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Interleukin-2 Promotes Angiogenesis by Activation of Akt and Increase of ROS

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Interleukin-2 plays a significant role in T cell proliferation. Here, we report the role of IL-2 in angiogenesis. IL-2 increased the ROS level and phosphorylation of Akt in human umbilical vein endothelial cells (HUVECs). IL-2 increased angiogenesis in an animal model and tube formation in HUVECs. The effect of IL-2 on angiogenesis and tube formation was mediated by ROS and Akt. This is the first report that IL-2 promotes angiogenesis.

Keywords: Angiogenesis, interleukin-2, reactive oxygen species, serine/threonine kinase Akt

Growth and metastasis of tumor depends on angiogenesis, which is the formation of new blood vessels from a pre-existing network of blood capillaries. Interleukin-2 (IL-2) is naturally produced by T cells, and serves as an important immunoregulatory cytokine. It drives T cell proliferation and differentiation [2, 3, 8, 14, 15]. It serves as an activator for macrophages, CTL, NK cells, and B lymphocytes [16, 17]. IL-2 is a glycoprotein that plays a role in the development of inflammation and apoptosis [4, 9]. Reportedly, T cells stimulated by IL-2 secrete VEGF and respond to it [13]. This suggests the implied role of IL-2 in angiogenesis. IL-2 also induces adhesion of T cells [1] and human umbilical vein endothelial cells (HUVECs) to the extracellular matrix. The effect of IL-2 on cellular adhesion is believed to be mediated by the CC chemokine receptor [11]. In association with its effect on cellular adhesion, IL-2 was shown to promote motility of dendritic cells [7] and NK cells [12]. All these reports point to the role of IL-2 in angiogenesis. Here, we report a novel role of IL-2 in angiogenesis and the mechanism associated with IL-2-promoted angiogenesis.

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Materials and Methods

Cell Culture

HUVECs were isolated from human umbilical cord veins by collagenase treatment and used in passages 3-6. The cells were grown in M199 medium supplemented with 20% fetal bovine serum, 100 U/ml penicillin G, 100 μg/ml streptomycin, 3 ng/ml bFGF (Upstate, MA, U.S.A.), and 5 U/ml heparin at 37°C under 5% CO_2/95% air.

Materials

Dichlorofluorescein diacetate (DCFH-DA) and Mitotracker Red CMXRos were purchased from Molecular Probes, Inc. (Eugene, OR, U.S.A.). Dulbecco's modified Eagle's medium, M199, penicillin, streptomycin, L-glutamate, and heparin were obtained from Invitrogen. Vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY, U.S.A.). Antibodies against Akt, p-Akt, and actin were purchased from Transduction Laboratories (Lexington, KY, U.S.A.). Recombinant human IL-2 was purchased from Sigma. All primers used in this study were commercially synthesized by Bionex Company (Korea).

Western Blot Analysis

Total cellular extracts were prepared with lysis buffer (62.5 mM Tris-HCl [pH 6.8], 2% SDS [w/v], 10% [w/v] glycerol, 50 mM DTT, 1 mM NaF, 1 mM sodium orthovanadate, and protease inhibitor cocktail). The samples were boiled for 5 min, and equal amounts of protein were electrophoresed onto 10% SDS-PAGE. After electrophoresis, proteins were transferred to nitrocelluose membrane and processed for immunoblot. The membrane containing transferred proteins was washed three times with 1× TBS-T buffer (TBS, 0.1% Tween 20). The membrane was incubated with blocking buffer (1× TBS, 5% nonfat dry milk, 0.1% Tween 20) for 1 h. After washing with TBS-T buffer, the membrane was incubated with primary antibody at an appropriate dilution. After washing with TBS-T buffer, the blot was further incubated with horseradish peroxidase-conjugated secondary antibody diluted at 1:2,000, and specific bands were visualized by chemiluminiscence (ECL, Amersham Corp., Arlington Heights, IL, U.S.A.). Primary antibodies to both Akt and phospho-Akt were used in a 1:1,000 dilution.

