN rats; instead, they displayed long, flat, and fusiform cell morphologies (Fig. 1C).

Under confocal microscopy, consecutive and compact vWF+ cells were found in the surface of the AN neck in the AN-EPO-treated rats. It was noted that the vWF layer in the AN rats was not continuous (Fig. 1D). There were significantly more vWF+ and KDR+ cells in the inner surface of the AN neck in the AN-EPO-treated rats than in the AN rats, p < .05 (Fig. 1D).

**Circulating EPC Detection and Peripheral Blood Changes**

On day 10 after surgery, the circulating EPC count was significantly elevated in the AN-EPO-treated rats compared with the MS and AN rats (p < .05). On day 20, the circulating EPC count significantly increased in the AN and AN-EPO groups compared with the MS group. There was no indicated superiority in the EPO treatment group. On day 30, the AN and AN-EPO-treated rats continued to show to increase EPC counts, and this increase was more obvious in the EPO-treated group (Fig. 2A).
We performed a peripheral blood examination to exclude the possible side effect of EPO elevating the red blood cell (RBC) count. We found no significant differences between the AN-EPO group and the AN group for the RBC count, hemoglobin, red blood cell specific volume, mean corpuscular volume, mean corpuscular hemoglobin, and the mean corpuscular hemoglobin concentration on days 10, 20, and 30 (Fig. 2B).

EPC Identification and Changes in EPO-Induced Cell Death, Viability, and Migration

We isolated EPCs from the femur marrow and found that many cells had a round, cobblestone-like morphology in the primary adherent cell culture. Furthermore, most of these primary cells were able to uptake both Dil-ac-LDL and FITC-UEA-I (Fig. 3A). Through flow cytometry, we demonstrated that 62.96 ± 4.48% of the cells were KDR+, 5.1 . 5 3 ± 3.65% of the cells were CD34+, and 3.91 ± 1.77% of the cells were KDR/CD34+. The flow cytometry analyses indicated that these KDR+/CD34+ cells were EPCs (Fig. 3B).

PI and calcein AM-labeled cells were observed by confocal microscopy. No increase in the number of dead cells was observed in the EPO-treated rats after 7 days of EPO treatment compared with the control rats (Fig. 3C).

We recorded the absorbance of EPCs in each well after their reaction with the reagents in a Cell Counting Kit-8. A significant increase in absorbance was observed on days 7 and 10 using high concentrations of EPO (150 μg/l and 15 μg/l), and an EPO concentration of 1.5 μg/l also presented a significant increase in absorbance on day 10. These findings indicate that EPCs presented improved cell viability when cultured with high concentrations of EPO for prolonged durations (Fig. 3D).
We performed a scratch assay to assess the degree of EPC migration and found that treatments with 150 μg/l, 15 μg/l, and 1.5 μg/l EPO for 10 days significantly increased EPC migration, \( p < .05 \) (Fig. 3E).

Cerebrovascular Cytokine Levels and Gene Expression After EPO Treatment

We tested cytokines and chemokines in the peripheral blood in both the AN and AN-EPO groups, including VEGF, TNF-\( \alpha \), and IL-6. The peripheral blood VEGF concentration in the AN-EPO group was elevated on days 20 and 30 compared with the AN group. There was no significant differences in the TNF-\( \alpha \) and IL-6 levels between the AN and AN-EPO-treated rats (Fig. 4A).

However, significant changes in cytokines and chemokines levels were observed in vitro. On day 1, the levels of VEGF from EPO-treated cells were significantly increased, and the IL-6 level was decreased (Fig. 4B). On day 3, the VEGF levels of the EPCs treated with 150 μg/l, 15 μg/l, 0.15 μg/l, and 0.015 μg/l EPO were...
significantly higher than those observed in the control group. The reducing effect of EPO on the IL-6 levels was not obvious, and only 150 \( \text{mg/l} \) EPO induced inhibition. There were no significant changes in the TNF-\( \alpha \) levels on either day 1 or day 3 (Fig. 4B).

qPCR analysis was used to determine the VEGF, TNF-\( \alpha \), and IL-6 gene expression levels. After 1 and 3 days of 150 \( \text{mg/l} \) EPO treatment, the expression of VEGF showed evident elevation, and IL-6 showed gradually decreasing trend. No significant differences were observed for the TNF-\( \alpha \) and IL-6 gene expression levels (Fig. 4C).

