Erythropoietin induces lymph node lymphangiogenesis and lymph node tumor metastasis

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Abstract

Cancer therapy often produces anemia in patients that is treated with erythropoietin (EPO) to stimulate red blood cell 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 co-treatment with specific inhibitors of PI3K or MAP kinase, under conditions where EPO increased Akt and ERK1/2 phosphorylation. Intraperitoneal administration of EPO stimulated peritoneal lymphangiogenesis and systemic treatment of EPO increased infiltration of CD11b+ macrophages in tumor draining lymph nodes. Lastly, 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). Anemia correction 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 cancer (8). In fact, EPO may accelerate tumor progression (6-8). Although 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). EPO 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 demonstrated 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.

Vascular endothelial growth factor (VEGF)-C is an angiogenic factor mainly involved in lymphatic endothelial cell growth. Macrophages produce, secrete VEGF-C, and play a critical role in lymphangiogenesis (19). VEGF-C from macrophage induces lymphangiogenesis in advanced ovarian cancer (20). In a mouse breast cancer model, the tumor progression is related with 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 murine model of melanoma and breast cancer and human lymphatic endothelial cells (hLECs). The molecular basis of EPO-mediated lymphangiogenesis and role of macrophages in lymphangiogenesis were also examined in 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 ERK1/2 dependent pathway.
Materials and Methods

(Full methods are described in the 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 (Manassas, VA) were inoculated into the left flank of female nude mice (Orient Bio Inc., Seoul, Korea; 18–20 g body weight) (22). EPO at a dose of 5,000 IU/kg was injected subcutaneously 3 times per week. After 14 d, 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 $1 \times 10^6$ B16-F10 cells (American Type Culture Collection) in a 100μL volume of PBS in the left flank. EPO at a dose of 5,000 IU/kg was injected subcutaneously 3 times per week. After 7 d, lymph node volume were 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 d.
**Immunofluorescence**

To determine the metastasis of MDA-MB-231 human breast cancer cells to mouse axillary lymph node, mouse lymph nodes were stained with an antibody against human mitochondria (Millipore, Billerica, MA), 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, Cambridge, MA) 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, Del Mar, CA), Prox-1 (ReliaTech, Braunschweig, Germany), and Podoplanin (R&D systems, Minneapolis, MN) to detect lymphatic endothelial cells in lymph nodes. An antibody against PECAM-1 (Millipore) was used to evaluate the changes of vascular endothelial cells in lymph node.

**Cell culture**

hLECs (Lonza, Basel, Switzerland) were incubated and maintained in EBM-2 medium (Lonza) with 5% (vol/vol) heat-inactivated fetal bovine serum (FBS) at 37°C in 5% CO₂/95% O₂ incubator. For bone marrow-derived macrophages, bone marrow was isolated from femurs and tibias, and cultured in DMEM supplemented with 10% FBS and 30% L929 conditioned media. Recombinant human EPO (Epokine®) was purchased from CJ Pharm. (Seoul, Republic of Korea). Recombinant human VEGF-A 165 was purchased from R&D Systems (Minneapolis, MN). Mitogen-activated protein kinase (MAPK)/ERK kinase (MEK) inhibitor, PD98059, the phosphatidylinositol 3'-kinase (PI3K) inhibitor, LY294002, wortmannin, gelatin, antibiotics and antimycotics were from Sigma-Aldrich (St. Louis, MO).
Migration assay

The migration assay with hLECs was performed using a modified Boyden chamber (NeuroProbe, Cabin John, MD) as described previously (25).

Capillary-like tube formation assay

*In vitro* tube formation assay was performed in a three-dimensional culture of hLECs on ECM gel (Sigma-Aldrich) (25).

Cell proliferation by XTT assay

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

Immunoblotting

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

Thoracic duct collection and three-dimensional lymphatic ring assay

Identification and harvest of thoracic ducts were performed as described previously (27). EPO (5, 10, or 20 IU/mL), control buffer, or recombinant mouse VEGF-A (R&D Systems) were added to the culture medium at the beginning of the experiment as appropriate. Anti-

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EPO (Santa Cruz biotechnology, Santa Cruz, CA) 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 (Miltenyi Biotec, Auburn, CA) and a magnetic cell sorter (Miltenyi Biotec) as described previously (24).

