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Activation of Endothelial Nitric Oxide Synthase is Critical for Erythropoietin-induced Mobilization of Progenitor Cells

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Abstract

The present study aimed to define the ability of erythropoietin (EPO) to mobilize hematopoietic stem cells (c-kit$^+$/sca-1$^+$/lin-1$^-$; KSL cells) and hematopoietic progenitor cells (CD34$^+$ cells), including vascular endothelial growth factor receptor 2 expressing hematopoietic progenitor cells (CD34$^+$/Flk-1$^+$ cells). We also sought to determine the role of endothelial nitric oxide synthase (eNOS) in EPO-induced mobilization. Wild type (WT) and eNOS$^{-/-}$ mice were injected biweekly with recombinant erythropoietin (EPO, 1000 U/kg, s.c.) for 14 days. EPO increased the number of KSL, CD34$^+$, CD34$^+$/Flk-1$^+$ cells in circulating blood of wild type mice. These effects of EPO were abolished in eNOS$^{-/-}$ mice. Our results demonstrate that, EPO stimulates mobilization of hematopoietic stem and progenitor cells. This effect of EPO is critically dependent on activation of eNOS.

1. Introduction

In 1997, Asahara and colleagues demonstrated that so-called circulating "endothelial progenitor cells (EPCs)" are mobilized from bone marrow to the site of neovascularization, and participate in the formation of new blood vessels in situ [4]. Currently, there is no consensus in the literature on the phenotypic characteristics of endothelial progenitors. However, prior studies demonstrate that the number of circulating endothelial progenitor cells is inversely correlated with cardiovascular risk factors such as diabetes, hypertension, hyperlipidemia and smoking [17,23,26–28], underscoring the importance of these cells in pathogenesis of vascular disease.

In peripheral blood, hematopoietic progenitor cells (CD34-positive cells) expressing receptor for vascular endothelial growth factor (VEGF), VEGFR2 or Flk-1, have been considered to represent the population of cells enriched with EPCs [1,12,26]. Although the number of CD34$^+$/Flk-1$^+$ cells has been inversely correlated with cardiovascular risk, Case and colleagues recently demonstrated that culturing human CD34$^+$AC133$^+$VEGFR2$^+$ cells or CD34$^+$VEGFR2$^+$ cells do not yield functional endothelial cells [7,8], arguing against their endothelial progenitor nature. Nevertheless, it has been suggested that cells expressing CD34$^+$/Flk-1$^+$ participate in therapeutic neovascularization [20]. It is also important to note that, hematopoietic stem cells, characterized by co-expression of stem cell antigen-1 (Sca-1)

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and receptor for stem cell factor, c-kit [21,22,24], posses the ability to differentiate into non-hematopoietic tissues, including cardiac myocytes [19].

Erythropoietin (EPO) is a hematopoietic cytokine that stimulates proliferation, survival and differentiation of erythroid precursor cells, as well as maturation of erythroid cells [15]. In addition to erythropoiesis, EPO stimulates proliferation of endothelial cells *in vitro* [3]. The importance of EPO for repair of injured endothelium and revascularization of ischemic tissues appears to depend on EPO-induced mobilization of circulating progenitor cells [5,12]. However, the exact mechanism underlying the effect of EPO on mobilization of circulating progenitors is unknown.

In the present study, we hypothesized that mobilization of stem and progenitor cells by EPO is critically dependent on activation of eNOS. To test this hypothesis, we enumerated circulating stem/progenitor cells in peripheral blood by flow cytometry and investigated the role of endothelial nitric oxide synthase (eNOS) in EPO-induced mobilization of hematopoietic stem/progenitor cells in wild type and eNOS$^{-/-}$ mice.

2. Methods

Male C57BL/6J (wild-type) and eNOS-deficient mice (C57BL/6J-*Nos3*^{tm1Unc}) (Jackson Laboratory, Bar Harbor, ME) were distributed into two groups: no treatment (n = 6 mice) and EPO treatment (n = 6 mice, recombinant human EPO 1000 U/kg body weight subcutaneous, bi-weekly, Amgen Thousand Oaks, CA)[10,12]. After 14 days, mice were anesthetized and euthanized by an intraperitoneal injection of pentobarbital (Nembutal Sodium, 60 mg/kg body weight). All the experimental protocols were approved by the Institutional Animal Care and Use Committee of the Mayo Clinic.

To enumerate circulating stem (c-kit$^+$/sca-1$^+$/lin-1$^-$, KSL) cells after EPO treatment, anticoagulated blood was collected by cardiac puncture in anesthetized mice. After removal of red blood cells by Ficoll Paque centrifugation, mononuclear cells in Hank's Balanced Salt Solution (HBSS, pH 7.4, Invitrogen) containing 0.5% bovine serum albumin and EDTA (1 mmol/l) were incubated at 4°C with rat anti-mouse antibodies (BD Biosciences) against FITC-conjugated Lin-1, PE-conjugated Sca-1, APC-conjugated c-kit (CD117) for 1 hour. IgG antibodies conjugated with the respective fluorochromes were incubated with mononuclear cells in parallel and served as controls. After incubation, cells were washed and fixed in 2% paraformaldehyde and cells were acquired using a FACS Calibur flow cytometer and data analyzed by Cell Quest Software (BD Biosciences, San Jose, CA).