**DISCUSSION**

In our study, EPO was administered after the induction of a coiled embolization AN to prevent recurrence. We successfully established a coiled AN rat model via vasotransplantation. Half of the AN rats were treated with EPO. This EPO-treated group showed a significant increase in the number of circulating EPCs in the rats with aneurysms after coiling, and the EPCs also participated in the aneurysm neck endothelialization. EPO promoted the AN neck integration.
Figure 4. Cerebrovascular cytokines level and gene expression after EPO treatment in cultured endothelial progenitor cells (EPCs). (A): Bar graphs demonstrate the peripheral serum levels of VEGF (Aa), TNF-α (Ab), and IL-6 (Ac). Data are means ± standard errors of the mean; n = 8 per group; *, p < .05, EPO-AN rats vs. untreated rats. (B): VEGF (Ba, Bd), TNF-α (Bb, Be), and IL-6 (Bc, Bf) from cultured EPCs were measured on days 1 and 3. Data are means ± standard errors of the mean; n = 3 per group; *, p < .05 compared with untreated group. (C): Bar graphs demonstrate the levels of VEGF (Ca), TNF-α (Cb), and IL-6 (Cc) expression of EPCs after 150 μg/l EPO treatments for 1 day and 3 days. Data are means ± standard errors of the mean; n = 3 per group; *, p < .05 compared with untreated group. Abbreviations: AN, aneurysm; EPO, erythropoietin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL-6, interleukin 6; TNF-α, tumor necrosis factor α; VEGF, vascular endothelial growth factor.
and endothelialization, as shown by SEM. We also found that in vitro bone marrow-derived EPCs could be enhanced after 7 days of EPO treatment. Our study showed that EPO increased VEGF levels in vivo and in vitro. The safety of short-term EPO treatment was indicated by showing fewer side effects on the AN rat peripheral blood work, such as increased RBC counts or EPC deaths in vitro.

Endothelialization in the AN neck has been observed in clinical studies [18, 19] and animal models [20]. Endothelial dysfunctions are regarded as the main contributor to the recurrence of postembolization cerebral AN. The promotion of endothelialization is key to preventing AN recurrence. Because EPCs were initially identified from human peripheral blood and found to express CD34 and KDR [21], EPC therapy was used to facilitate vascular repair and homoeostasis [21, 22]. The autologous transfusion of bone marrow-derived EPCs could be used for stroke treatment [23]. There is a growing interest in using circulating EPCs in clinical trials for ischemia, stroke, and vascular injury [24–27]. Our previous study [16] also showed that bone marrow-derived EPCs play a crucial role in the closure and reconstruction of the aneurysm neck orifice after aneurysm coiling.