**Reverse-transcription polymerase chain reaction (RT-PCR) of lymphangiogenic factors**

The semiquantitative and quantitative RT-PCR of total RNA isolated from hLEC, MACS-enriched CD11b^+ cells and bone marrow-derived macrophages was performed.

**Statistical analysis**

Data are expressed as mean ± standard deviation (S.D.). Student’s *t* test or one-way ANOVA was performed 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 performed, 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 node 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 (Figures 1A and 1B). 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 (Figure 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 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 were increased in the lymph nodes from mice treated with EPO compared to those of control mice (Figures 1D and 1E). We also found that EPO treatment increased the density of human mitochondria–positive cells in mouse sentinel lymph node compared to that in mouse sentinel lymph node treated with control buffer, indicating that the human breast tumor cells had metastasized to the lymph nodes (Figure 1D). The mean hematocrit of EPO-treated mice was significantly higher than that of mice treated with control buffer (Figure 1F).
EPO also promotes sentinel lymph node lymphangiogenesis and lymph node tumor metastasis in melanoma cancer model

To ensure the above observations that EPO induced tumor-draining lymph node lymphangiogenesis and lymph node metastasis, a melanoma tumor mouse model was examined. Treatment the tumor-injected mice with EPO increased the volume of lymph nodes compared to those of the mice treated with control buffer (Figures 2A and 2B). LYVE-1– and Prox-1–positive lymphatic vessels were increased in sentinel lymph nodes of EPO-treated mice (Figure 2C). EPO increased the density of LYVE-1–positive lymphatic or PECAM-1–positive vessels in the lymph nodes of mice compared to those of control mice (Figures 2D and 2E). EPO treatment also increased the density of pan cytokeratin–positive melanoma cells in sentinel lymph nodes (Figure 2D). The mean hematocrit of EPO-treated mice was significantly higher than that of mice treated with control buffer (Figure 2F).

LYVE-1, Prox-1, Podoplanin and EPOR are expressed in hLECs

To confirm the identity of lymphatic endothelial cell and to evaluate whether EPOR is expressed on hLECs, lymphatic endothelial cell makers were examined by immunostaining using respective antibody. The results showed that the lymphatic endothelial cell markers, LYVE-1, Podoplanin and Prox-1 were expressed in hLEC (Supplementary Figure 1), and EPORs were also expressed on hLEC (Supplementary Figure 2).

EPO induces migration, capillary-like tube formation and proliferation of hLECs

To investigate whether EPO has in vitro lymphangiogenic activity, we performed migration,
capillary-like tube formation and proliferation assays with hLECs. EPO increased the number of migrating hLECs in a dose-dependent manner (Figure 3A). Since 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 h treatment with EPO, tube formation was increased in a dose-dependent manner (Figures 3B and 3C). 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 hLEC proliferation with an XTT assay. In hLECs treated with EPO for 48 h, EPO significantly increased cell proliferation over that of control (Figure 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 as early as 5 min and produced a maximal effect at 10 min after EPO treatment and then the phosphorylation was returned to the control level at 15 min (Figure 4A). The maximum mean increase in ERK1/2 phosphorylation was 2.2-fold. EPO also increased ERK1/2 phosphorylation in a dose-dependent manner (Figure 4B). Pretreatment with a MEK inhibitor, PD98059 significantly decreased EPO-induced ERK1/2 phosphorylation at 10 min. EPO also increased Akt phosphorylation in a time-dependent manner (Figure 4C). Treatment of hLECs with EPO (20 IU/mL) produced maximal phosphorylation of Akt (1.4-fold over control) at 10 min (Figure 4C). The maximum mean increase in Akt phosphorylation was 1.4-fold. EPO increased Akt phosphorylation in a dose-dependent manner (Figure 4D). Pretreatment with an PI3K inhibitor LY294002 or wortmannin significantly decreased the EPO-induced Akt phosphorylation at 10 min.
Treatment with the control buffer alone did not show any significant effects on the phosphorylation of ERK1/2 and Akt in hLECs (Supplementary figure 6).