RT-PCR

Total RNA from cells treated without or with IL-2 (100 units/ml) was isolated by using Trizol (Gibco BRL). Total RNA was converted into cDNA by superscript reverse transcriptase. RT-PCR was carried out according to the instruction manual provided by the manufacturer (Gibco BRL).

The following primer pairs were used for PCR: VEGF, 5'-CAG CTA CTG CCA TCC AAT GGA-3' and 5'-ACG CTC CAG GAC TTA TAC CGG-3'; PDGF, 5'-AAG CAT GTG CCG GAG AAG CG-3' and 5'-TCC TCT AAC CTC ACC TGG AC-3'; IL-2Rα, 5'-ATC AGC GTC CTC CTC CTG AGT-3' and 5'-CAA GCA CAA CGG ATG TCT-3'; IL-2Rβ, 5'-CCA GAC ACC CAG TAT GAG TT-3' and 5'-CAC TTC AGG ACC TTC TTC AG-3'; amd β-actin, 5'-TAA CCA ACT GGG ACG ATA TG-3' and 5'-ATA CAG GGA CAG CAC AGC CT-3'. The PCR conditions were as follows: VEGF: 95°C for 45 s, 58°C for 45 s, 72°C for 60 s, and 35 cycles; PDGF: 95°C for 45 s, 55°C for 45 s, 72°C for 60 s, and 33 cycles; β-actin: 94°C for 60 s, 58°C for 45 s, 72°C for 45 s, and 20 cycles: IL-2Rα and -β: 94°C for 45 s, 58°C for 45 s, 72°C for 60 s, and 33 cycles.

Tube Formation Assays

The formation of tubelike structure by HUVEC on growth factor-reduced Matrigel was performed. The 24-well culture plates were coated with Matrigel according to the manufacturer's instructions. HUVECs were incubated in M199 medium containing 1% FBS for 6 h, plated onto the layer of Matrigel at a density of 2.5×10^5 cells/well, and followed by the addition of IL-2 without or with chemical inhibitor. Matrigel cultures were incubated at 37°C for 24 h. Tube formation was observed using an inverted phase contrast microscope. Images were captured with a video graphic system. The degree of tube formation was quantified by measuring the length of tubes in five randomly chosen low-power fields (×100) from each well using the Image-Pro plus v4.5 (Media Cybernetics, San Diego, CA, U.S.A.).

***In Vivo* Angiogenesis Assays**

Male BABL/c mice (6–8 week old) were obtained from Daehan Biolink (Seoul, Korea). Mice were maintained at the specific pathogen-free housing facility at the School of Medicine, Kangwon National University. Angiogenesis was assessed *in vivo* as described previously [6]. The mice were anesthetized by 1.5% isofurane and O_2-N_2O using a vaporizer (Surgivet, Waukesha, WI, U.S.A.), and abdominal wall windows were implanted. A titanium circular mount with eight holes on the edge was inserted between the skin and abdominal wall. Growth factor-reduced Matrigel containing VEGF (50 ng/ml) or IL-2 (100 units/ml) was applied to the space between the windows, and a circular glass cover slip was placed on top and fixed by a snap ring. After 4 days, the animals were anesthetized and injected intravenously with 50 μl of 25 ng/ml fluorescein isothiocyanate-labeled dextran (molecular weight, M_r ~2,000,000) *via* the tail vein. The mice were then placed on a Zeiss Axiovert 200 M microscope. The epi-illumination microscopy setup included a 100-W mercury lamp and filter set for blue light. Fluorescence images were recorded in random locations of each window by a electron-multiplying charge-coupled device camera (Photo Max 512, Princeton Instruments, Trenton, NJ, U.S.A.) and digitalized for subsequent analysis using the Metamorph program (Universal Imaging, Downingtown, PA, U.S.A.). The assay was scored from 0 (negative) to 5 (most positive) in a double-blinded manner.