Medications, including statins, angiotensin converting enzyme inhibitors, and cancer drugs, have been studied to determine their effects on EPC mobilization. EPO has been shown to reduce the formation and progression of cerebral aneurysms in rats [28]. In this study, we found that the number of circulating EPCs was significantly increased at 10 days after EPO treatment in coiled AN rats compared with nontreated AN rats, which could be caused by mobilization promoted by EPO during early stages. On day 20, both the AN and AN-EPO-treated rats induced EPC mobilization and homeing after vascular injury. During this stage, the effect of EPO did not appear to be significant. Late stages of endothelialization were evident on day 30. In these stages, the number of AN neck EPCs decreased, and the circulating EPC levels recovered to a relatively high level. During this period, the EPO-treated rats maintained an obviously higher number of circulating EPCs. Previous studies have shown strong evidence indicating that EPO can mobilize EPCs from bone marrow to increase the amount of circulating EPCs [28, 29]. During the AN neck endothelialization, the circulating EPCs of the AN-EPO group were significantly increased significantly on day 10. With the completion of endothelialization, the circulating EPCs showed a decrease trend on days 20 and 30. In contrast, the circulating EPCs of the rats belonging to the AN group showed a significant increase on day 20 compared with the EPCs of the MS group and showed a decreasing trend in day 30. These findings indicate that EPO may lead to earlier mobilization. However, it is possible that the time interval between EPO administration and blood collection may partially affect the circulating EPCs.

EPO is a hormone secreted by the kidney in response to hypoxia and plays a cardinal role in regulating plasma hemoglobin concentrations. According to routine blood tests, the short term use of EPO did not show any side effects in the AN-EPO rats. In in vitro EPC cultures, EPO did not show significant cytotoxicity. In a previous study, EPO showed vascular protection and endothelium-promoting properties. These effects were primarily mediated by inhibiting apoptosis, mobilizing endothelial progenitor cells, inhibiting the migration of inflammatory cells, and promoting angiogenesis [12]. Previous studies on EPO-mediated endothelialization and its molecular pathways have focused on antiapoptotic and survival signals, including the phosphatidylinositol 3-kinase pathway and the endothelial nitric oxide synthase pathway [30–32]. In our study, we assessed the levels of VEGF, TNF-α and IL-6. We found that VEGF increased after EPO treatment in vivo and in vitro. The modulation of VEGF by EPO is thought to be one of the most important factors in promoting AN neck endothelialization after coil embolization treatment. EPO modulation plays key roles via VEGF and VEGF receptors in many vascular diseases [32–35].

In EPC cultures treated with EPO for 1 and 3 days, the levels of IL-6 showed a decreasing trend. This may be attributed to the anti-inflammatory effect of EPO. However, the in vivo IL-6 levels of AN rat sera and the in vitro gene expression profile of IL-6 did not correlate with this observation. This phenomenon may be induced by complex cytokine regulation and would require further study. Furthermore, no strong evidence or discernible trend was observed linking EPO and TNF-α in coiled AN rat sera or cultured EPCs.

**CONCLUSION**

In its recombinant form, EPO has been tested in clinical trials and proven to be beneficial in cerebral vascular diseases by providing vascular protection, inhibiting inflammation, and promoting endothelialization [36–38]. This study represents the first use of recombinant rat EPO to promote coiled AN neck endothelialization. We showed that EPO enhanced the endothelialization of coiled AN neck via VEGF modulation. EPO or its analogs may provide new therapeutic alternatives in preventing recurrence in coiled cerebral AN. However, there remain limitations. The peripheral blood cytokine levels may fluctuate in part owing to the deficient interval between administration and blood collection. No extensive dose response studies were performed in animal models. The exact and detailed mechanism through which EPO affects endothelialization remains unclear. Other important factors in addition to VEGF may be involved, such as hypoxia inducible factor 1 and stromal-derived factor 1, which are also known to mobilize EPCs under conditions of vascular disease and injury [39–41]. Further studies are required to explore the mechanism responsible for the EPO-based endothelialization of aneurysm necks.

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**AUTHOR CONTRIBUTIONS**

P.L.: conception and design of the study, provision of study materials and animal models, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript; Y.Z.: animal model creation, blood sample analysis, histological examination, discussion of manuscript; Q.A.: animal model analysis, histological examination; Y.S.: data analysis and interpretation, manuscript writing; X.C.: database input, data interpretation; G.-Y.Y.: provision of study material or patients, revision and final approval of manuscript; W.Z.: conception and design, revision and final approval of manuscript.

**DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST**

The authors indicated no potential conflicts of interest.
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