**EPO induces in vitro lymphangiogenesis of hLEC 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 figure 3A). These inhibitors also significantly decreased EPO-induced capillary tube formation of hLECs and partially suppressed EPO-induced sprouting formation (Supplementary figures 3B and 3C). In addition, LY294002, wortmannin or PD98059 significantly inhibited EPO-induced proliferation of hLECs (Supplementary figure 3D). Treatment with the inhibitors alone did not show any significant effects on migration, capillary tube formation or proliferation sprouting of hLECs (Supplementary figure 3).

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

Lymphatic ring assay was used to assess the spreading of lymphatic endothelial cells (LEC) from a pre-existing 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 d, EPO increased the number of LEC sprouts approximately 10.4-fold (Figures 5A and 5B). A positive control, VEGF-A increased the number of LECs sprouts approximately 16.9-fold. Treatment with an antibody against EPO or VEGF reversed the effect of EPO or VEGF-A on sprouting of LECs, respectively (Figures 5A and 5B).
Intraperitoneal administration of EPO induces peritoneal lymphangiogenesis

We administered EPO to mice intraperitoneally once daily for 7 d. The mice were then sacrificed and the diaphragm was harvested. To evaluate whether EPO increased the number of lymphatic endothelial cells 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 (Figures 5C, 5D and 5E).

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 performed 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, FGF-2 after treatment of hLEC with EPO. None of these genes had changed their expression levels after treatment hLECs with EPO for 4 h (Supplementary Figure 4).

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 section 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 (Figure 6). EPO treatment alone had no effect on the number of CD11b+ macrophages in draining lymph node compared to the number in mice treated with control buffer alone (Figures 6B and 6D).

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

Since VEGF-C expression is increased in tumor-associated macrophages, we therefore evaluated whether EPO increases VEGF-C mRNA levels in CD11b+ macrophages from lymph nodes of melanoma tumor mice (24, 29, 30). Quantitative real-time RT-PCR analysis revealed that EPO increased VEGF-C mRNA levels (approximately 1.6-fold) compared to that of mice treated with control buffer in melanoma tumor model (Figure 7A). To evaluate whether EPO increases VEGF-C mRNA levels in bone marrow–derived macrophages, we performed a quantitative real-time RT-PCR 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 (Figures 7B and 7C). The expression of VEGF-C protein also increased in a time- and dose-dependent manner after treatment with EPO (Figures 7D and 7E).
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 demonstrated that EPO increases lymph node lymphatic endothelial cell density and tumor metastasis in lymph node. 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 contribute 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 associated with tumor metastasis. We demonstrated 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 (Figures 1D, 1E, 2D and 2E). EPO treatment also increased lymph node volume compared to that of control (Figures 1A, 1B, 2A and 2B). 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 lesion in mice (39). However, our results showed that mRNA levels of these lymphangiogenic factors in hLECs are not changed by treatment with EPO (Supplementary figure 4). 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 demonstrated that EPO increases proliferation and migration of breast cancer cells through activation of ERK1/2, Akt and c-Jun-NH2-kinase (SAPK/JNK) pathway (41). EPO-induced angiogenesis in retinal endothelial cell is associated with PI3K/Akt-dependent pathway (42). Consistent with these observations, our results showed that EPO induces migration, proliferation, tube formation of hLECs and sprouting formation through ERK1/2- and PI3K-dependent pathway (Figure 4).

Macrophages are associated with tumor angiogenesis, tumor metastasis, and immune regulation (43). Macrophages also produce VEGF-C, a lymphangiogenic factor (24). In this study, the results revealed that EPO increases VEGF-C mRNA level from lymph node CD11b+ macrophages of mice injected melanoma tumor and in bone marrow-derived macrophages in a time- and dose-dependent manner (Figures 6 and 7). 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 lymphatic endothelial cells and indirectly via VEGF-C production by macrophages.

