Erythropoietin Induces Lymph Node Lymphangiogenesis and Lymph Node Tumor Metastasis

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Abstract

Cancer therapy often produces anemia, which is treated with erythropoietin (EPO) to stimulate erythrocyte production. However, concerns have recently arisen that EPO treatment may promote later tumor metastasis and mortality. The mechanisms underlying such effects are unknown, but it is clear that EPO has pleiotropic effects in cell types other than hematopoietic cells. In this study, we investigated how EPO affects lymphangiogenesis and lymph node tumor metastasis in mouse models of breast cancer and melanoma. In these models, EPO increased lymph node lymphangiogenesis and lymph node tumor metastasis in a manner associated with increased migration, capillary-like tube formation, and dose- and time-dependent proliferation of human lymphatic endothelial cells. EPO increased sprouting of these cells in a thoracic duct lymphatic ring assay. These effects were abrogated by cotreatment with specific inhibitors of phosphoinositide 3-kinase or mitogen-activated protein kinase, under conditions in which EPO increased Akt and extracellular signal-regulated kinase 1/2 phosphorylation. Intraperitoneal administration of EPO stimulated peritoneal lymphangiogenesis, and systemic treatment of EPO increased infiltration of CD11b+ macrophages in tumor-draining lymph nodes. Finally, EPO increased VEGF-C expression in lymph node-derived CD11b+ macrophages as well as in bone marrow-derived macrophages in a dose- and time-dependent manner. Our results establish that EPO exerts a powerful lymphangiogenic function and can drive both lymph node lymphangiogenesis and nodal metastasis in tumor-bearing animals.

Introduction

Erythropoietin (EPO) is a specific stimulator of erythropoiesis, exhibiting pleiotropic effects in various cell types other than hematopoietic cells (1–3). Anemia is associated with low quality of life in cancer patients who receive chemotherapy and with poor response to cancer treatment (4). Treatment of anemia with EPO may increase quality of life and prevent complications associated with blood transfusions in cancer patients. EPO treatment increases radiosensitivity of tumor, therewith increasing survival rate among patients with cancer (5). However, clinical trial data have indicated that EPO treatment does not increase the survival rate of cancer patients with head and neck (6), lung (7), or breast (8) cancer. In fact, EPO may accelerate tumor progression (6–8). Although the exact mechanism of EPO-induced tumor progression remains to be understood, several mechanisms of EPO-induced tumor progression have been proposed. EPO may increase EPO receptor (EPOR) expression in preexisting tumor cells, thereby promoting tumor metastasis (6, 8–10). It increases the growth of tumors lacking EPOR through tumor angiogenesis (11). Thus, increased peritumoral angiogenesis through direct or indirect effects on endothelial cells has been introduced as another mechanism in EPO-induced tumor progression (9, 12–14).

In addition, malignant tumors, including melanoma and breast cancers, preferentially metastasize via lymphatic vessels to regional lymph nodes, and peritumoral lymphangiogenesis predicts lymph node metastasis (15). Lymph node lymphangiogenesis has also been shown to be associated with lymphatic metastases in animal models (16, 17). It has been shown that the lymphangiogenesis in the sentinel lymph node is initiated before tumor metastasis in the lymph nodes (18, 19). Thus, lymph node lymphangiogenesis can be an important factor in lymph node tumor metastasis. However, a direct link between EPO and lymph node lymphangiogenesis has not been reported.
VEGF-C is an angiogenic factor mainly involved in lymphatic endothelial cell (LEC) growth. Macrophages produce and secrete VEGF-C and play a critical role in lymphangiogenesis (19). VEGF-C from macrophages induces lymphangiogenesis in advanced ovarian cancer (20). In a mouse breast cancer model, the tumor progression is related to macrophage infiltration in the primary tumors (21). Despite these correlations between macrophage and tumor lymphangiogenesis, the effect of EPO on macrophages in promoting tumor and lymph node lymphangiogenesis is largely unexplored.

