Nuclear Translocation of Phosphorylated STAT3 Is Essential for Vascular Endothelial Growth Factor-induced Human Dermal Microvascular Endothelial Cell Migration and Tube Formation*

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Vascular endothelial growth factor (VEGF) is a potent, multifunctional, endothelial-cell-specific growth factor. It stimulates proliferation and migration of endothelial cells. Characterization of intracellular signal transduction after VEGF and VEGF receptor (VEGFR) interaction has demonstrated the involvement of the mitogen-activated protein kinase pathway. However, several studies indicated that signal transducers and activators of transcription (STAT) is another important pathway downstream of VEGF/VEGFR interaction. Therefore, we studied the role of STAT3 in the migration and tube formation of the human dermal microvascular endothelial cells (HDMEC). HDMEC expressed phosphorylated forms of STAT1, STAT3, and STAT5, and a marked increase of phosphorylated STAT3 in the nuclear fraction after addition of VEGF was observed by Western blot and immunohistochemical staining. To verify the functional implication of STAT3 phosphorylation in HDMEC migration, we introduced a dominant-negative STAT3 using adenovirus vector system. Dominant-negative STAT3 abolished the VEGF-induced translocation of phosphorylated STAT3 and inhibited HDMEC migration completely. Dominant-negative STAT3 also suppressed VEGF-induced HDMEC tube formation on Matrigel and on collagen gel. These data demonstrate that STAT3 and its phosphorylation are involved in the downstream pathway of VEGF/VEGFR interaction and regulate VEGF-induced HDMEC migration and tube formation.

Vascular endothelial growth factor (VEGF) is a member of the platelet-derived growth factor superfamily and an endothelial-cell-specific growth factor. It stimulates vasodilation and cell proliferation, increases permeability and migration, and promotes endothelial cell survival (1–4). VEGF plays an important role as a regulator of blood-vessel growth and development (5–14). VEGF exerts its effects by interacting with two high affinity membrane receptors, VEGF receptor (VEGFR) 1 (Flt-1) (15, 16) and VEGFR2 (Flk-1/KDR) (17, 18). VEGFR1 and VEGFR2 are tyrosine kinase receptors that trans-phosphorylate and, in turn, phosphorylate on specific tyrosine residues of SH2 domain-containing signaling molecules (19, 20). VEGFR1 undergoes weak ligand-dependent tyrosine phosphorylation, whereas VEGFR2 has a strong response. This difference in signal transduction properties corresponds to diverse functions of VEGF (16, 19). Namely, VEGFR1 mediates cell migration and differentiation, whereas VEGFR2 mediates cell proliferation and survival (7, 8, 16, 21, 22).

It has been suggested that the major signaling pathway downstream of VEGF/VEGFR is the serine/threonine kinase mitogen-activated protein kinase (20). However, an involvement of signal transducers and activators of transcription (STAT) proteins in the VEGF signaling pathway has also been reported (23). STAT proteins are activated in response to a number of cytokines, growth factors, and hormones (24–26). After the binding of ligands to their receptors, STAT proteins are activated, dimerize, translocate to the nucleus, and bind to specific target gene promoters. So far, seven STAT proteins (STAT1, -2, -3, -4, -5A, -5B, and -6) have been identified.

Induction of endothelial cell migration is one of the major biological functions of VEGF. Interestingly, a recent report showed that STAT3 plays crucial roles in the migration of keratinocytes, the hair cycle, and wound healing, using keratinocyte-specific STAT3 knockout mice (27). Therefore, we hypothesized that VEGF-induced endothelial cell migration involves the STAT3 signaling pathway. To test this hypothesis, we treated cultured human dermal microvascular endothelial cells (HDMEC) with recombinant VEGF and studied the role of STAT3 on HDMEC migration and tube formation using an adenovirus vector carrying a dominant negative STAT3. We show here the first evidence that STAT3 and its phosphorylation regulates VEGF-induced HDMEC migration and tube formation.