In summary, our results have revealed 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 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.
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Figure legends

Figure 1. EPO promotes lymph node lymphangiogenesis and lymph node tumor metastasis in breast tumor model. (A) Gross findings of axillary lymph nodes from mice treated with EPO or control buffer (CB). (B) Volume of axillary lymph nodes. Nine mice (one lymph node from one mouse) in each group from breast tumor animal model. *, $P < 0.05$ versus CB-treated mice (C) Immunofluorescence of Prox-1 and LYVE-1 in lymph nodes from mice treated with CB or EPO. Bar=50μm. Note the increase in Prox-1− and LYVE-1− positive lymphatic endothelial cells in the lymph nodes after treatment with EPO. (D-E) Combination of images in lymph nodes. Triple-immunostaining of LYVE-1, PECAM-1 and human mitochondria (for human breast cancer cells) was carried out using frozen lymph node sections. EPO increased lymph node lymphangiogenesis and also increased lymph node metastasis of tumor cells compared with control mice. Bar=50μm. (E) Quantification of LYVE-1-positive lymphatic vessels, PECAM-1-positive endothelial cells and human mitochondria density. Metastatic sentinel lymph nodes of mice treated with EPO have increased density of LYVE-1−positive cells compared with control mice. Bars represent the means ± S.D. from five mice in each group. *, $P < 0.05$ versus PECAM-1 in CB-treated mice; #, $P < 0.05$ versus human mitochondria in CB-treated mice; **, $P < 0.01$ versus LYVE-1 in CB-treated mice (F) Hematocrit of mice treated with CB or EPO. Bars represent the means ± S.D from five mice in each group. **, $P < 0.01$ versus CB-treated mice.

Figure 2. EPO promotes axillary lymph node lymphangiogenesis and lymph node tumor metastasis in melanoma cancer model. (A) Gross findings of axillary lymph nodes from mice treated with EPO or control buffer (CB). (B) Volume of axillary lymph nodes. Nine mice (one lymph node from one mouse) in each group from melanoma tumor animal model.
*, $P<0.05$ versus CB-treated mice (C) Immunofluorescence of Prox-1 and LYVE-1 in lymph nodes from mice treated with CB or EPO. Bar=50µm. Note the increase of Prox-1− and LYVE-1−positive lymphatic endothelial cells in lymph nodes after treatment with EPO. (D-E) Combination of images in lymph nodes. Triple-immunostaining of LYVE-1, PECAM-1 and pan-cytokeratin (for melanoma tumor cells) was carried out using frozen lymph node sections. EPO increased lymph node lymphangiogenesis and also increased lymph node metastasis of tumor cells compared with control mice. Bar=50µm. (E) Quantification of LYVE-1-positive lymphatic vessels, PECAM-1-positive endothelial cells and pan-cytokeratin density. Metastatic sentinel lymph nodes of mice treated with EPO have increased density of enlarged LYVE-1−positive cells compared with control mice. Bars represent the means ± S.D. from five mice in each group. *, $P<0.05$ versus PECAM-1 in CB-treated mice; #, $P<0.05$ versus cytokeratin in CB-treated mice; **, $P<0.01$ versus LYVE-1 in CB-treated mice (F) Hematocrit of mice treated with CB or EPO. Bars represent the means ± S.D. from five mice in each group. **, $P<0.01$ versus CB-treated mice.

**Figure 3. EPO increases migration, tube formation and proliferation of hLECs.** (A) Control buffer (CB), EPO (5, 10 and 20 IU/mL), or VEGF-A (30 ng/mL) in EBM-2 containing 1% bovine serum albumin were placed in the bottom wells of the chamber. Cells that migrated through to the lower chamber were stained with Diff-Quik solution and counted at 200× magnification as described in “Materials and Methods.” Bars represent means ± S.D. from 4 independent experiments. (B) Representative phase-contrast photographs of capillary-like tube formation in ECM gel. Gels were incubated for 16 h in the presence of the indicated reagents. Capillary-like tube formation was assayed in three-dimensional matrices of ECM gel as described in “Materials and Methods.” Bar at lower
right =50 μm. (C) Quantification of capillary-like tube formation. Tube formation was quantified by the number of tubes using phase contrast microscopy. (D) hLECs were incubated in the presence of CB, EPO (5, 10 or 20 IU/mL) or VEGF-A (30 ng/mL) as indicated. After 24 h-incubation, hLEC proliferation was measured with an XTT assay. Bars represent means ± S.D. from 5 independent experiments. Bars represent means ± S.D. of 5 independent experiments. *, P <0.05 versus CB; **, P <0.01 versus CB; ***, P <0.001 versus CB.