In this study, we investigated an involvement of EPO in lymph node lymphangiogenesis, using a murine model of melanoma and breast cancer and human lymphatic endothelial cells (hLEC). The molecular basis of EPO-mediated lymphangiogenesis and role of macrophages in lymphangiogenesis were also examined in vitro and ex vivo. The results showed that EPO induces lymphangiogenesis through increasing VEGF-C expression in lymph node CD11b⁺ macrophages and bone marrow–derived macrophages and that EPO-induced lymphangiogenesis is mediated through the Akt- and extracellular signal–regulated kinase (ERK) 1/2-dependent pathway.

Materials and Methods

Methods are described in greater detail in Supplementary Materials and Methods.

Animal experiments

**Breast cancer mouse model.** The animal experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Chonbuk National University. MDA-MB-231 cells (American Type Culture Collection) were inoculated into the left flank of female nude mice (Orient Bio Inc.; 18–20 g body weight; ref. 22). EPO at a dose of 5,000 IU/kg was injected s.c. 3 times per week. After 14 days, lymph node volume was measured as previously described (23) and processed for immunofluorescent staining (24).

**Melanoma cancer mouse model.** C57BL/6 mice (Orient Bio Inc.) were injected with 1 x 10⁶ B16-F10 cells (American Type Culture Collection) in a 100-μL volume of PBS in the left flank. EPO at a dose of 5,000 IU/kg was injected s.c. 3 times per week. After 7 days, lymph node volume was measured as previously described (23) and harvested for immunofluorescent staining and isolation of CD11b⁺ macrophages (24).

**Peritoneal lymphangiogenesis.** For EPO-induced peritoneal lymphangiogenesis, the indicated dose of EPO (5,000 IU/kg) in 200 μL of PBS was injected into the peritoneal cavity daily for 7 days.

Immunofluorescence

To determine the metastasis of MDA-MB-231 human breast cancer cells to mouse axillary lymph nodes, mouse lymph nodes were stained with an antibody against human mitochondria (Millipore), which specifically acts on human cells to evaluate lymph node metastasis of breast cancer. Mouse lymph node sections were immunostained with an antibody against pan-cytokeratin (Abcam) to evaluate the metastasis of melanoma cancer cells to mouse axillary lymph nodes. Frozen sections of lymph nodes were immunostained with antibodies against LYVE-1 (AngioBio), Prox-1 (ReliaTech), and Podoplanin (R&D Systems) to detect LECs in lymph nodes. An antibody against PECAM-1 (Millipore) was used to evaluate the changes of vascular endothelial cells in lymph nodes.

Cell culture

hLECs (Lonza) were incubated and maintained in endothelial basal medium-2 (EBM-2) medium (Lonza) with 5% (v/v) heat-inactivated FBS at 37°C in a 5% CO₂/95% O₂ incubator. For bone marrow–derived macrophages, bone marrow was isolated from femurs and tibias, and cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS and 30% L929 conditioned media. Recombinant human EPO (Epokine) was purchased from CJ Pharm. Recombinant human VEGF-A 165 was purchased from R&D Systems. Mitogen-activated protein kinase (MAPK)/ERK kinase (MEK) inhibitor, PD98059, the phosphoinositide 3-kinase (PI3K) inhibitor, LY294002, wortmannin, gelatin, antibiotics, and antifungicides were from Sigma-Aldrich.

Migration assay

The migration assay with hLECs was conducted using a modified Boyden chamber (NeuroProbe) as described previously (25).

Capillary-like tube formation assay

In vitro tube formation assay was conducted in a 3-dimensional culture of hLECs on ECM gel (Sigma-Aldrich; ref. 25).

Cell proliferation by XTT assay

After 48 hours of treatment with EPO (5, 10, or 20 IU/mL), proliferation of hLECs was measured using a Cell Proliferation Kit II (XTT; Roche) in accordance with the manufacturer’s protocol.

