Oncogene (2000) 19, 2138–2146

www.nature.com/onc

Roles of two VEGF receptors, Flt-1 and KDR, in the signal transduction of VEGF effects in human vascular endothelial cells

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Vascular endothelial growth factor (VEGF) is a principal regulator of vasculogenesis and angiogenesis. VEGF expresses its effects by binding to two VEGF receptors, Flt-1 and KDR. However, properties of Flt-1 and KDR in the signal transduction of VEGF-mediated effects in endothelial cells (ECs) were not entirely clarified. We investigated this issue by using two newly developed blocking monoclonal antibodies (mAbs) against Flt-1 and KDR. VEGF elicits DNA synthesis and cell migration of human umbilical vein endothelial cells (HUVECs). The pattern of inhibition of these effects by two mAbs indicates that DNA synthesis is preferentially mediated by KDR. In contrast, the regulation of cell migration by VEGF appears to be more complicated. Flt-1 regulates cell migration through modulating actin reorganization, which is essential for cell motility. A distinct signal is generated by KDR, which influences cell migration by regulating cell adhesion via the assembly of vinculin in focal adhesion plaque and tyrosine-phosphorylation of focal adhesion kinase (FAK) and paxillin. *Oncogene* (2000) **19,** 2138–2146.

Keywords: VEGF; Flt-1; KDR; migration; proliferation

Introduction

Vascular endothelial growth factor (VEGF) is a principal regulator of vasculogenesis and angiogenesis (Ferrara and Davis-Smyth, 1997). VEGF binds to two receptor-type tyrosine kinases, Flt-1 (VEGF receptor-1) (Shibuya *et al*., 1990; de Vries *et al*., 1992) and KDR/Flk-1 (VEGF receptor-2) (Quinn *et al*., 1993), and to membrane protein neuropilin-1, which does not contain a tyrosine kinase domain (Soker *et al*., 1998). Flt-1 and KDR/Flk-1 have seven immunoglobulin-like repeats in the extracellular domain, a single transmembrane region, and a tyrosine kinase domain in the intracellular domain that is interrupted by a kinase-insert domain. Flt-1 has a higher affinity with K_d of approximately 10–20 pmol/L (de Vries *et al*., 1992), while the K_d of KDR is approximately 75–125 pmol/L (Terman *et al*., 1992). Although neuropilin-1 lacks the signaling domain within its molecule, it may enhance the effects of VEGF by transferring VEGF to the tyrosine kinase receptors (Soker *et al*., 1998).

Flt-1 and KDR/Flk-1 have distinct functions in vascular development in embryos. Flk-1 knockout mice, which die by embryonic day 8.5 (E8.5), lack both ECs and hematopoietic cells (Shalaby *et al*., 1995). Indeed, KDR is shown to be a key marker defining hematopoietic stem cells in human postnatal hematopoietic tissue (Ziegler *et al*., 1999). In contrast, Flt-1 knockout mice, which also die around E8.5, have abundant ECs, which do not assemble in functional vessels (Fong *et al*., 1995). In spite of these observations, properties of KDR/Flk-1 and Flt-1 in normally differentiated ECs are controversial. It has been reported that KDR but not Flt-1 plays a principal role in the transduction of VEGF-mediated effects in porcine aortic ECs lacking endogenous VEGF receptors which are transfected with either Flt-1 or KDR gene (Waltenberger *et al*., 1994; Kroll *et al*., 1997). The importance of KDR/Flk-1 is further emphasized by using mutant VEGF which only binds to KDR (Keyt *et al*., 1996) or KDR antisense oligonucleotides (Bernatchez *et al*., 1999). However, Flt-1 does play a role in VEGF-stimulated migration of monocytes/macrophages which express only Flt-1 (Shen *et al*., 1993; Barleon *et al*., 1996; Clauss *et al*., 1996).

In the present study, we investigated the roles of two VEGF receptors by using newly developed blocking monoclonal antibodies (mAbs). Our results indicate that the Flt-1-mediated signal elicits migration of HUVECs by actin reorganization. A distinct signal is generated by the KDR. This signal influences cell migration by regulating phosphorylation of focal adhesion kinase (FAK) and paxillin and the assembly of vinculin in focal adhesion plaque. Moreover, this latter signal regulates DNA synthesis.

Results

We first characterized the specificity and blocking activity of newly developed mAbs, KM1750 and KM1992, as follows. As shown in Figure 1a, KM1750 selectively reacted with soluble Flt-1 and NIH3T3-Flt-1 cells, but not with soluble KDR and NIH3T3-KDR cells (Figure 1a,b). In contrast, KM1992 selectively reacted with soluble KDR and NIH3T3-KDR cells, but not with soluble Flt-1 and NIH3T3-Flt-1 cells (Figure 1a,b). Thus, there is no cross-reactivity between KM1750 and KM1992. Immunoprecipitation followed by Western blot analysis

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Received 18 November 1999; revised 22 February 2000; accepted 22 February 2000