ROS Measurement

Serum-starved HUVECs on cover slips were incubated with 10 μM DCFH-DA for 20 min. For the last 15 min of treatment, IL-2 (100 units/ml) was added to measure ROS. Cells were then immediately observed by laser scanning confocal microscopy (LSM 410, Carl Zeiss). The samples were excited by 488 nm Ar laser and images were filtered To determine the effects of antioxidants on ROS in HA-treated melanoma cells, serum-starved melanoma cells on cover slips were pretreated with NAC (100 μM) for 1 h followed by incubation with DCFH-DA. The samples were excited by 488 nm Ar laser and images were filtered by a long-pass 515 nm filter. To determine whether IL-2 enhances peroxynitrite, HUVECs were treated with 10 nM Mitotracker Red CMXRos (Molecular Probes) and IL-2 (100 units/ml), and incubated for 10 min at room temperature. Dihydrorhodamine 123 (DHR, 10 μM) was then added, incubation was continued for 5 min, and cells were washed three times with PBS. The confocal images were acquired and analyzed.

Results and Discussion

IL-2 Increases ROS in HUVECs

ROS are responsible for angiogenesis [6, 10]. Therefore we examined whether IL-2 affected ROS in HUVEC cells. For this, HUVECs were treated with IL-2 (100 units/ml) for various time intervals. DCFH-DA was added to measure the level of H_2O_2. IL-2 increased H_2O_2 in a time-dependent manner (Fig. 1A). *N*-Acetyl-L-cysteine (NAC), a ROS blocker, suppressed induction of H_2O_2 in HUVEC cells treated with IL-2 (Fig. 1A). VEGF, a potent angiogenic factor, also increased ROS in HUVECs (Fig. 1B). Next, the level of peroxynitrite ($ONOO^-$) was measured. For this, DHR123 was added. IL-2 increased the level of $ONOO^-$ in a time-dependent manner (Fig. 1C).

IL-2 Increases Angiogenic Factors in HUVECs

It is well known that VEGF and PDGF are involved in angiogenesis. The effect of IL-2 on these angiogenic factors was investigated. For this, HUVECs were treated with IL-2 (100 units/ml) or $ONOO^-$ (100 μM) for various time intervals. Both IL-2 and $ONOO^-$ increased expression levels of angiogenic factors, such as VEGF and PDGF (Figs. 2A and 2B). This suggests that IL-2 may promote angiogenesis. We also found that IL-2 increased the expression level of the IL-2 receptor (data not shown). This suggests that IL-2 functions through its own receptor.

IL-2 Promotes Angiogenesis

Since IL-2 increased angiogenic factors, it was necessary to examine whether IL-2 promotes angiogenesis. *In vivo* angiogenesis assays were carried out. To examine whether IL-2 promoted *in vivo* angiogenesis, growth factor-reduced matrigel containing VEGF (50 ng/ml), IL-2 (100 units/ml), or in combination was placed on the abdomen of a