Immunoblotting

hLECs treated with various drugs were harvested, homogenized, and then immunoblotted as described previously (26).

Thoracic duct collection and 3-dimensional lymphatic ring assay

Identification and harvest of thoracic ducts were conducted as described previously (27). EPO (5, 10, or 20 IU/mL), control buffer, or recombinant mouse VEGF-A (R&D Systems) was added to the culture medium at the beginning of the experiment, as appropriate. Anti-EPO (Santa Cruz Biotechnology) or anti-VEGF-A (ReliaTech) was used to evaluate the blocking effect of EPO or VEGF-A on sprouting of LECs.

Isolation of CD11b⁺ cells from draining lymph nodes by MACS

CD11b⁺ macrophages in the lymph nodes were enriched using anti-mouse CD11b⁺ antibody–coupled MicroBeads.
Reverse transcriptase-PCR of lymphangiogenic factors
The semiquantitative and quantitative reverse transcriptase-PCR (qRT-PCR) of total RNA isolated from hLECs, MACS-enriched CD11b\(^+\) cells, and bone marrow–derived macrophages was conducted.

Statistical analysis
Data are expressed as mean ± SD. Student t test or one-way ANOVA was conducted to compare the means of normally distributed continuous variables. Because the lymph node volume represented a skewed distribution in this experiment, a Box–Cox transformation was conducted, converting the lymph node volumes into log\(_{10}\)-transformed values (28). The level of statistical significance was set at \(P < 0.05\).

Results

EPO promotes sentinel lymph node lymphangiogenesis and lymph node tumor metastasis in breast cancer model
We evaluated the effects of EPO on lymph nodes in a breast cancer model by determining the volume, lymphangiogenesis, and tumor lymph node metastasis. Lymph nodes from the mice treated with EPO had larger volumes than those from the mice treated with control buffer (Fig. 1A and B). The weight of lymph nodes from the mice treated with EPO was heavier than that from the mice treated with control buffer (data not shown).

To examine whether EPO treatment increases lymph node lymphangiogenesis, we immunostained lymph node sections using antibodies against LYVE-1 and Prox-1. An increase in LYVE-1- and Prox-1–positive lymphatic vessels was found in sentinel lymph nodes of EPO-treated mice (Fig. 1C).

To evaluate the effect of EPO on LYVE-1–positive lymphatics and PECAM-1–positive vessels in tumor-draining lymph nodes, we also immunostained the lymph nodes by using LYVE-1 and PECAM-1 antibodies. Immunofluorescent staining of the sentinel lymph nodes showed that LYVE-1–positive lymphatic or PECAM-1–positive vessels in the lymph nodes of mice compared with those of control mice (Fig. 2D and E). EPO treatment also increased the density of pan-cytokeratin–positive melanoma cells in sentinel lymph nodes (Fig. 2D). The mean hematocrit level of EPO-treated mice was significantly higher than that of mice treated with control buffer (Fig. 2F).

LYVE-1, Prox-1, Podoplanin, and EPOR are expressed in hLECs
To confirm the identity of LECs and to evaluate whether EPOR is expressed in hLECs, LEC makers were examined by immunostaining using respective antibody. The results showed that the LEC markers LYVE-1, Podoplanin, and Prox-1 were expressed in hLECs (Supplementary Fig. S1), and EPORs were also expressed in hLECs (Supplementary Fig. S2).

EPO induces migration, capillary-like tube formation, and proliferation of hLECs
To investigate whether EPO has in vitro lymphangiogenic activity, we conducted migration, capillary-like tube formation, and proliferation assays with hLECs. EPO increased the number of migrating hLECs in a dose-dependent manner (Fig. 3A). Because our data indicated that EPO is a relatively strong inducer of migration, we examined the effect of EPO on capillary-like tube formation of hLECs in ECM gel. After 16 hours of treatment with EPO, tube formation was increased in a dose-dependent manner (Fig. 3B and C). EPO at 20 IU/mL increased capillary-like tube formation approximately 18.5-fold over that of control buffer–treated hLECs. We estimated the degree of hLECs proliferation with a 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)–5-carboxanilide inner salt (XTT) assay. In hLECs treated with EPO for 48 hours, EPO significantly increased cell proliferation over that of control (Fig. 3D). VEGF-A was used as a positive control.