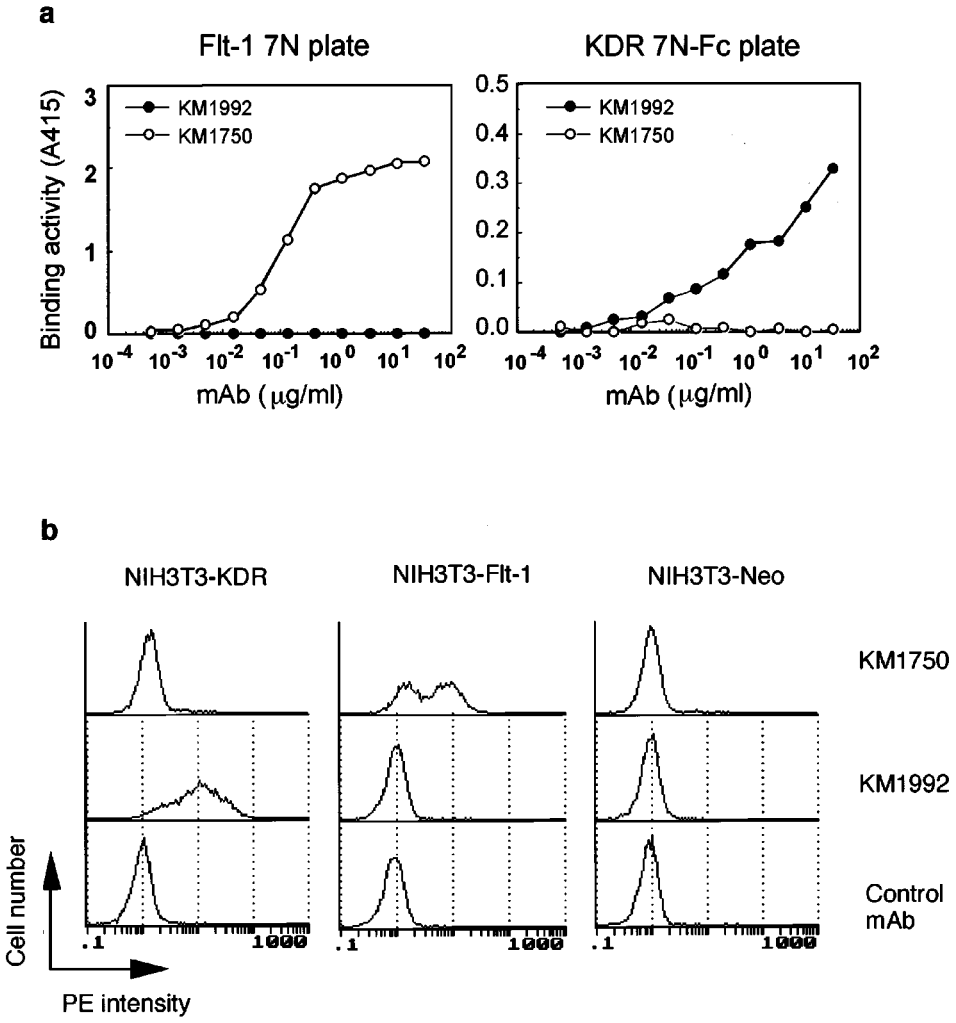

Figure 1 Specificity of KM1750 and KM1992. (**a**) Reactivity of KM1750 (αFlt-1 mAb) and KM1992 (αKDR mAb) with soluble VEGF receptors, Flt-1 7N and KDR 7N-Fc was analysed by ELISA. Serially diluted KM1750 and KM1992 were added to wells coated with Flt-1 7N or KDR 7N-Fc, and the reactivity was determined by ELISA. (**b**) Flow cytometric analysis of the cell binding of KM1750 and KM1992. NIH3T3-Flt-1, NIH3T3-KDR and NIH3T3-neo cells were reacted with biotin labeled mAbs. The cells were stained with Streptoavidin-R-Phycoerythrin and analysed by flow cytometry. KM1750 (30 μg/ml) only reacted with NIH3T3-Flt-1, whereas KM1992 (30 μg/ml) only reacted with NIH3T3-KDR

showed that KDR (230 kDa) and Flt-1 (210 kDa) were tyrosine-phosphorylated by the treatment of HUVECs with VEGF (Figure 2a). Placenta growth factor (PlGF) only binds to Flt-1 (Park *et al*., 1994). PlGF tyrosine-phosphorylated Flt-1 but not KDR (Figure 2b). KM1750 at 1 μg/ml completely inhibited tyrosine phosphorylation of 210 kDa protein (Figure 2b). KM1992 inhibited tyrosine phosphorylation of 230 kDa protein in a dose-dependent manner, partially at 10 μg/ml and completely at 30 μg/ml (Figure 2b). By using these two mAbs, we evaluated the roles of Flt-1 and KDR in the transduction of VEGF-mediated signals in HUVECs.

VEGF stimulated DNA synthesis of HUVECs, with the maximal stimulation being observed at 50 ng/ml (Figure 3a). KM1992 inhibited VEGF-stimulated DNA synthesis partially (10 μg/ml) or completely (30 μg/ml), whereas KM1750 exhibited no inhibition at 1 μg/ml (Figure 3b). Equivalent concentration of PlGF (1 nM) did not augment DNA synthesis of HUVECs (Figure 3c). Interestingly, the combination of KM1750 (1 μg/ml) and KM1992 (10 μg/ml) almost completely inhibited VEGF-stimulated DNA synthesis (Figure 3b). This combined effect of KM1750 and KM1992 might suggest KDR/Flt-1 heterodimerization. However, we could not reproducibly point out KDR/Flt-1 heterodimerization.

VEGF stimulated migration of HUVECs. The maximal stimulation observed at 10 ng/ml with a typical bell-shaped curve (Figure 4a). In contrast to DNA synthesis, 1 μg/ml of KM1750 completely inhibited VEGF-stimulated migration of HUVECs, whereas KM1992 (up to 30 μg/ml) showed 50% inhibition (Figure 4b). Equivalent concentration of PlGF (0.2 nM) stimulated migration of HUVECs, however it was not as potent as VEGF (Figure 4c). The effect of KM1750 observed in this assay was not influenced by cell proliferation, since the same concentration of KM1750 had no effect on DNA synthesis of HUVECs (Figure 3b).

Cell migration is a complex phenomenon which requires cytoskeleton-regulated cell motility and cell adhesion (Lauffenburger and Horwitz, 1996). VEGF elicited actin reorganization and vinculin assembly in focal adhesion plaque of HUVECs (Figure 5). One μg/ml of KM1750 completely abrogated actin reorganiza-

tion, but failed to affect vinculin assembly (Figure 5). PlGF elicited actin reorganization but not vinculin assembly (Figure 5). Conversely, KM1992 inhibited vinculin assembly partially (10 μg/ml) or completely (30 μg/ml), but failed to affect actin reorganization even at 30 μg/ml (Figure 5). Therefore, Flt-1 regulates actin reorganization, whereas KDR regulates vinculin assembly.