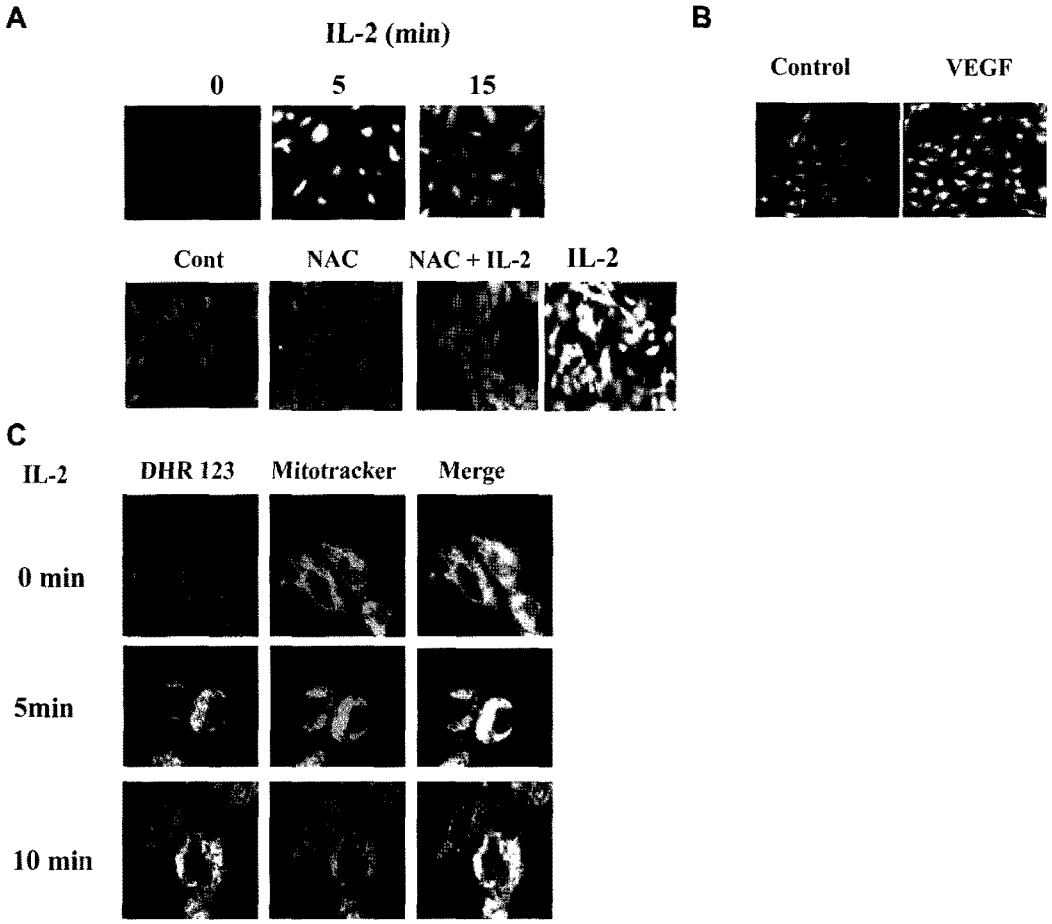

Fig. 1. Interleukin-2 increases ROS in HUVECs.
A. Serum-starved HUVECs on cover slips were treated without or with IL-2 (100 units/ml) for various time intervals. Ten μM of DCFH-DA was then added to measure ROS. DCFH fluorescence intensities of melanoma cells treated with IL-2 were compared with those treated without HA (upper panel). Serum-starved HUVECs on cover slips were pretreated without or with *N*-acetyl-L-cysteine (100 μM) for 15 min. Cells were then treated without or with IL-2 (100 units/ml) for 15 min. ROS measurement was carried out as above (Lower panel). **B.** Serum-starved HUVECs on cover slips were treated without or with VEGF (20 ng/ml) for 15 min. ROS measurement was carried out as above. **C.** Melanoma cells were treated with IL-2 (100 units/ml) for 10 min. IL-2 was added along with Mitotracker Red CMXRos and incubation continued for 20 min followed by incubation with DHR123 for 10 min. ROS level was analyzed by confocal microscopy.

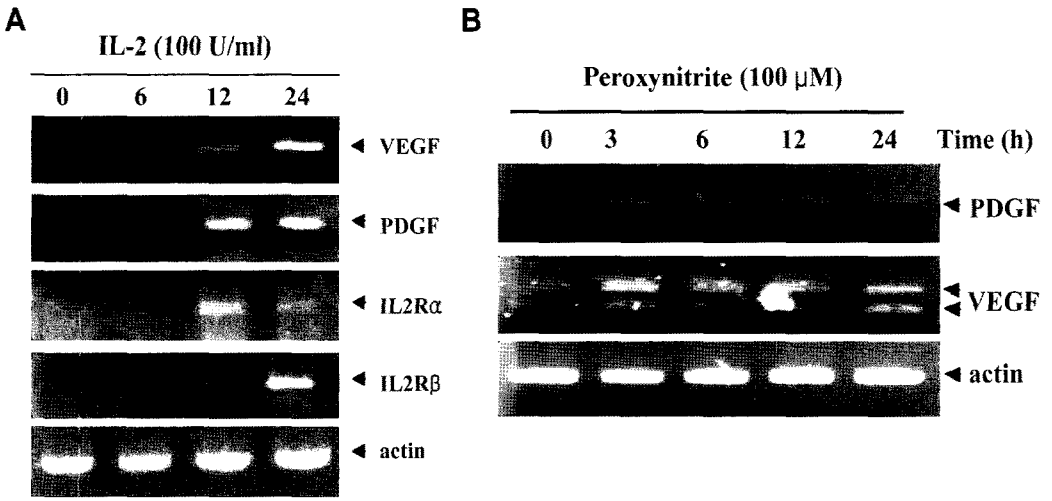

Fig. 2. IL-2 increases expression of angiogenic factors.
A. HUVECs were treated with IL-2 (100 units/ml). Total RNA was isolated at the indicated time point. RT-PCR was carried out. **B.** HUVECs were treated with peroxynitrite (100 μM). Total RNA was isolated at the indicated time point. RT-PCR was carried out.