EPO increases phosphorylation of MAPK and Akt in hLECs
To determine whether the MAPK and Akt signaling pathway is involved in EPO-induced lymphangiogenesis, we examined the phosphorylation of ERK1/2 (p44/p42 MAPK) and Akt. EPO increased ERK1/2 phosphorylation in as short a time as 5 minutes and produced a maximal effect at 10 minutes after EPO treatment, and then phosphorylation returned to the control level at 15 minutes (Fig. 4A). The maximum mean increase in ERK1/2 phosphorylation was 2.2-fold. EPO also increased ERK1/2 phosphorylation in a dose-dependent manner (Fig. 4B). Pretreatment with an MEK inhibitor, PD98059, significantly decreased EPO-induced ERK1/2 phosphorylation at 10 minutes. EPO also increased...
Akt phosphorylation in a time-dependent manner (Fig. 4C). Treatment of hLECs with EPO (20 IU/mL) produced maximal phosphorylation of Akt (1.4-fold) over control at 10 minutes (Fig. 4C). The maximum mean increase in Akt phosphorylation was 1.4-fold. EPO increased Akt phosphorylation in a dose-dependent manner (Fig. 4D). Pretreatment with a PI3K inhibitor, LY294002, or wortmannin significantly decreased the EPO-induced Akt phosphorylation at 10 minutes. Treatment with the control buffer alone did not show any significant effects on the phosphorylation of ERK1/2 and Akt in hLECs (Supplementary Fig. S6).

**EPO induces in vitro lymphangiogenesis of hLECs through PI3K- and MEK-dependent pathway**

We evaluated whether EPO is capable of inducing lymphangiogenesis, migration, capillary tube formation, and sprouting formation of hLECs through PI3K- and MEK-dependent pathway. Inhibition of Akt or ERK1/2 pathway with...
LY294002, wortmannin, or PD98059 significantly suppressed EPO-induced migration of hLECs (Supplementary Fig. S3A). These inhibitors also significantly decreased EPO-induced capillary tube formation of hLECs and partially suppressed EPO-induced sprouting formation (Supplementary Fig. S3B and C). In addition, LY294002, wortmannin, or PD98059 significantly inhibited EPO-induced proliferation of hLECs (Supplementary Fig. S3D). Treatment with the inhibitors alone did not show any significant effects on migration, capillary tube formation, or proliferation sprouting of hLECs (Supplementary Fig. S3).

**EPO induces sprouting in a lymphatic ring assay**

Lymphatic ring assay was used to assess the spreading of LECs from a preexisting vessel, cell proliferation, migration, and differentiation into capillaries (27). An effect of EPO on sprouting of LECs from mouse thoracic ducts was evaluated using a lymphatic ring assay. After 7 days, EPO increased the
number of LEC sprouts by approximately 10.4-fold (Fig. 5A and B). A positive control, VEGF-A, increased the number of LEC sprouts by approximately 16.9-fold. Treatment with an antibody against EPO or VEGF reversed the effect of EPO or VEGF-A, respectively, on sprouting of LECs (Fig. 5A and B).

**Intraperitoneal administration of EPO induces peritoneal lymphangiogenesis**

We administered EPO to mice intraperitoneally once daily for 7 days. The mice were then sacrificed and the diaphragm was harvested. To evaluate whether EPO increased the number of LECs in peritoneum, we immunostained the diaphragm with an anti–LYVE-1 antibody. LYVE-1 immunostaining of the diaphragm from control buffer–treated mice revealed the typical distribution of lymphatic vessels on the peritoneal sides of the diaphragm. The density of LYVE-1–positive lymphatic vessel on the peritoneal side of the diaphragm muscle and in the central tendon was higher in EPO-treated mice than in control mice (Fig. 5C–E).