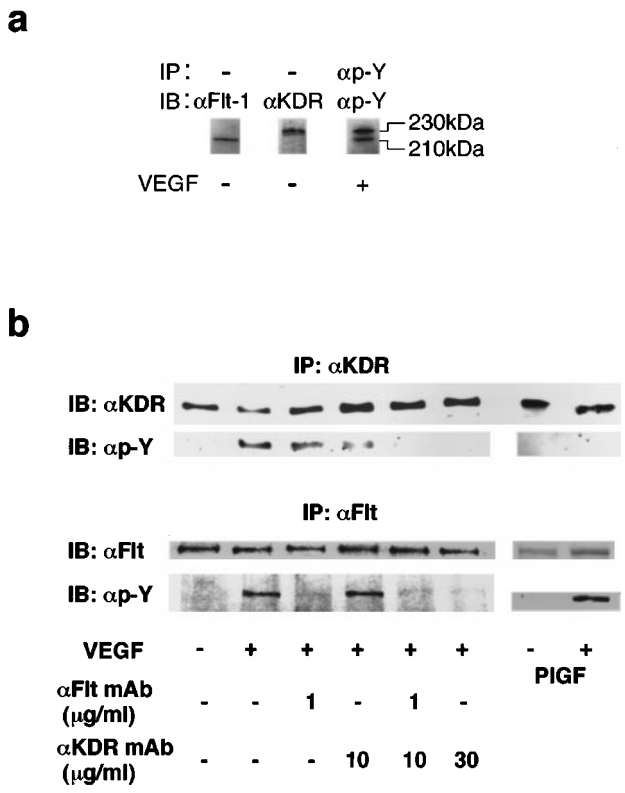

Figure 2 Blocking effect of KM1750 and KM1992. (**a**) Tyrosine phosphorylation of KDR and Flt-1 after the stimulation of HUVECs with VEGF (10 ng/ml) was analysed by immunoprecipitation followed by Western blot analysis. IP: antibodies used for immunoprecipitation, IB: antibodies used for Western blotting. (**b**) HUVECs were left untreated or exposed to indicated concentrations of KM1750 (αFlt-1 mAb) and/or KM1992 (αKDR mAb) 15 min prior to the addition of VEGF. Then the cultures were incubated with or without 10 ng/ml of VEGF for 5 min and tyrosine phosphorylation of KDR and Flt-1 was analysed by immunoprecipitation followed by Western blot analysis. For the immunoprecipitation of Flt-1 in this particular experiment, we used rabbit polyclonal anti-Flt-1 antibody prepared by Dr Shibuya's laboratory

Cell spreading correlates with F-actin formation (Lewis *et al*., 1996). VEGF stimulated spreading of HUVECs (Figure 6). One μg/ml of KM1750 completely abrogated this effect of VEGF, whereas 30 μg/ml of KM1992 did not. Vinculin assembly in focal adhesion plaque is correlated with tyrosine phosphorylation of FAK and paxillin (Gumbiner, 1996). VEGF induced tyrosine phosphorylation of FAK and paxillin (Figure 7). KM1750 exhibited no effect on phosphorylation of FAK or paxillin, whereas 30 μg/ml of KM1992 completely inhibited phosphorylation of FAK and paxillin (Figure 7).

The intracellular signal transduction of Flt-1 and KDR was further evaluated. VEGF activated MAP kinases, i.e., ERK1/2 and p38 MAP kinase in HUVECs (Figure 8). The activation of ERK1/2 was completely inhibited by anti-KDR mAb (30 μg/ml), whereas anti-Flt-1 mAb (1 μg/ml) exhibited no inhibition (Figure 8). In contrast, the activation of p38 MAP kinase was completely abrogated by anti-Flt-1 mAb (1 μg/ml), whereas anti-KDR mAb exhibited no inhibition (Figure 8). PlGF activated p38 MAP kinase but not ERK1/2 (Figure 8). PD98059, a specific inhibitor of MEK1, completely inhibited augmentation of DNA synthesis (Figure 9a). However, PD98059 slightly inhibited cell migration (Figure 9b), and the inhibition of phosphorylation of FAK and paxillin and assembly of vinculin by PD98059 was incomplete (Figures 9c and 10). GF109203X, an inhibitor of PKC, completely inhibited the activation of ERK1/2 but not that of p38 MAP kinase, and completely inhibited DNA synthesis (Figure 9a,d). Moreover, GF109203X inhibited cell migration by 50% (Figure 9b), and completely inhibihted VEGF-mediated phosphorylation of FAK and paxillin (Figure 9d), and the assembly of vinculin (Figure 10). SB203580, a specific

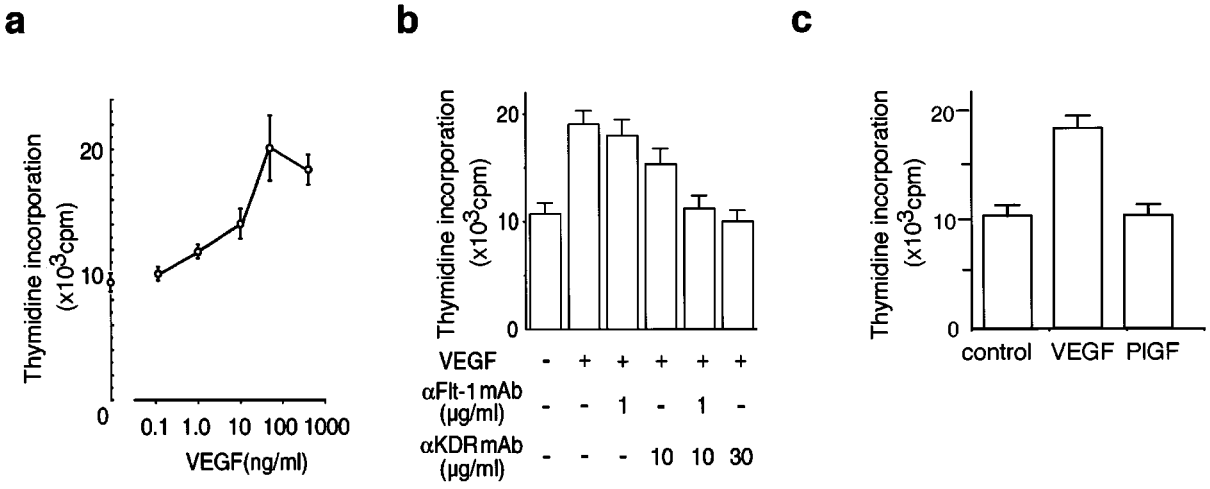

Figure 3 Regulation of DNA synthesis by KDR. (**a**) HUVECs in a 96-well culture plate were stimulated with indicated concentrations of VEGF. Thereafter, indicated concentrations of VEGF and 1.0 μCi [methyl^{3}H]thymidine were added to the cultures, and the cells were incubated for another 24 h. Incorporation of [methyl^{3}H]thymidine into cells was determined by liquid scintigraphy. (**b**) HUVECs were left untreated or exposed to indicated concentrations of KM1750 (αFlt-1 mAb) and/or KM1992 (αKDR mAb) 15 min prior to the addition of VEGF. (**c**) Fifty ng/ml of VEGF or 30 ng/ml of P1GF was added to the cultures, and the cells were incubated for 24 h. Values in **a**, **b**, and **c** are the means and s.d.s of eight samples