EXPERIMENTAL PROCEDURES

Cell Culture—Human dermal microvascular endothelial cells (HDMEC) were isolated from human foreskin by modification of previously published methods (28). HDMEC were grown in medium 199 (Invitrogen, Tokyo, Japan) with 10% fetal calf serum (FCS), 10 ng/ml of recombinant human basic fibroblast growth factor (a generous gift from Raken Pharmaceutical Inc., Tokyo, Japan), and kanamycin. HDMEC show typical cobblestone morphology of confluent monolayers in the absence of contaminating fibroblasts. Cells at the sixth passage were used for all experiments. These cells were pre-incubated in endothelial...
Collagen Endothelial Cell Tube Formation Assays—HDMEC were seeded on type I collagen-coated filters, and then various concentrations of VEGF were added to the medium. After 7 h, VEGF enhanced HDMEC migration in a dose-dependent manner (Fig. 1A). At 3 ng/ml, VEGF enhanced HDMEC migration 2-fold, and at 33 ng/ml, migration was enhanced 2.7-fold compared with control. At a higher concentration of 100 ng/ml, HDMEC migration reached a plateau. Therefore, we used VEGF at 33 ng/ml to stimulate HDMEC in the following experiment.

Expression Profile of STAT in HDMEC and Translocation of Phosphorylated STAT3 into the Nucleus by VEGF—Because STAT protein expression differs depending on cell types, we investigated STAT protein expression in HDMEC. Western blot analysis showed that HDMEC expressed STAT1, STAT3, and STAT5, but not STAT2, STAT4, or STAT6 (Fig. 1B). Next, we examined whether STAT1, -3, and -5 were phosphorylated in response to stimulation with VEGF. Although phosphorylated STAT1, -3, and -5 were detected in HDMEC, their levels of phosphorylation were not altered by VEGF treatment of HDMEC (Fig. 1B). Because phosphorylated STAT proteins translocate into the nucleus, we examined the expression of phosphorylated STAT1, STAT3, and STAT5 in the nuclear fraction. Phosphorylated STAT3 was increased remarkably in the nuclear fraction at 45 and 60 min after addition of VEGF (Fig. 1C).

In contrast, VEGF did not affect the localization of phosphorylated STAT1 or -5 in HDMEC (Fig. 1C). To further confirm nuclear translocation of phosphorylated STAT3, we investigated the localization of phosphorylated STAT1, -3, and -5 in HDMEC immunohistochemically by confocal microscopy. Phosphorylated STAT3 was detected in the cytoplasm, but not in the nucleus of unstimulated HDMEC. However, phosphorylated STAT3 was detected predominantly in the perinuclear region or within the nuclear compartment 30 min after addition of VEGF. After 50 min, phosphorylated STAT3 was found mainly in the nuclear region (Fig. 2). Taken together, VEGF induced the translocation of phosphorylated STAT3 from the cytoplasm to the nucleus, although VEGF did not alter the total amount of phosphorylated STAT3 in HDMEC.

Because it has been reported that the phosphorylation of STAT3 is regulated by two pathways, the Janus kinase (JAK) and intrinsic tyrosine kinase of growth factor receptors (23), we examined whether HDMEC express JAK1, JAK2, and Tyk2. Western blot showed that HDMEC express JAK1, JAK2, and Tyk2; however, none of them were phosphorylated after addition of VEGF (data not shown).

Inhibition of VEGF-induced Migration and Nuclear Translocation of STAT3 by a Dominant-negative Mutant of STAT3—Because STAT3 deficiency is lethal in mice during early embryogenesis (32), it is actually impossible to analyze the role of STAT3 about skin angiogenesis using mouse model. In this case, we used pAxCAw. Ad containing the CA promoter and STAT3F (AxSTAT3F) was generated by the COS-TPC method (31). The cosmid DNA was mixed with the EcoT22I-digested DNA-terminal protein complex of Ad5-dlX, and used to cotransfect 293 cells. Recombinant viruses were generated through homologous recombination in these cells. Virus stocks were prepared by a standard procedure (31). Concentrated, purified virus stocks were prepared by the CsCl gradient method, and the virus titer was checked with a plaque-formation assay. HDMEC were infected with adenovirus vectors at a multiplicity of infection (m.o.i.) of 15.