Figure 4. EPO induces phosphorylation of ERK1/2 and Akt in hLECs hLECs were incubated for 16 h in EBM-2 containing 1% serum, then incubated with EPO (10 IU/mL) for the indicated times (A and C) and at the indicated concentrations for 10 min (B and D). The cells were also treated with PD98059 (PD; 50 μmol/L), LY294002 (LY; 10 μmol/L), or wortmannin (WT; 30 nmol/L) as indicated. After treatment, cell lysates were harvested. Each lane contains 20 μg of total protein from the cell lysates. Blots were probed with an anti–phospho-ERK1/2 antibody or anti–phospho-Akt (Ser473) antibody. The membranes were stripped and reprobed with an anti–ERK1 or anti–Akt antibody, respectively. Densitometric analyses are presented as the relative ratio of phospho-ERK1/2 to ERK1 or phospho-Akt to Akt. The relative ratio measured at time 0, or the ratio relative to control buffer (CB) is arbitrarily presented as 1. Numbers represent the means ± S.D. from 3 independent experiments. *, P <0.05 versus time 0 or CB; **, P <0.01 versus time 0 or control buffer; #, P <0.05 versus 10 min time point for p-ERK1/2 or p-Akt.
Figure 5. EPO induces sprouting in LECs and lymphangiogenesis on the peritoneal side of the diaphragm. (A) Representative phase-contrast photographs of sprouting activity. Thoracic ducts were placed in ECM gel. Gels were incubated in the presence of control buffer (CB), EPO (5, 10 IU/mL), EPO (10 IU/mL)+anti-EPO (4 μg/mL), VEGF-A (30 ng/mL) or VEGF-A+anti-VEGF-A (10 μg/mL) as indicated. Bar = 50 μm. (B) Quantification of the sprouting activities in the lymphatic ring assay. Total LEC sprouts from a thoracic duct were counted after 6 d. Data are means ± SD from 10 independent experiments. *, P<0.05 versus CB; **, P<0.01 versus CB; ***P<0.001 versus CB; ##, P<0.01 versus EPO (10 IU/mL); $$$, P<0.001 versus VEGF-A (C) Representative images of LYVE-1⁺ lymphatic vessels (black arrows) on the peritoneal side of the diaphragm muscle and central tendon. C57BL/6 mice were treated intraperitoneally with EPO (5,000 IU/mL) for 7 d and diaphragms were immunostained for LYVE-1 (brown) and visualized with DAB. (D and E) Densities of LYVE-1⁺ lymphatic vessels in the diaphragm muscle (D) and central tendon (E) were measured in each given area (18.4 mm²) and values are presented as a percentage of each area (n=4). Bars represent mean ± S.D. **, P<0.01 versus CB.

Figure 6. EPO increases CD11b⁺ macrophage infiltration in draining lymph node. (A and C) Immunofluorescence stain of LYVE-1 and CD11b in the lymph node. C57BL/6 mice were injected with B16-F10 cells (A) in a 100μL volume of control buffer (CB) in the left flank and MDA-MB-231 cells (C) were inoculated into the left flank of female nude mice with or without EPO. EPO at a dose of 5,000 IU/kg was injected subcutaneous 3 times per week. CB alone or EPO alone was injected in control mice. Axillary draining lymph nodes were dissected 7 d after injection cells and lymph node sections were co-immunostained for
LYVE-1 and CD11b and merged. (B and D) Quantitative score of CD11b-positive macrophages in lymph node. Bars represent the means ± S.D. from 4 mice in each group. Note that EPO increased CD11b⁺ macrophage infiltration compared to melanoma+CB or breast cancer+CB. Bar=20μm. **, $P<0.01$ versus CB alone; #, $P<0.05$ versus CB+breast cancer; ##, $P<0.01$ versus CB+melanoma