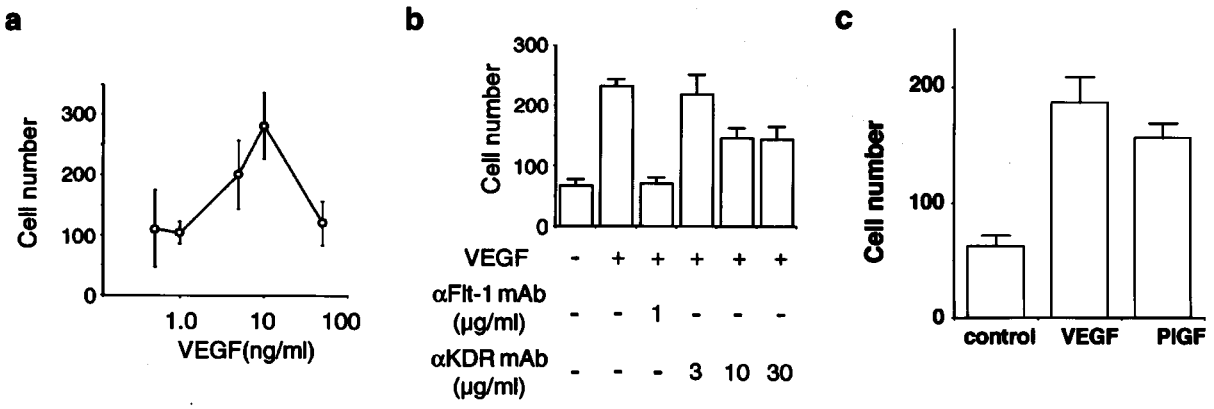

Figure 4 Regulation of cell migration by Flt-1 and KDR. (**a**) Confluent monolayers of HUVECs on 35 mm type-I collagen coated dishes were wounded with a razor blade and further incubated for 24 h with indicated concentrations of VEGF. (**b**) Confluent monolayers of HUVECs were wounded with a razor blade and were left untreated or exposed to indicated concentrations of KM1750 (αFlt-1 mAb) and/or KM1992 (αKDR mAb) for 15 min prior to the addition of VEGF. The cultures were further incubated for 24 h with or without 10 ng/ml of VEGF. (**c**) Confluent monolayers of HUVECs were wounded with a razor blade and further incubated for 24 h with 10 ng/ml of VEGF (0.2 nmol/L) or 6 ng/ml (0.2 nmol/L) of P1GF. Values in **a**, **b**, and **c** are the means and s.d.s of eight samples

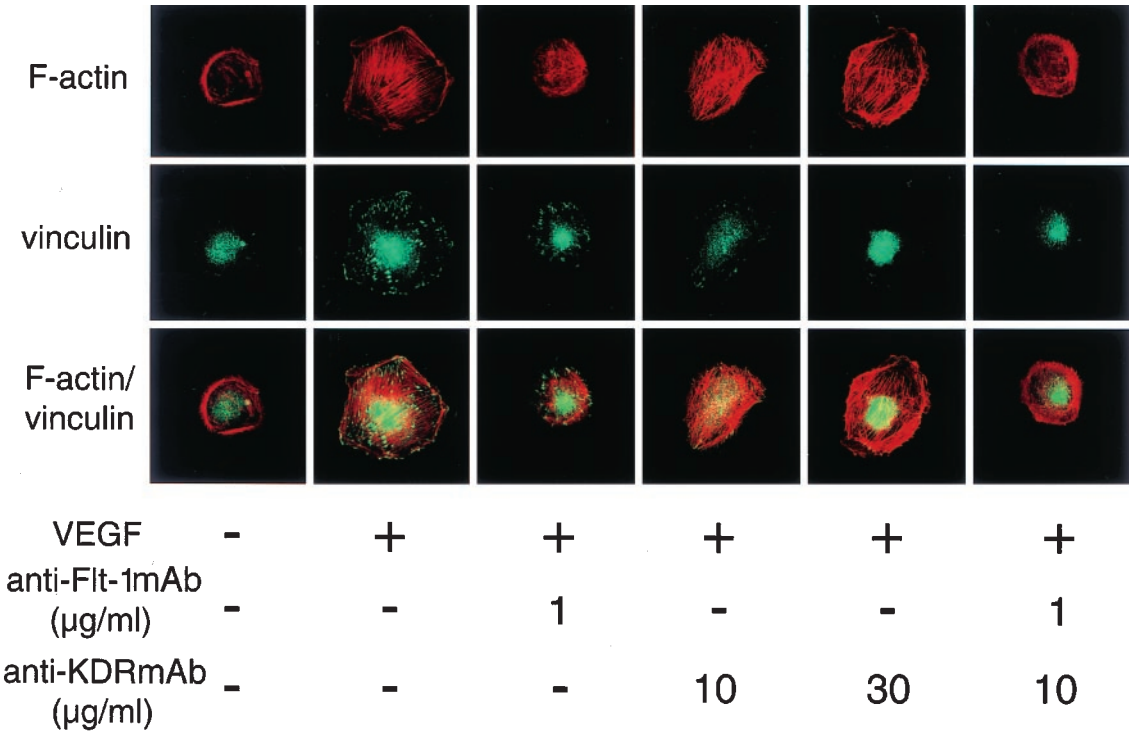

Figure 5 Regulation of actin reorganization by Flt-1 and vinculin assembly to focal adhesion plaque by KDR. HUVECs were plated on type-I collagen coated dishes in M-199 containing 5% FCS, and were incubated for 2 h at 37°C. Thereafter, HUVECs were left untreated or exposed to indicated concentrations of KM1750 (αFlt-1 mAb) and/or KM1992 (αKDR mAb) for 15 min. Ten ng/ml of VEGF was added to the cultures, which were further incubated for 15 min. After the incubation, cells were fixed and analysed

inhibitor of p38 MAP kinase, completely inhibited augmentation of cell migration (Figure 9b), and actin reorganization by VEGF (Figure 10).

Discussion

Here we examined the role of two VEGF receptors, Flt-1 and KDR, in the signal transduction of VEGF effects by newly developed blocking monoclonal antibodies. Figure 11 summarizes the findings of the present study. Although the blocking anti-KDR mAb has been reported (Zhu *et al*., 1998), the blocking anti-Flt-1 mAb is the first to be described. KDR is reported to play a principal role in the signal transduction of VEGF effects, and the participation of Flt-1 in ECs is controversial (Waltenberger *et al*., 1994; Kroll *et al*., 1997). Our present results clearly indicate that both Flt-1 and KDR play roles in the signal transduction of VEGF in normally differentiated human ECs, and the roles of these two receptors are distinct.

Anti-KDR mAb but not anti-Flt-1 mAb completely inhibited the stimulatory effect of VEGF on DNA synthesis. This result is in good agreement with

previous reports (Waltenberger *et al*., 1994; Keyt *et al*., 1996; Kroll *et al*., 1997; Bernatchez *et al*., 1999), and further confirms that VEGF stimulates DNA synthesis of ECs preferentially via KDR. KDR-mediated signal preferentially modulates DNA synthesis of HUVECs via the activation of ERK1/2. It is generally accepted that the activation of ERK1/2 is required for DNA synthesis (Rousseau *et al*., 1977; Dudley *et al*., 1995). This effect of ERK1/2 is thought to be mediated via the induction of cyclin D1 (Weber *et al*., 1997). In contrast to DNA synthesis, the regulation of cell migration by VEGF appears to be more complicated. The most important findings in our present experiments are that cytoskeleton-regulated cell motility and cell adhesion on cell migration are regulated by distinct receptor systems.