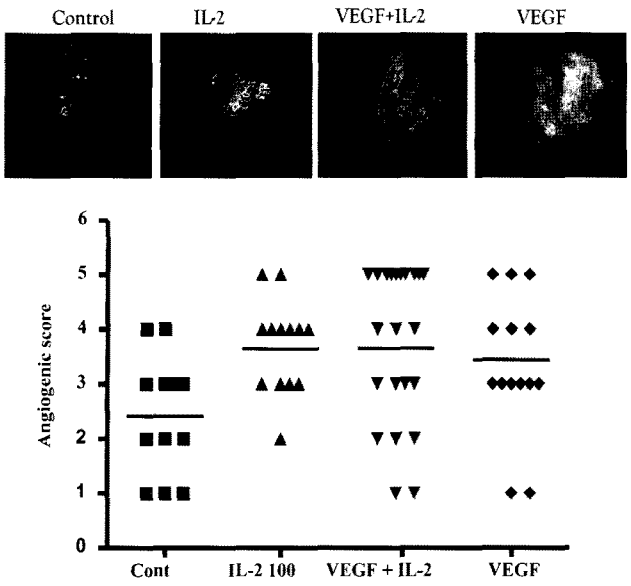

Fig. 3. IL-2 promotes angiogenesis.
In vivo angiogenic activity recorded under intravital microscopy. IL-2 (100 units/mouse), VEGF (50 ng/mouse), or in combination was intravenously injected into the abdominal window of each mouse.

BALB/c mouse. IL-2 (100 units/ml) promoted angiogenesis (Fig. 3). The effect of IL-2 was comparable to that of VEGF. It seems that combined treatment increases the number of animals with the highest angiogenic scores (Fig. 3). Next, we determined factors affecting IL-2-promoted angiogenesis. IL-2 activates Akt to regulate the expression of several genes, including human telomerase reverse transcriptase [5]. We found that IL-2, H_2O_2, and VEGF increased phosphorylation of Akt (Fig. 4A). We also found that peroxynitrite increased phosphorylation of Akt (data not shown). These data suggest that Akt mediates IL-2-promoted angiogenesis. The addition of LY294002, an inhibitor of Akt, decreased the angiogenic function of IL-2 (Fig. 4B). These results indicate that IL-2 promotes angiogenesis *via* an Akt-dependent manner.

IL-2 Promotes Tube Formation *via* ROS- and Akt-Dependent Manner

We further examined the angiogenic potential of IL-2 by performing tube formation. Both IL-2 and VEGF increased tube formation, as expected (Fig. 5A). *N*-Acetyl-L-cysteine (100 μM), an inhibitor of ROS formation, exerted the negative effect on tube formation by IL-2 (Fig. 5B). The inhibition of Akt by LY294002 also exerted a negative effect on tube formation (Fig. 5C). These results indicate that IL-2 increases tube formation *via* ROS- and Akt-dependent manner.

In this study, we found a novel role of IL-2 in angiogenesis. ROS and Akt were shown to be necessary