Figure 7. EPO increases increases VEGF-C expression in CD11b⁺ macrophage from lymph nodes and bone marrow–derived macrophages. (A) VEGF-C mRNA levels in the lymph node macrophages as determined by real-time PCR. CD11b⁺ cells from the draining lymph node 7 d after B16-F10 cells in melanoma tumor model were enriched by MACS and quantitative real-time RT-PCR of VEGF-C was performed. Data are presented as the relative fold to CB after normalization with GAPDH. Bars represent the means ± S.D. from 3 independent experiments. (B and C) VEGF-C mRNA levels in bone marrow–derived macrophages as determined by real-time PCR. Bone marrow–derived macrophages were incubated with EPO (10 IU/mL) for the indicated times and doses. Quantitative real-time RT-PCR of VEGF-C was performed. Data are presented as the relative fold to CB after normalization with GAPDH. Bars represent the means ± S.D. from 3 independent experiments. (D and E) Immunoblotting analyses for VEGF-C. Bone marrow–derived macrophages were incubated with EPO (10 IU/mL) for the indicated times and doses and cell lysates were harvested. Each lane contains 40 μg of total protein from the cell lysates. Blots were probed with an anti–VEGF-C antibody. The membranes were stripped and reprobed with an anti-actin. Densitometric analyses are presented as 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 represent the means ± S.D. from 4 independent experiments. *, $P<0.05$ versus
CB or time 0; **, \( P < 0.01 \) versus CB or time 0
Figure 1. Lee AS et al.

A. Photographs showing lymph node volume comparison between CB and EPO.

B. Bar graph illustrating the lymph node volume (mm³) comparison between CB and EPO.

C. Immunofluorescence images showing Prox-1 and LYVE-1 expression in CB and EPO.

D. Confocal microscopy images displaying LYVE-1, PECAM-1, mitochondria, and merged images for CB and EPO.

E. Graph depicting the density (%) comparison between CB and EPO for PECAM-1, human mitochondria, and LYVE-1.

F. Bar graph showing hematocrit (%) comparison between CB and EPO for control and breast cancer conditions.
Figure 3. Lee AS et al.

A

B

C

D

EPO (IU/mL)  VEGF-A

EPO (IU/mL)  VEGF-A

EPO (IU/mL)  VEGF-A

EPO (IU/mL)  VEGF-A
Figure 4. Lee AS et al

A

**

B

**

C

*

D

*
Figure 5. Lee AS et al.

A

B

C

D

E

Peritoneal side of muscle

Peritoneal side of central tendon

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Figure 6. Lee AS et al.

A

Melanoma+CB | Melanoma+EPO

B

Number of CD11b-positive cell/ unit area

<table>
<thead>
<tr>
<th>CD11b/CD11b/LYVE-LYVE-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>CB</td>
</tr>
<tr>
<td>0</td>
</tr>
<tr>
<td>15</td>
</tr>
</tbody>
</table>

C

Breast cancer+CB | Breast cancer+EPO

D

Number of CD11b-positive cell/ unit area

| CB | EPO |
| 0  | 5   |
| 10 | 15  |

Melanoma+CB vs Melanoma+EPO

Breast cancer+CB vs Breast cancer+EPO

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Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.
Figure 7. Lee AS et al.

A

VEGF-C mRNA

Fold to CB

CB EPO

Melanoma tumor

B

VEGF-C mRNA

Fold to CB

CB 6 12 24 h

EPO (10 IU/mL)

C

VEGF-C mRNA

Fold to CB

CB 5 10 20 IU/mL

EPO (6 h)

D

mVEGF-C

EPO (10 IU/mL)

0 6 12 24 h

Actin

Relative ratio

E

mVEGF-C

EPO (12 h)

CB 5 10 20 IU/mL

Actin

Relative ratio
Erythropoietin induces lymph node lymphangiogenesis and lymph node tumor metastasis

Ae Sin Lee, Duk Hoon Kim, Jung Eun Lee, et al.

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