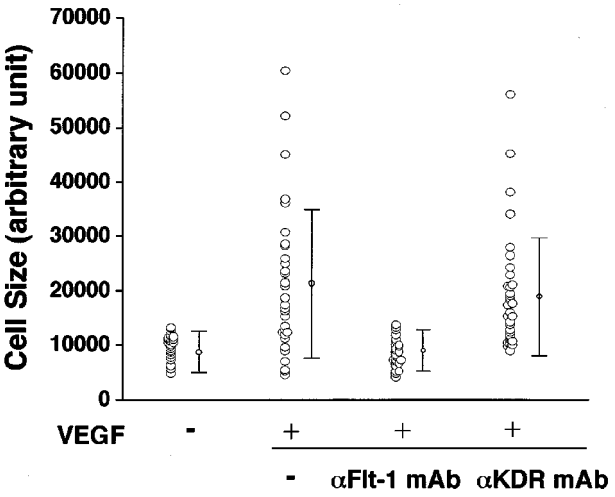

Figure 6 Regulation of cell spreading by Flt-1. After the fixation of HUVECs as described in Figure 5, cell size was analysed with NIH image analyzer soft

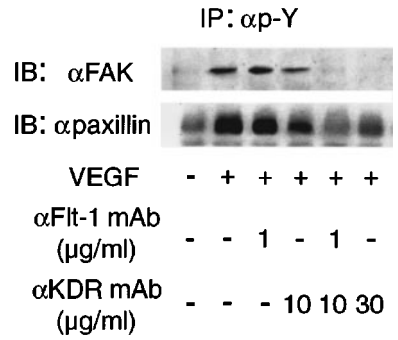

Figure 7 Tyrosine phosphorylation of FAK and paxillin by KDR. HUVECs were pretreated with 0.1 mmol/L of sodium orthovanadate for 1 h, and were left untreated or exposed to indicated concentrations of KM1750 (αFlt-1 mAb) and/or KM1992 (αKDR mAb) 15 min prior to the addition of VEGF. Then the cultures were incubated with or without 10 ng/ml of VEGF for 15 min. Immunoprecipitation followed by Western blotting was performed. IP: antibodies used for immunoprecipitation, IB: antibodies used for Western blotting

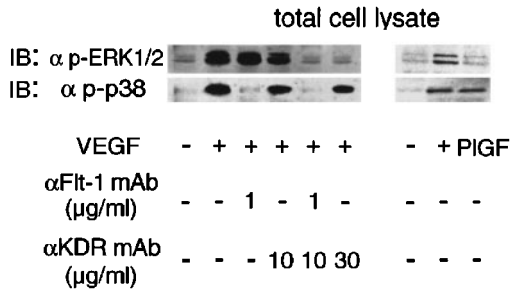

Figure 8 Activation of ERK1/2 was mediated via KDR, whereas that of p38 was mediated via Flt-1. HUVECs were left untreated or exposed to indicated concentrations of KM1750 (αFlt-1 mAb) and/or KM1992 (αKDR mAb) 15 min prior to the addition of VEGF. Then the cultures were incubated with or without 10 ng/ml of VEGF for 5 min. Activation of ERK1/2 or p38 was determined by immunoblotting with anti-dually phosphorylated ERK1/2 antibody (αp-ERK1/2) or anti-dually phosphorylated p38 antibody (αp-p38)

Our results indicate that Flt-1-mediated signal preferentially modulates actin reorganization of HUVECs via the activation of p38 MAP kinase. This is in good agreement with previous report that VEGF activates p38 MAK kinase in HUVECs and transduces the signal for cell migration (Rousseau *et al*., 1997). Actin reorganization is essential for cell motility (Lauffenburger and Horwitz, 1996), thus the blockade of Flt-1-mediated signals completely stopped migration of HUVECs. The mechanism by which p38 MAK kinase modulates actin reorganization in response to VEGF remains to be elucidated. The best characterized molecules, which are essential for actin reorganization and cell motility, are rho family low molecular weight G proteins including rac, CDC44, and rho A (Hall, 1998). We are currently examining whether rho family low molecular weight G proteins participate in the signal transduction of Flt-1 and p38 MAK kinase.

The cell adhesion-related molecules such as FAK and paxillin are generally activated by two systems, namely, growth factor and integrin (Craig and Johnson, 1996). Regarding growth factor-mediated regulation in Swiss3T3 cells, tyrosine phosphorylation of growth factor receptor is followed by subsequent tyrosine phosphorylation of FAK and paxillin, and recruitment of talin and vinculin to focal adhesion plaque (Abedi and Zachary, 1997). Thus, VEGF-mediated system in HUVECs may be comparable to that in Swiss3T3 cells, and this system is preferentially mediated by KDR. However, ERK1/2 may not play a major role in this system, since specific inhibition of ERK1/2 by PD98059 exhibited only a marginal effect on vinculin assembly and phosphorylation of FAK and paxillin. Previous reports indicate that VEGF, by binding to KDR, activates ERK1/2 via a phospholipase Cγ (PLCγ)-PKC-dependent pathway (Seetharam *et al*., 1995; Guo *et al*., 1995; Takahashi and Shibuya, 1997). We observed that specific inhibition of PKC by GF109203X completely abrogated vinculin assembly as well as DNA sysnthesis. It has been reported that activation of PKC regulates phosphorylation of FAK and paxillin in ECs as well as other cell types (Abedi and Zachary, 1997; Lewis *et al*., 1996; Slack, 1998). Taken together, phosphorylation of FAK and paxillin and the assembly of vinculin were regulated preferentially via a PKC-dependent and ERK1/2-independent pathway, while DNA synthesis was regulated via a PKC-dependent and ERK1/2-dependent pathway.