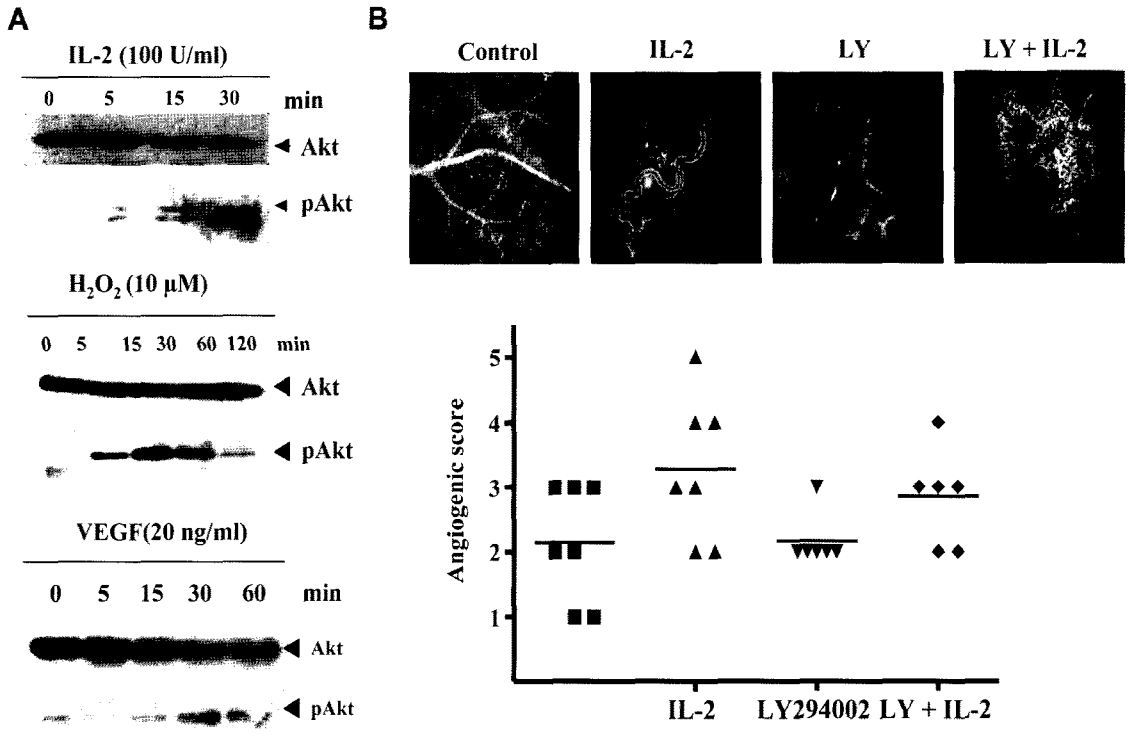

Fig. 4. Akt mediates IL-2-promoted angiogenesis.
A. HUVECs were treated with IL-2 (100 units/ml), H_2O_2 (10 μM), or VEGF (20 ng/ml) for various time intervals. Cell lysates were prepared at each time point and subjected to Western blot analysis. **B.** *In vivo* angiogenesis assays were carried out using Matrigel containing IL-2 (100 units/ml) without or with LY294002 (10 μM).