The number of CD34-positive (CD34$^+$) cells in mice was measured by BD Procount Progenitor Cell Enumeration Kit (BD Biosciences, San Jose, CA) according to manufacturer's instructions. Briefly, 100 µl of mouse blood was divided into two BD Trucount™ tubes containing the Control reagent (IgG_1) and CD34 reagent (mouse monoclonal CD34) respectively, and incubated in dark for 15 minutes at room temperature. Subsequently, 450 µl FACS lysing solution was added to each tube, and the cells were acquired using a FACS Calibur flow cytometer and analyzed by Cell Quest Software (BD Biosciences, San Jose, CA).

Percentage of cells positive for both CD34$^+$ and Flk-1$^+$ in the mononuclear fraction was measured by incubating mononuclear cells with rat anti-mouse antibodies (BD Biosciences) against CD34 and Flk-1 for 1 hour in dark and analyzed as described for KSL cells.

The profile of blood cells in wild type and eNOS-deficient mice treated with EPO were performed with ABAXIS VetScan HMII Hematology System (Union City, CA), as reported in our previous study [10].

Results are expressed as means ± SEM and "n" indicates the number of animals. For comparisons between two groups, an un-paired Student's t-test was used where appropriate. Multiple comparisons were performed by one way ANOVA followed by Bonferroni post hoc test. A value of $P<0.05$ was considered significant.

3. Results

Bi-weekly treatment with EPO for 14 days significantly increased the number of reticulocytes (3 fold), erythrocytes (1.2 fold), hematocrit (1.3 fold) and hemoglobin levels (1.2 fold), while circulating white blood cells and platelets remained unchanged (Table 1). The representative dot plots for quantification of KSL-cells are presented in Supplemental Figure S1. The percentage of KSL-cells was significantly elevated in blood after EPO treatment in wild type mice (Figure 1a), while absence of eNOS abolished the EPO-induced mobilization of KSL-cells. Treatment with EPO produced five-fold increase in circulating levels of $CD34^+$ cells in wild type mice (Supplemental Figure S2 and Figure 1b). The elevation in $CD34^+$ levels by EPO treatment was significantly attenuated in eNOS-deficient mice (Figure 1b). The representative dot plots for quantification of $CD34^+/Flk\text{-}1^+$ cells are presented in Supplemental Figure S3.The number of cells expressing both CD34 and Flk-1 (VEGFR2), were also significantly increased on EPO-treatment (Figure 1c). In eNOS-deficient mice, EPO failed to elevate the number of circulating $CD34^+/Flk\text{-}1^+$ cells (Figure 1c).

4. Discussion

Our findings demonstrate that activation of eNOS is a critical mechanism responsible for the stimulatory effect of EPO on elevation of KSL, $CD34^+$ and $CD34^+/Flk\text{-}1^+$ cells in circulating blood. These observations offer important mechanistic insights into the vascular and tissue protective effects of EPO.

In addition to representing hematopoietic stem cells, $c\text{-}Kit^+/Sca\text{-}1^+/Lin\text{-}1^-$ (KSL) cells in circulating blood have also been considered vascular progenitor cells derived from bone marrow [21,22,24]. $CD34^+$ cells (hematopoietic progenitors) are population of cells enriched with EPCs [4,18], and $CD34^+/Flk\text{-}1^+$ cells participate in therapeutic neovascularization [20]. Recombinant human EPO mobilizes $CD34^+/Flk\text{-}1^+$ cells from the bone marrow in animals as well as in humans [5,12]. Darbepoetin, an analogue of EPO, also elevated the circulating levels of $CD34^+$ cells in patients with renal anemia [6]. Consistent with these reports, we demonstrated that bi-weekly treatment with EPO significantly mobilized hematopoietic progenitor cells, including VEGFR2-expressing progenitor cells.

Endothelial nitric oxide synthase (eNOS), the enzyme responsible for nitric oxide (NO) production in endothelium, plays an essential role in stem and progenitor cell mobilization in response to VEGF, statins and estrogen [1,13,16]. Impaired VEGF-induced mobilization of EPCs was observed in eNOS-deficient mice [1,11]. Based on the ability of EPO to activate PI3K/Akt pathway (like VEGF and estrogen), it was speculated that mobilization of progenitor cells by EPO was likely to be mediated by eNOS [2,26]. We present evidence demonstrating that indeed, EPO-induced mobilization of progenitor cells is dependent on activation of eNOS. Pro-angiogenic population of cells has now been identified in almost all hematopoietic cell lineages [15]. The ability of EPO to stimulate mobilization of both hematopoietic stem and progenitor cells, irrespective of their phenotypic character, is indicative of the unique ability of EPO to participate in angiogenesis as well as repair of injured endothelium.