Our present study clearly indicates that Flt-1 does participate in the signal transduction of VEGF effects in normally differentiated human ECs. However, Flt-1 lacking the tyrosine kinase domain has recently been

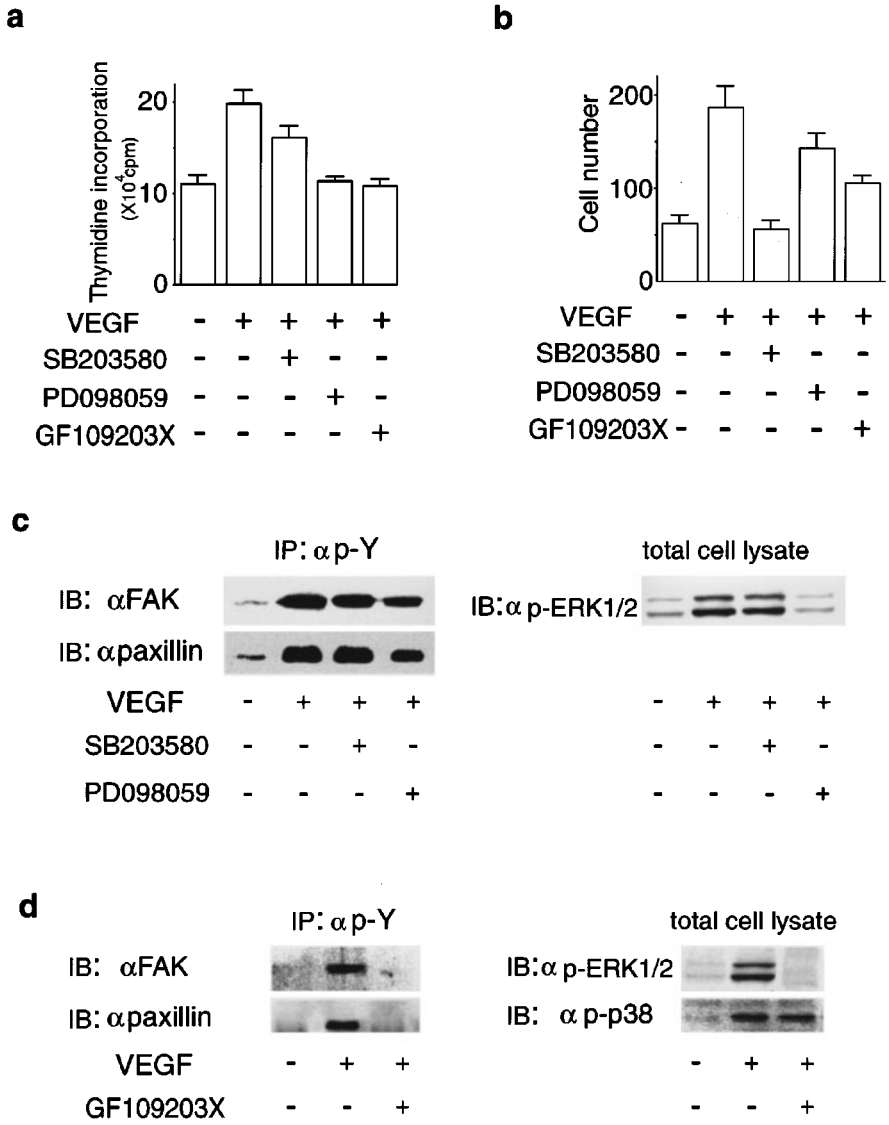

Figure 9 PD98059, GF109203X, and SB203580 differently inhibited VEGF-mediated effects in HUVECs. (**a**) HUVECs in a 96-well culture plate were left untreated or exposed to SB203580 (3 μM), PD98059 (25 μM), or GF109203X (3 μM) for 60 min prior to the addition of VEGF. The cultures were incubated with or without 50 ng/ml of VEGF and with 1.0 μCi [methyl^{3}H]thymidine for 24 h, and incorporation of [methyl^{3}H]thymidine into cells was then determined by liquid scintigraphy. (**b**) Confluent monolayers of HUVECs on 35 mm type-I collagen coated dishes were left untreated or exposed to SB203580 (3 μM), PD98059 (25 μM), or GF109203X (3 μM) for 60 min prior to the addition of VEGF. The cultures were wounded with a razor blade. (**c**) HUVECs were pretreated with 0.1 mM of sodium orthovanadate for 1 h, and were left untreated or exposed to SB203580 (3 μM) or PD98059 (25 μM) for 60 min prior to the addition of VEGF. Thereafter the cultures were incubated with or without 10 ng/ml of VEGF for 15 min. Immunoprecipitation followed by Western blotting was performed. IP: antibodies used for immunoprecipitation, IB: antibodies used for Western blotting. Activation of ERK1/2 or p38 MAP kinase was determined by Western blotting with anti-dually phosphorylated ERK1/2 Ab (αp-ERK1/2) or anti-dually phosphorylated p38 MAP kinase Ab (αp-p38). (**d**) Same experiments were done with GF109203X (3 μM). Values in **a** and **b** are the means and s.d.s of eight samples

shown to be sufficient for the normal development of vasculature in the murine embryo (Hiratsuka *et al*., 1998). This discrepancy raises several questions. Is kinase negative Flt-1 really negative in terms of the signal transduction? The signal transduction systems of ECs in those mice expressing tyrosine kinase negative Flt-1 need to be determined. It is possible that some other signaling molecules may associate with tyrosine kinase negative Flt-1. Alternately, the role of Flt-1 in ECs of developing vasculature in embryo and in differentiated ECs of postnatal state or of adult may not be identical. In any case, our present results provide important information for understanding the function of two VEGF receptors in human differentiated ECs.

Materials and methods

Materials

[Methyl-^{3}H]thymidine was purchased from Amersham (Buckinghamshire, England); type-I collagen-coated dishes from Iwaki Glass Co., Ltd. (Chiba, Japan); rabbit polyclonal Anti-ACTIVE™ MAPK antibody, and Anti-ACTIVE™ p38 MAP kinase antibody from Promega Corporation (Madison, WI, USA); rabbit polyclonal anti-FAK, anti-KDR, and anti-Flt-1 antibodies from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA); monoclonal anti-vinculin antibody from Seikagaku Corp. (Tokyo, Japan); rhodamine-conjugated phalloidin from Molecular Probes, Inc. (Eugene, OR, USA); monoclonal anti-phosphotyrosine (PY20) and anti-paxillin from Transduction Laboratories (Lexington, KY, USA); SB203580, a specific