**EPO does not increase mRNA expression of other lymphangiogenic factors in hLECs**

To evaluate the mechanism of the lymphangiogenic effect of EPO through other possible lymphangiogenic factors, we...
conducted a series of RT-PCRs of VEGF-A, VEGF-C, VEGF-D, angiopoietin (Ang)-1, Ang-2, platelet-derived growth factor (PDGF)-A, PDGF-B, fibroblast growth factor (FGF)-1, and FGF-2 after treatment of hLECs with EPO. None of these genes had changed their expression levels after treatment of hLECs with EPO for 4 hours (Supplementary Fig. S4).

**EPO increases CD11b⁺ macrophage infiltration in draining lymph nodes from mice with melanoma and breast cancer**

The expression of VEGF-C increased in tumor-associated macrophages, thereby increasing lymphatic vessel growth (29, 30). In a melanoma or breast cancer model used in this study, the lymphangiogenesis was observed in tumor-draining lymph nodes. Therefore, we investigated whether EPO increases the number of CD11b⁺ lymph node macrophages in the melanoma or breast tumor model by immunostaining lymph node sections with a CD11b⁺ antibody. Our data revealed that EPO increased CD11b⁺ macrophage infiltration in draining lymph nodes of melanoma and breast tumor mice by 1.7-fold and 1.3-fold, respectively, over those in draining lymph nodes of mice treated with melanoma and breast tumor (Fig. 6). EPO treatment alone had no effect on the number of CD11b⁺ macrophages in draining lymph nodes compared with the number in mice treated with control buffer alone (Fig. 6B and D).

**EPO increases VEGF-C expression in CD11b⁺ macrophages from lymph nodes and bone marrow-derived macrophages**

Because VEGF-C expression is increased in tumor-associated macrophages, we evaluated whether EPO increases VEGF-C mRNA levels in CD11b⁺ macrophages from lymph nodes of melanoma tumor mice (24, 29, 30). Quantitative real-time PCR (qRT-PCR) analysis revealed that EPO increased VEGF-C mRNA levels (~1.6-fold) compared with those of mice treated with control buffer in the melanoma tumor model.
To evaluate whether EPO increases VEGF-C mRNA levels in bone marrow–derived macrophages, we conducted a qRT-PCR analysis and immunoblotting with bone marrow–derived macrophages after treatment with or without EPO. The results showed that EPO treatment significantly increased VEGF-C mRNA expression in a time- and dose-dependent manner in bone marrow–derived macrophages (Fig. 7B and C). The expression of VEGF-C protein also increased in a time- and dose-dependent manner after treatment with EPO (Fig. 7D and E).
Discussion

Several mechanisms of EPO involvement in tumor progression have been suggested: EPO increases EPOR expression in preexisting tumor cells, thereby increasing tumor growth and metastasis, or directly promotes tumor vascular angiogenesis (6, 8–10). In this study, we showed that EPO increases LEC density and tumor metastasis in lymph nodes. Therefore, EPO-induced lymph node lymphangiogenic effects may have a role in tumor progression.

It is well known that lymphangiogenesis in peritumoral area and lymph node contributes to tumor metastasis through tumor-draining lymph nodes (31, 32). EPO is a growth factor with pleiotropic effects. Therefore, it can be suggested that EPO may induce lymphangiogenesis and is associated with tumor metabolism. We showed that EPO induces sentinel lymph node lymphangiogenesis and is associated with lymph node tumor metastasis in a mouse human breast cancer xenograft and melanoma tumor model (Figs. 1D and E and 2D and E). EPO treatment also increased lymph node volume compared with that of control (Figs. 1D and E and 2A and B). These results suggest that EPO-induced lymph node lymphangiogenesis is a new mechanism of lymph node tumor metastasis.