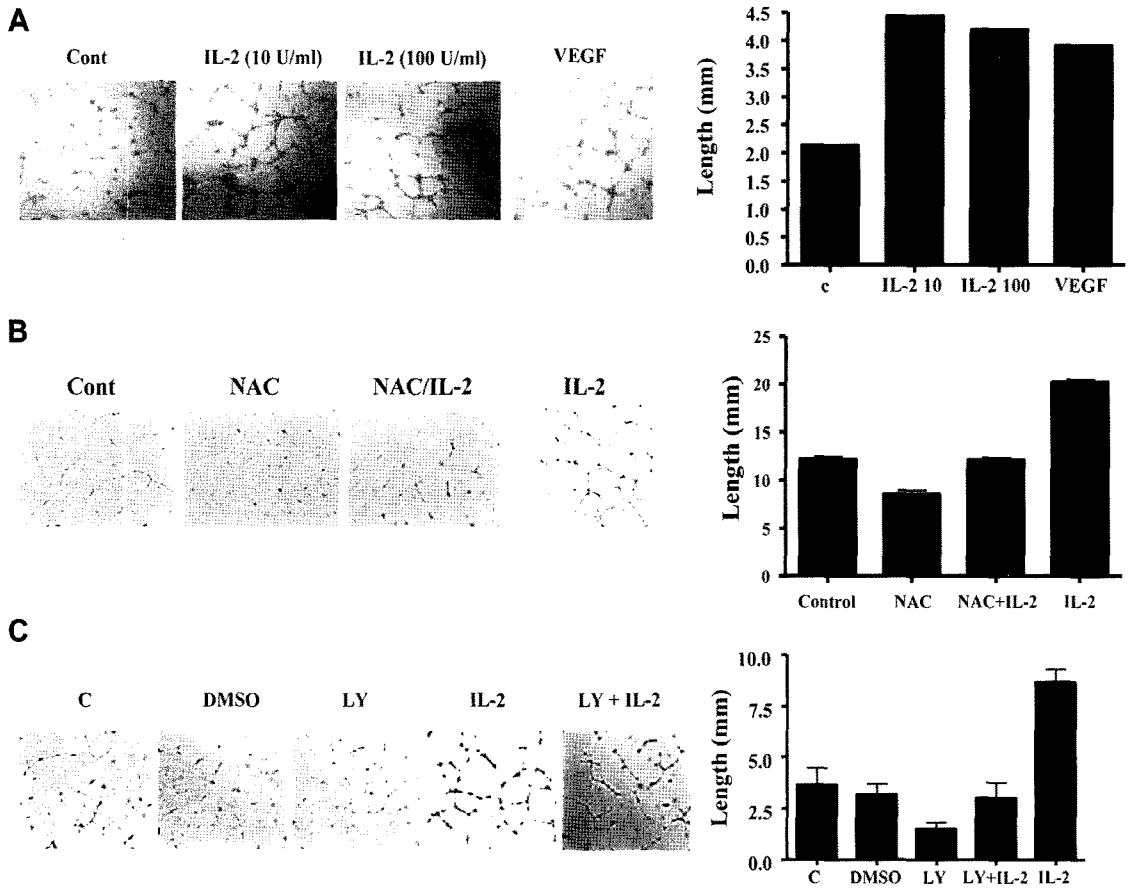

Fig. 5. IL-2 increases tube formation *via* Akt- and ROS-dependent manner.
A. HUVECs treated with IL-2 or VEGF were subjected to tube formation assays after 20 h of incubation at 37°C. An inverted phase contrast microscope was used, and images were captured with a graphic system. Area covered by the tube network was quantitated using ImagePro Plus software. **B.** HUVECs treated with IL-2 (100 units/ml) in the absence or presence of NAC (100 μM) were subjected to tube formation assays. **C.** HUVECs treated with IL-2 (100 units/ml) in the absence or presence of LY294002 (10 μM) were subjected to tube formation assays.

for IL-2-promoted angiogenesis. Based on the fact that IL-2 increased the expression of the IL-2 receptor, it is likely that IL-2 functions through the IL-2 receptor. The underlying mechanism has not been elucidated in detail. Therefore, further identification of downstream targets of IL-2 would be necessary. It would be also necessary to identify the domain of IL-2 that promotes angiogenesis.

Acknowledgments

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References

1. Ariel, A., R. Hershkoviz, I. Altbaum-Weiss, S. Ganor, and O. Lider. 2001. Cell surface-expressed moesin-like receptor regulates T cell interactions with tissue components and binds an adhesion-modulating IL-2 peptide generated by elastase. *J. Immunol.* **166:** 3052–3060.
2. Chen, C. J. and J. E. Shively. 2004. The cell-cell adhesion molecule carcinoembryonic antigen-related cellular adhesion molecule 1 inhibits IL-2 production and proliferation in human T cells by association with Src homology protein-1 and down-regulates IL-2 receptor. *J. Immunol.* **172:** 3544–3552.
3. Fung, M. M., F. Rohwer, and K. L. McGuire. 2003. IL-2 activation of a PI3K-dependent STAT3 serine phosphorylation pathway in primary human T cells. *Cell Signal* **15:** 625–636.
4. Healy, A. M., E. Izmailova, M. Fitzgerald, R. Walker, M. Hattersley, M. Silva, E. Siebert, J. Terkelsen, D. Picarella, M. D. Pickard, B. LeClair, S. Chandra, and B. Jaffee. 2006. PKC-theta-deficient mice are protected from Th1-dependent antigen-induced arthritis. *J. Immunol.* **177:** 1886–1893.
5. Kawauchi, K., K. Ihjima, and O. Yamada. 2005. IL-2 increases human telomerase reverse transcriptase activity transcriptionally and posttranslationally through phosphatidylinositol 3'-kinase/