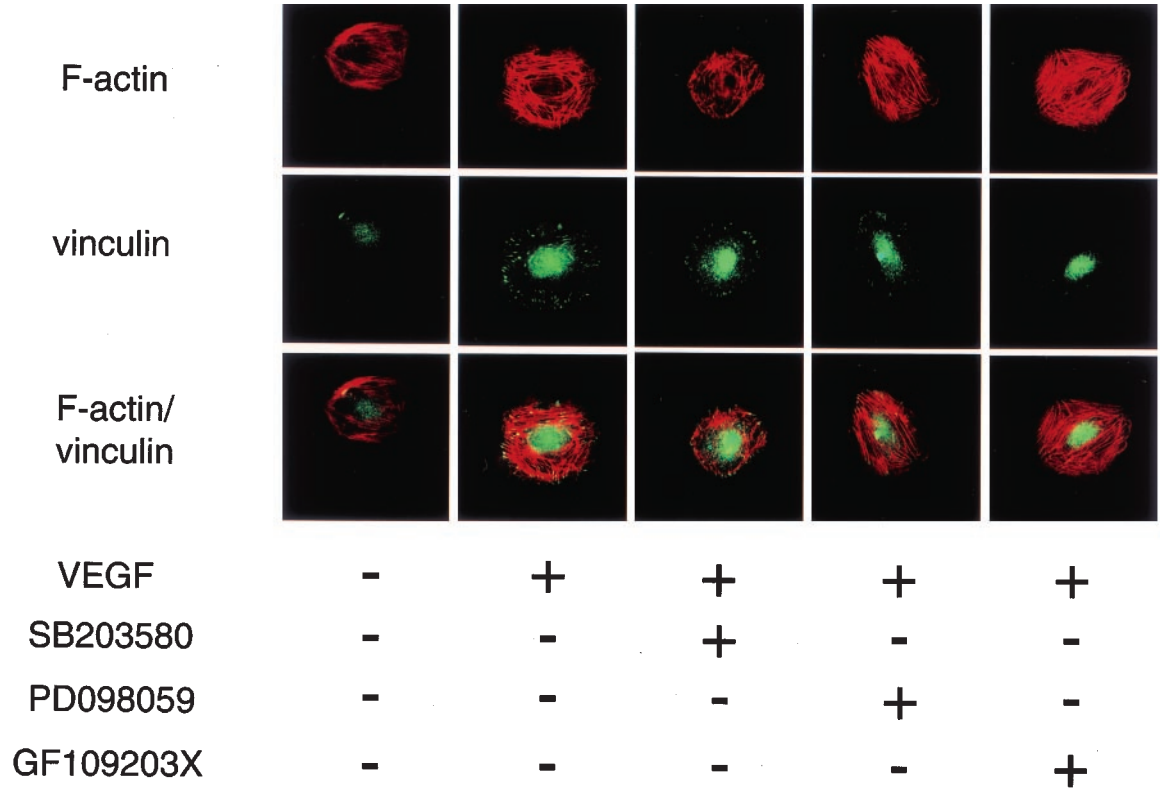

Figure 10 VEGF-mediated phosphorylation of FAK and paxillin was mediated via a PKC-dependent and ERK1/2-independent pathway. HUVECs were plated and incubated for 2 h at 37°C on type-I collagen coated dishes (35 mm) in M-199 containing 5% FCS without any supplements. HUVECs were left untreated or exposed to SB203580 (3 μM), PD98059 (25 μM), or GF109203X (3 μM) for 60 min. Thereafter, VEGF (10 ng/ml) was added to the cultures, which were incubated for 15 min. After the incubation, cells were fixed and analysed

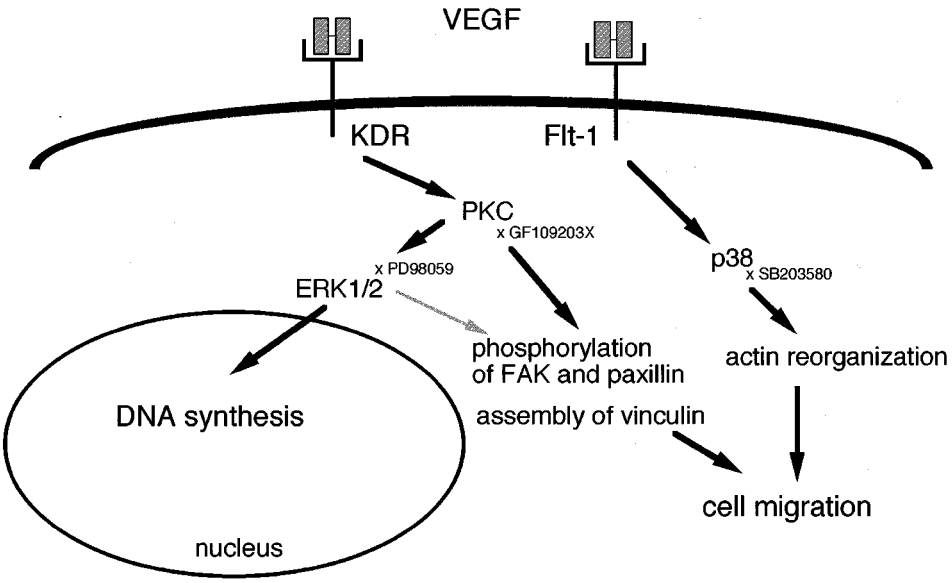

Figure 11 The intracellular signal transduction of VEGF-mediated effects in HUVECs. KDR mediates the signals for DNA synthesis, phosphorylation of FAK and paxillin, and vinculin assembly. The signals for DNA synthesis is mediated via PKC-dependent and ERK1/2 dependent pathway, whereas those for phosphorylation of FAK and paxillin and vinculin assembly are preferentially mediated via PKC-dependent and ERK1/2-independent pathway. Flt-1 activates p38 MAP kinase, and activated p38 regulates cell migration via actin reorganization

inhibitor of p38 MAP kinase, and GF109203X, a protein kinase C inhibitor from Calbiochem (La Jolla, CA, USA); PD098059 from New England Biolabs, Inc. (Beverly, MA, USA); horseradish peroxidase conjugated-protein G from Bio-Rad Laboratories (Hercules, CA, USA); and PlGF from R&D systems Inc. (Minneapolis, MN, USA). Human $VEGF_{165}$ was synthesized with the baculovirus/insect cell system as previously described (Sawano *et al*., 1996).

Cell culture

HUVECs were obtained from KURABO (Osaka, Japan) and routinely cultured on type-I collagen coated dishes in endothelial basal medium containing endothelial cell growth supplements (Clonetics Corp., Walkersville, MD, USA) and 5% FCS (Summit Biotechnology, Ft. Collins, CO, USA). Prior to the following experiments, HUVECs were preincubated overnight (confocal microscopy experiment) or for 24 h (the other experiments) in M-199 (Nissui Pharmaceutical Co., Ltd. Tokyo, Japan) containing 5% FCS in the absence of growth supplements.

Flt-1 overexpressing NIH3T3 cells (NIH3T3-Flt-1), KDR overexpressing NIH3T3 cells (NIH3T3-KDR) and NIH3T3 control transfectant cells (NIH3T3-neo) established by Sawano *et al*. (1996) were cultured in DMEM containing 200 μg/ml G418 and 10% FCS (Nissui, Tokyo, Japan).