Under physiologic conditions, only low numbers of progenitor cells circulate, and mobilization of progenitor cells is assumed to be stimulated by ischemic insults [9]. In accordance, EPO stimulated ischemia-induced angiogenesis and repair of injured arterial wall by mobilization

of progenitor cells [5,12,25]. In the present study, we demonstrate that EPO stimulates mobilization of hematopoietic progenitor cells through an Enos-dependent mechanism even in the absence of ischemia. The observed effects of EPO are similar to the effects of VEGF and estrogen. The ability to activate PI3K/Akt pathway provides a plausible signaling mechanism underlying EPO-induced mobilization of progenitor cells [2]. In conclusion, the present study presents evidence that EPO stimulates mobilization of cells known to have beneficial effect on angiogenesis and endothelial repair, and this effect is critically dependent on activation of eNOS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Figure 1a

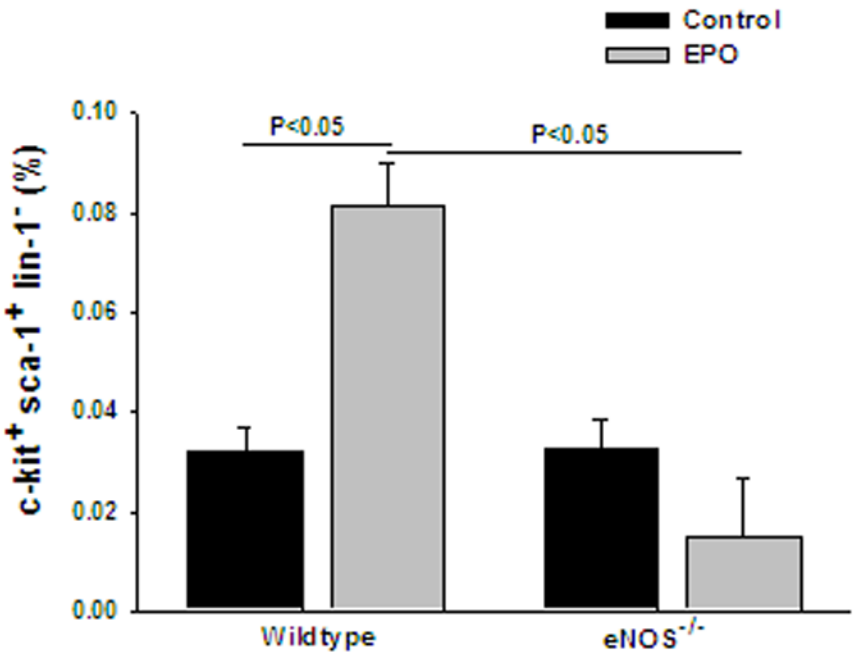

Figure 1.

(a) Bar diagram representing c-kit^{+}/sca-1^{+}/lin-1^{-} (KSL) cell population in wild type and eNOS$^{-/-}$ mice after treatment with EPO. Gated mononuclear cells that stained negative for lin-1-FITC were further analyzed for co-expression of sca-1-PE (FL2) and c-kit-APC (FL4), and KSL cell population were represented as percentage of gated events. **(b)** Bar diagram representing enumeration of CD34^{+} cells by Procount progenitor cell enumeration kit (BD Biosciences). Events gated in region R3 are cells expressing CD34. **(c)** Bar diagram representing cells co-expressing CD34^{+} (FITC, FL-1) and Flk-1 (PE, FL-2), in wild type and eNOS$^{-/-}$ in the presence of EPO. EPO-mediated increase in KSL cells, CD34^{+} cells and CD34^{+}/Flk-1^{+} cells were abolished in eNOS$^{-/-}$ mice. Data are represented as mean ± SEM of six mice in each group.

Table 1

Profile of Blood Cells in wild type and eNOS-deficient mice after EPO treatment for 14 days

Parameters	Wild type mice		eNOS$^{-/-}$ mice	
	Control	EPO	Control	EPO
White blood cells, 10^3/mm^3	11.44 ± 0.62	12.18 ± 0.90	11.01 ± 1.23	10.63 ± 1.17
Lymphocytes, 10^3/mm^3	10.47 ± 0.57	11.13 ± 0.81	9.27 ± 0.89	9.74 ± 0.91
Granulocytes, 10^3/mm^3	0.72 ± 0.10	0.44 ± 0.09	1.41 ± 0.58	0.38 ± 0.25
Monocytes, 10^3/mm^3	0.31 ± 0.07	0.82 ± 0.25	0.32 ± 0.09	0.54 ± 0.31
Red blood cells, 10^6/mm^3	10.47 ± 0.20	12.96 ± 0.33***	10.60 ± 0.22	11.56 ± 0.12
Hematocrit, %	44.04 ± 0.71	55.47 ± 0.93***	44.76 ± 0.59	51.68 ± 0.29***
Hemoglobin, g/dL	15.11 ± 0.17	18.96 ± 0.34***	15.23 ± 0.33	20.17 ± 0.61***
Platelet, 10^3/mm^3	859.4 ± 35.1	764.8 ± 31.1	808.0 ± 47.9	681.0 ± 41.3

Data are represented as mean ± SEM of 4–8 mice.

*** P<0.001 in EPO-treated mice as compared to wild type controls.