X-Gal Staining—HDMEC were infected with AdLacZ at m.o.i. 15. After 48 h, X-gal staining was performed by β-galactosidase staining kit (Active Motif, Carlsbad, CA) according to the manufacturer's instructions.
study, we used an adenovirus vector containing a mutant STAT3 that has a dominant-negative effect. Adenovirus vector has an advantage over other methods of transfection for endothelial cells. Transfection efficiency of adenovirus vector for endothelial cell is almost 100%. To assess the requirement for STAT3 phosphorylation in VEGF-induced endothelial cell migration directly, we exploited the effect of a dominant-negative mutant of STAT3 named STAT3F. In this mutant, the tyrosine residue at amino acid position 705 is mutated to phenylalanine. This results in the blockade of Tyr705 phosphorylation of STAT3, which is required for dimerization and nuclear translocation (33). Ad expressing /H9252-galactosidase (AxLacZ) was used as controls to exclude the effect of Ad itself. We infected HDMEC with AxSTAT3F or control Ad at an m.o.i. of 15. AxSTAT3F as well as AxLacZ showed no significant morphological change (Fig. 3a). We confirmed gene expression in almost all of HDMEC using control virus (AxLacZ) at same m.o.i. (i.e. 15) by X-gal staining (Fig. 3b), and AxSTAT3F had no effect on HDMEC proliferation (Fig. 3c). However, AxSTAT3F inhibited the VEGF-induced HDMEC migration by 100% (Fig. 3d). Furthermore, AxSTAT3F inhibited nuclear translocation of phosphorylated STAT3 after VEGF addition as confirmed by confocal microscopy (Fig. 4).

Blockade of Tube Formation on Matrigel and Collagen Gels by a Dominant-negative Mutant of STAT3—On GFR-Matrigel, HDMEC began to rearrange or align themselves in an organized manner within 1 or 2 h without exogenous stimulation. In the absence of stimulation, HDMEC formed only a small number of short, incomplete tubes. The majority of the tubes were not linked to one another but remained close to the cell body from which they were derived. Untreated HDMEC formed significantly fewer tubes compared with stimulated cells. After a 12-h incubation, the cells treated with VEGF further differentiated into an expansive tube network, whereas most of the control cells remained as individual clusters or ovoid colonies. By 16 h, the boundaries of the stimulated tubes were sharply defined, elongated, and more extended. Furthermore, a majority of the HDMEC formed tubes. Overall, HDMEC treated with VEGF produced a more extensive network of interconnecting tubes compared with untreated HDMEC (Fig. 5a). The total tube length was calculated by Image-Pro Plus software. Compared with control, the total length of tubes was 1.7-fold longer.
in VEGF-treated cells. These VEGF-induced tube formation was decreased by the treatment of dominant negative mutant form of STAT3, AxSTAT3F, by 74% (Fig. 5b). This result shows that VEGF-induced HDMEC tube formation occurred through the STAT3 signaling pathway.

To examine tube formation on an extracellular matrix that is inherently less conducive to rapid neovascularization, HDMEC were cultured on collagen gels in the presence or absence of stimulants. Confluent cultures of HDMEC on the collagen gels formed a monolayer of interlocking cells. Eight hours after the addition of VEGF, the cells became elongated and spindle shaped. After 48 h, numerous tubes were visible in the wells treated with VEGF. The control cells remained in the characteristic monolayer without any discernible tube growth. Like the tube formation on Matrigel, AxSTAT3F decreased VEGF-induced tube formation on collagen gel (Fig. 5c). This result shows that also in the VEGF-induced HDMEC tube formation on collagen gel was through STAT3 signaling pathway.

**DISCUSSION**

Blood vessel regeneration is an important process in skin wound healing and regulated by various cytokines and growth factors. The most important of them is VEGF. It has been shown that blood vessel formation is directly induced by VEGF in vivo (34, 35) and that vascular endothelial cell migration and blood vessel regeneration are induced in a similar manner in vitro (36, 37). VEGF strongly induces migration of human umbilical vein endothelial cells, even at low concentrations compared with basic fibroblast growth factor (38). Therefore, induction of endothelial cell migration is one of the major functions of VEGF.