Anti-VEGF receptors mAbs

Soluble VEGF receptor proteins, Flt-1 7N carrying the N-terminal 1st to 7th and Flt-1 3N carrying 1st to 3rd Ig-like domains of Flt-1, were obtained as described previously (Tanaka *et al*., 1997). KDR 7N carrying the N-terminal 1st to 7th Ig-like domains and KDR 5 δ 1N carrying the 2nd to 5th Ig-like domains of KDR fused with the Fc portion of an IgG (KDR 7N-Fc and KDR 5 δ 1N-Fc, respectively) were obtained as described previously (Shinkai *et al*., 1998). The production of anti-human Flt-1 mAbs including KM1750 (mouse IgG2b) and anti-human KDR mAbs including KM1992 (mouse IgG1) will be described elsewhere (M Ito *et al*., in preparation). Briefly, B3C3F1 mice (Charles River Japan, Kanagawa, Japan) at 6–8-weeks-old were immunized by i.p. injection of Flt-1 7N or KDR 5 δ 1N-Fc. Spleen cells were fused with mouse myeloma cells and hybridoma cells producing the mAbs to Flt-1 3N and KDR 7N-Fc, were selected.

Elisa

ELISA was performed as described previously (Hanai *et al*., 1986). Briefly, 96-well immunoplates (Greiner, Frickenhausen, Germany) were coated with 50 μl/well of Flt-1 7N or KDR 7N-Fc (2 μg/ml in PBS) for overnight at 4°C, and were blocked with 1% BSA in PBS. Fifty μl of anti-VEGF receptors mAbs were added to each well and incubated at room temperature for 2 h. After washing three times with 0.05 % Tween-20 in PBS, 50 μl of peroxidase-labeled rabbit anti-mouse immunoglobulin (Dakopatts, Glosrup, Denmark) were allowed to react for 2 h at room temperature. After washing six times with 0.05% Tween-20 in PBS, 50 μl of 1 mmol/L 2,2-azinobis (3-ethyl benzothiazoline-6-sulfonic acid) diammonium solution were added to each well and incubated at room temperature for 15 min. The reaction was stopped with 5% sodium dodecyl sulfate solution and the absorbance at 415 nm was measured by microtiter plate analyzer (E-max, Molecular Devices, Menlo Park, CA, USA).

Flow-cytometric analysis

Biotinylation of mAb was performed as described (Shitara *et al*., 1987). In indirect immunofluorescence, the cells (1×10^6) were incubated with biotinylated mAbs (30 μg/ml) for 30 min at 4°C. After the reaction with 5 μg/ml Streptoavidin-R-Phycoerythrin (Gibco, Gaithersburg, MD, USA), the reactivity was analysed in an EPICS Elite flow cytometer (Coulter Corporation, Hialeah, FL, USA).

DNA synthesis

Ten thousand HUVECs preincubated for 24 h in M-199 containing 5% FCS were incubated with indicated concetration of VEGF and 1.0 μCi [methyl ^{3}H]thymidine for another 24 h. Incorporation of [methyl ^{3}H]thymidine into cells was determined by liquid scintigraphy. For the blocking experiments, indicated concentrations of antibodies or inhibitors were added to the culture 15 min prior to the addition of VEGF (50 ng/ml).

Wound migration assay

Confluent monolayers of HUVECs on 35 mm type-I collagen coated dishes were wounded with a razor blade as described (Sato and Rifkin, 1988), and incubated for 24 h with or without indicated concentrations of VEGF. After the incubation, cells that had migrated across the edge of the wound were counted. For the blocking experiments, indicated concentrations of antibodies or inhibitors were added to the culture 15 min prior to the addition of VEGF (10 ng/ml).

Western blot analysis

For Western blotting of Flt-1 and KDR, HUVECs were cultured in M-199 containing 5% FCS and not treated with VEGF. For the blocking experiments, antibodies or inhibitors were added to HUVECs 15 min prior to the addition of VEGF. Thereafter, 10 ng/ml of VEGF was added to the cultures, which were incubated for 15 min. Cells were washed with PBS, collected in modified RIPA buffer (2 mmol/L sodium orthovanadate, 50 mmol/L NaF, 20 mmol/L HEPES, 150 mmol/L NaCl, 1.5 mmol/L $MgCl_2$, 5 mmol/L sodium pyrophosphate, 10% glycerol, 0.2% Triton X-100, 5 mmol/L EDTA, 1 mmol/L PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin), and allowed to lyse for 30 min on ice. Samples were clarified by centrifugation for 15 min at 15 000 r.p.m. at 4°C. Western blotting was performed as described previously (Iwasaka *et al*., 1996). Briefly, the samples containing equal amounts of protein were separated on SDS polyacrylamide gel under reducing conditions and transferred to nitrocellulose membranes. The blots were incubated for 1 h at room temperature with the indicated primary antibodies, followed by incubation for 1 h with horseradish peroxidase-conjugated protein G. Immunoreactive bands were visualized by chemiluminescence with a LAS-1000 Image Analyzer (Fuji, Tokyo, Japan).

Immunoprecipitation followed by Western blot analysis

HUVECs cultured in M-199 containing 5% FCS were preincubated with 0.1 mmol/L of sodium orthovanadate for 1 h. Then, antibodies or inhibitors were added to the cultures 15 min prior to the addition of VEGF. Thereafter, HUVECs were incubated with 10 ng/ml of VEGF for 15 min (for FAK, paxillin) or 5 min (for Flt-1 and KDR). Samples were prepared as mentioned in Western blot analysis and incubated with the indicated antibodies and protein A-Sepharose for 24 h at 4°C. The immunoprecipitates were washed four times in modified RIPA buffer, boiled for 5 min in reducing buffer, and electrophoresed on SDS polyacrylamide gels; Western blotting was then performed.

Actin reorganization and vinculin assembly

HUVECs on type-I collagen coated dishes (3×10^4 cells/35 mm dish) were incubated for 2 h at 37°C in M199 containing 5% FCS, and further incubated with or without KM1750 and/or KM1992 for 15 min at 37°C. In some experiments, cells were preincubated with PD98059 (25 μM), SB203580 (3 μM), GF109203X (3 μM), or wortmannin (100 nM) for 60 min. Thereafter, 10 ng/ml of VEGF was added to the cultures, which were further incubated for 15 min. After the incubation, cells were fixed with 3.7% formaldehyde and permeabilized with 0.1% NP40 in PBS. Non-specific binding sites were blocked with 1% BSA and

10% skim milk in PBS. F-actin was detected with rhodamine-conjugated phalloidin. Vinculin was detected by indirect immunofluorescence using monoclonal anti-vinculin antibody and FITC-labeled secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). The cells were observed with a confocal microscope (LSM410) (Carl Zeiss Jena GmbH, Jena, Germany). Cell size was analysed with NIH Image Analyzer soft.

Acknowledgments
This work was supported by a Grant-in-Aid for Scientific Research on Priority Areas from the Japanese Ministry of Education, Science, Sports and Culture.

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