Malignant tumors can increase lymphangiogenesis and metastasize through the newly formed lymphatic system. The growth factors associated with tumor lymphangiogenesis are VEGF-A (16), VEGF-C (33), VEGF-D (34), FGF-2 (35), Ang-1 (36), Ang-2 (37), and PDGF-BB (38). Recently, it has also been shown that EPO-induced release of PDGF-BB increases smooth muscle cell–rich vascular lesions in mice (39). However, our results showed that mRNA levels of these lymphangiogenic factors in LECs are not changed by treatment with EPO (Supplementary Fig. S4). These findings suggest that other lymphangiogenic growth factors are not involved in lymphangiogenesis.

EPO-mediated invasion, migration, and adhesion of rat mammary cells are regulated through activation of PI3K/Akt and ERK (40). It has also been shown that EPO increases proliferation and migration of breast cancer cells through
activation of ERK1/2, Akt, and stress-activated protein kinase/c-Jun-NH2-kinase (SAPK/JNK) pathway (41). EPO-induced angiogenesis in retinal endothelial cell is associated with the PI3K/Akt-dependent pathway (42). Consistent with these observations, our results showed that EPO induces migration, proliferation, tube formation of hLECs, and sprouting through ERK1/2- and the PI3K-dependent pathway (Fig. 4).

Macrophages are associated with tumor angiogenesis, tumor metastasis, and immunoregulation (43). Macrophages also produce VEGF-C, a lymphangiogenic factor (24). In this study, the results revealed that EPO increases VEGF-C mRNA levels from lymph node CD11b+ macrophages from lymph nodes and bone marrow-derived macrophages. A, VEGF-C mRNA levels in the lymph node macrophages as determined by qRT-PCR. CD11b+ cells from the draining lymph nodes 7 days after B16-F10 cells in melanoma tumor model were enriched by MACS and qRT-PCR of VEGF-C was conducted. Data are presented as the relative fold to CB after normalization with glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Bars, means ± SD from 3 independent experiments. B and C, VEGF-C mRNA levels in bone marrow-derived macrophages as determined by qRT-PCR. Bone marrow-derived macrophages were incubated with EPO (10 IU/mL) for the indicated times and doses. qRT-PCR of VEGF-C was conducted. Data are presented as the relative fold to CB after normalization with GAPDH. Bars, means ± SD from 3 independent experiments. D and E, immunoblot analyses for VEGF-C. Bone marrow-derived macrophages were incubated with EPO (10 IU/mL) for the indicated times and doses. Bars, means ± SD from 3 independent experiments. D and E, immunoblot analyses for VEGF-C. Bone marrow-derived macrophages were incubated with EPO (10 IU/mL) for the indicated times and doses. Bar graph represents the relative ratio of VEGF-C to actin. The relative ratio measured at time 0, or the ratio relative to CB, is arbitrarily presented as 1. Numbers, means ± SD from 4 independent experiments. *, P < 0.05 versus CB or time 0; **, P < 0.01 versus CB or time 0.

These observations suggest that EPO treatment in cancer patients may increase the production of VEGF-C protein in macrophages in lymph nodes, and this increased expression of VEGF-C may induce lymphangiogenesis. Thus, EPO can increase tumor lymph node metastasis through a direct lymphangiogenic effect on LECs and indirectly via VEGF-C production by macrophages.

In summary, our results reveal that systemic administration of EPO increases lymph node volume, axillary lymph node diameter, lymph node tumor metastasis, and lymph node lymphangiogenesis in a mouse model of a human breast cancer xenograft and melanoma tumor model of mice. EPO induces migration, capillary-like tube formation, and proliferation of hLECs as well as lymphatic endothelial sprouting via the PI3K/Akt and ERK1/2 signaling pathway.
Peritoneal administration of EPO also induces lymphangiogenesis. EPO also increased VEGF-C expression in bone marrow–derived macrophages. All of these data suggest that EPO can be a new lymphangiogenic factor and that EPO-induced lymphangiogenesis may be associated with lymph node tumor metastasis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


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