Akt, heat shock protein 90, and mammalian target of rapamycin in transformed NK cells. *J. Immunol.* **174:** 5261–5269.
6. Kim, Y. M., K. E. Kim, G. Y. Koh, Y. S. Ho, and K. J. Lee. 2006. Hydrogen peroxide produced by angiopoietin-1 mediates angiogenesis. *Cancer Res.* **66:** 6167–6174.
7. Kradin, R. L., W. Xia, M. Pike, H. R. Byers, and C. Pinto. 1996. Interleukin-2 promotes the motility of dendritic cells and their accumulation in lung and skin. *Pathobiology* **64:** 180–186.
8. Kunitomi, A., T. Hori, A. Imura, and T. Uchiyama. 2000. Vascular endothelial cells provide T cells with costimulatory signals *via* the OX40/gp34 system. *J. Leukoc. Biol.* **68:** 111–118.
9. Lesur, O., M. Brisebois, A.Thibodeau, F. Chagnon, D. Lane, and T. Fullop. 2004. Role of IFN-gamma and IL-2 in rat lung epithelial cell migration and apoptosis after oxidant injury. *Am. J. Physiol. Lung Cell Mol. Physiol.* **286:** 4–14.
10. Liu, L. Z., X. W. Hu, C. Xia, J. He, Q. Zhou, X. Shi, J. Fang, and B. H. Jiang. 2006. Reactive oxygen species regulate epidermal growth factor-induced vascular endothelial growth factor and hypoxia-inducible factor-1alpha expression through activation of AKT and P70S6K1 in human ovarian cancer cells. *Free Radic. Biol Med.* **41:** 1521–1533.
11. Loetscher, P., M. Seitz, M. Baggiolini, and B. Moser. 1996. Interleukin-2 regulates CC chemokine receptor expression and chemotactic responsiveness in T lymphocytes. *J. Exp. Med.* **184:** 569–577.
12. Matera, L., A. Galetto, M. Bello, C. Baiocco, I. Chiappino, G. Castellano, A. Stacchini, M. A. Satolli, M. Mele, S. Sandrucci, A. Mussa, G. Bisi, and T. L. Whiteside. 2006. *In vivo* migration of labeled autologous natural killer cells to liver metastases in patients with colon carcinoma. *J. Transl. Med.* **4:** 49.
13. Mor, F., F. J. Quintana, and I. R. Cohen. 2004. Angiogenesis-inflammation cross-talk: Vascular endothelial growth factor is secreted by activated T cells and induces Th1 polarization. *J. Immunol.* **172:** 4618–4623.
14. Pae, H. O., G. S. Oh, B. M. Choi, S. C. Chae, Y. M. Kim, K. R. Chung, and H. T. Chung. 2004. Carbon monoxide produced by heme oxygenase-1 suppresses T cell proliferation *via* inhibition of IL-2 production. *J. Immunol.* **172:** 4744–4751.
15. Taguchi, Y., T. Kondo, M. Watanabe, M. Miyaji, H. Umehara, Y. Kotutumi, and T. Okazaki. 2004. Interleukin-2-induced survival of natural killer (NK) cells involving phosphatidylinositol-3 kinase-dependent reduction of ceramide through acid sphingomyelinase, sphingomyelin synthase and glucosylceramide synthase. *Blood* **104:** 3285–3293.
16. Takeuchi, T., N. Konno-Takahashi, Y. Kasuya, T. Ogushi, H. Nishimatsu, and T. Kitamura. 2004. Interleukin-2 blocks the antitumour activity caused by depletion of CD25 cells in a murine renal adenocarcinoma model. *BJU Int.* **94:** 171–176.
17. Zhou, J., J. Zhang, M. G. Lichtenheld, and G. G. Meadows. 2002. A role for NF-kappa B activation in perforin expression of NK cells upon IL-2 receptor signaling. *J. Immunol.* **169:** 1319–1325.