Intracellular signal transduction has been studied in many cytokines and growth factors, including VEGF. The major signaling pathway downstream of VEGF and VEGFR interaction has been supposed to be mitogen-activated protein kinase cascade (39). However, several groups have suggested the involvement of another signaling pathway, namely the STAT pathway.
in VEGF-induced signal transduction, although the implication of STAT in VEGF function has not been fully elucidated. Sano et al. (27) have shown that the migration of keratinocytes induced by epidermal growth factor family growth factors was severely impaired in STAT3-disrupted keratinocytes. STAT3 is phosphorylated and activated by the interleukin-6 family, epidermal growth factor, platelet-derived growth factor, hepatocyte growth factor, granulocyte colony-stimulating factor, and leptin in various cell types (25, 42). These reports suggest the important role of STAT3 in cell migration. VEGF up-regulated HDMEC migration, but the downstream signaling pathway remained unclear. Therefore, we hypothesized that the STAT3 might be involved in HDMEC migration and angiogenesis might be induced by VEGF.

To understand the role of STAT, it is important to clarify the mechanism of phosphorylation and nuclear translocation of phosphorylated STAT. Tyrosine phosphorylation of STAT is necessary but not sufficient for transcriptional activity. Because STAT protein lacks a nuclear localization signal, it is supposed that chaperone proteins are required to assist the nuclear translocation of STAT (43). We showed that STAT1, STAT3, and STAT5 are expressed in HDMEC confirmed by Western blot and immunohistochemistry. STAT1, -3, and -5 were phosphorylated to some extent even at the steady state, and their levels were not altered by VEGF addition. However, phosphorylated STAT3, but not phosphorylated STAT1 and STAT5, is translocated into nucleus after VEGF stimulation, and impairment of translocation of phosphorylated STAT3 by a dominant-negative STAT3 abolished VEGF-induced HDMEC migration and tube formation. These data indicate that translocation of phosphorylated STAT3 into nuclei is essential for triggering HDMEC migration. In aortic endothelial cells, phosphorylation of STAT1, STAT3, and STAT6 by VEGF was reported (23). In contrast to HDMEC, phosphorylated STAT1 and STAT6, but not phosphorylated STAT3, are translocated into nuclei (23). Therefore, this complicated relationship between phosphorylation and nuclear translocation in various STATs seems to contribute to diverse function corresponding to different cell types, even among endothelial cells.

Two pathways regulate the phosphorylation of STAT: phosphorylation by the Janus kinase and intrinsic tyrosine kinase of growth factor receptors (23). We found that HDMEC express Jak1, Jak2, and Tyk2 and that none of them were phosphorylated after addition of VEGF. In bovine aortic endothelial cells, involvement of the intrinsic tyrosine kinase activity of VEGFR2 was reported in STAT1 tyrosine phosphorylation (23). This suggests that intrinsic tyrosine kinase activity of VEGFR is a major pathway in STAT phosphorylation of endothelial cells.

VEGF and its two receptors, Flt-1 (VEGFR-1) and KDR/Flik-1 (VEGFR-2), have been demonstrated to be an essential regulatory system for blood vessel formation in mammals. KDR/Flik-1 (−/−) homozygous mice died at embryonic day 8.5 (E8.5) from a severe deficiency in vascular formation associated with a strong hematopoietic impairment (7). Like KDR null mice, the Flt-1 (−/−) homozygous mice also showed embryonic lethality at almost the same stage (E8.5−9.0) (8). On the other hand, the STAT3−/− mice embryos were being degenerated with no sign of mesoderm formation by E7.0. And by E7.5, STAT3−/− embryos were completely resorbed, indicating that STAT3−/− embryos die around E7.0, the day at which gastrulation initiates (32). It is suspected that the lethality might be the result of a defect in functions of visceral endoderm, such as nutritional insufficiency, although angiogenesis and vascular

\[\text{STAT3 in HDMEC Migration}\]
formation have not been fully investigated. We show here that the dominant-negative STAT3 suppressed HDMEC tube formation on Matrigel and the VEGF-induced tube formation as well as HDMEC migration. These findings suggest that VEGF-KDR/FH-STAT3 is one of the most important pathways in developmental angiogenesis

In conclusion, we first demonstrated that the nuclear translocation of phosphorylated STAT3 is essential for VEGF-induced HDMEC migration and tube formation. This VEGF-induced tube formation was fully inhibited in cells expressing the dominant-negative mutant form of STAT3, AxSTAT3F, to levels below those of controls. It gives a new insight into the role of VEGF-STAT3 signaling in wound healing and angiogenesis of the skin as well as developmental angiogenesis.

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