

PDF issue: 2024-10-05

Activation and translocation of PKC δ is necessary for VEGF-induced ERK activation through KDR in HEK293T cells

Kuriyama, Masamitsu

<mark>(Degree)</mark> 博士(医学)

(Date of Degree) 2005-02-28

(Date of Publication) 2013-02-26

(Resource Type) doctoral thesis

(Report Number) 甲3231

(URL) https://hdl.handle.net/20.500.14094/D1003231

※ 当コンテンツは神戸大学の学術成果です。無断複製・不正使用等を禁じます。著作権法で認められている範囲内で、適切にご利用ください。



Activation and translocation of PKCδ is necessary for VEGF-induced ERK activation through KDR in HEK293T

HEK293T 細胞における KDR を介した VEGF 誘導の ERK の活性化に PKCô の活性化とトランスロケーションが必要である

Masamitsu Kuriyama¹, Taizo Taniguchi¹, Yasuhito Shirai¹, Atsuo Sasaki², Akihiko Yoshimura², Naoaki Saito¹

¹Laboratory of Molecular Pharmacology, Biosignal Research Center, Kobe University, 1-1 Rokkodai-cho, Nada-ku, Kobe 657-8501, Japan

²Division of Molecular and Cellular Immunology, Medical Institute of Bioregulation, Kyushu University, 3·1·1 Maidashi, Higashi-ku, Fukuoka 812·8582, Japan

Keywords: Vascular endothelial growth factor-A; Receptor Tyrosine Kinase;
 Phospholipase C γ; Diacylglycerol; Protein kinase C; ERK; GFP;
 Myristoylated alanine-rich C kinase substrate; C1B domain

* Manuscript

Activation and translocation of PKC δ is necessary for VEGF-induced ERK activation through KDR in HEK293T cells

Masamitsu Kuriyama¹, Taizo Taniguchi¹, Yasuhito Shirai¹, Atsuo Sasaki², Akihiko Yoshimura², Naoaki Saito^{1*}

¹Laboratory of Molecular Pharmacology, Biosignal Research Center, Kobe University, 1-1 Rokkodai-cho, Nada-ku, Kobe 657-8501, Japan ²Division of Molecular and Cellular Immunology, Medical Institute of Bioregulation, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan

*Address correspondence to : Naoaki Saito Laboratory of Molecular Pharmacology, Biosignal Research Center, Kobe University, 1-1 Rokkodai-cho, Nada-ku, Kobe 657-8501, Japan. Tel: +81-78-803-5961 Fax: +81-78-803-5971 E-mail: <u>naosaito@kobe-u.ac.jp</u>

Abstract

VEGF-KDR/Flk-1 signal utilizes the phospholipase C·y (PLC-y) -protein kinase C (PKC)-Raf-MEK-ERK pathway as the major signaling pathway to induce gene expression and cPLA2 phosphorylation. However, the spatio-temporally activation of a specific PKC isoform induced by VEGF-KDR signal has not been clarified. We used HEK293T (human embryonic kidney) cells expressing transiently KDR to examine the activation mechanism of PKC. PKC specific inhibitors and human PKC8 knock-down using siRNA method showed that PKC^δ played an important role in VEGF-KDR-induced ERK activation. Myristoylated alanine-rich C-kinase substrate (MARCKS) translocates from the plasma membrane to the cytoplasm depending upon phosphorylation by PKC. Translocation of MARCKS-GFP induced by VEGF-KDR stimulus was blocked by rottlerin, a PKC8 specific inhibitor, or human PKC8 siRNA. VEGF-KDR stimulation did not induce ERK phosphorylation in human PKCô-knockdown HEK293T cells, but co-expression of rat PKCô-GFP recovered the ERK phosphorylation. Y311/332F mutant of rat PKC&GFP which can not be activated by tyrosine-phosphorylation but activated by DAG recovered the ERK phosphorylation, while C1B-deletion mutant of rat PKCô-GFP, which can be activated by tyrosine-phosphorylation but not by DAG, failed to recover the ERK phosphorylation in human PKCô-knockdown HEK293T cell. These results indicate that PKCô is involved in VEGF-KDR-induced ERK activation via C1B domain.

Keywords

Vascular endothelial growth factor-A; KDR/Flk-1; HEK293T cells; Phospholipase C % Protein kinase C; ERK; GFP; Myristoylated alanine-rich C kinase substrate; C1B domain

Introduction

Recent studies have revealed that vascular endothelial growth factor-A (VEGF), also referred to vascular permeability factor (VPF) is essential for many angiogenic processes in normal and abnormal states such as diabetic retinopathy, tumor vascularization and rheumatic arthropathy [9, 10, 31]. VEGF exhibits two major activities, the capacity to stimulate vascular endothelial cell proliferation [3, 8, 25] and the ability to increase microvessel permeability to macromolecules [21, 43]. In addition VEGF stimulates migration [2], protease production [39, 54], cell survival [12], generation of nitric oxide and prostacyclin [30, 53, 55] in endothelial cells and migration of peripheral blood monocytes [1, 45]. The signaling mechanisms underlying these diverse process are still not well characterized.

VEGF binds two tyrosine kinase receptors, Flt-1 and KDR/Flk-1 (also known as VEGF receptor-1 and -2, respectively) with high affinity [5, 29]. Flt-1 and KDR/Flk-1 belong to a receptor gene family distantly related to the Fms/Kit/platelet-derived growth factor receptor (PDGFR). In addition, they share seven immunoglobulin(Ig)-like domains in the extracellular domain and a long kinase insert in the middle of the kinase domain [28, 46, 52]. Since the mRNA of the Flt-1 and KDR are specifically expressed in the vascular endothelial cells [6, 17, 18, 40, 57], they are considered to play a crucial roles in the vascular system.

Unlike other representative growth factor receptor tyrosine kinases that mainly mediate Ras to activate Raf-MEK-ERK pathway, KDR/Flk-1 utilizes the phospholipase C- γ (PLC- γ)-protein kinase C (PKC)-Raf-MEK-ERK pathway but not Ras-mediated pathway [50, 51]. In an agreement with this notion, Ras-GTP complex is below detectable levels after VEGF stimulation and dominant-negative Ras does not suppress the VEGF signaling. The precise mechanism of VEGF-KDR signaling to ERK phosphorylation is still controversial.

PKC plays a pivotal role in many signaling pathway, and the existence of multiple isofroms suggests that different isoforms have various functions. The PKC superfamily consists of at least 10 isoforms and is divided into three group, classical PKC (cPKC; α , β I, β II, γ), novel PKC (nPKC; δ , ε , η , θ), and atypical PKC (aPKC; ζ , ι/λ), based on their structure and biochemical properties [32, 33]. Each PKC isoform has differential sensitivity to activators and shows tissue-specific and cell-specific expression, suggesting that each isoform plays a specific role in various signal transduction pathway and in the regulation of numerous cellular processes [32-34]. Recently, we demonstrated using GFP-tagged PKC that various stimuli induce specific translocation and activation of each PKC isoform, and suggested that each isoform has specific translocation mechanism and function in response to various stimuli respectively [19, 36, 41, 48]. Several investigators have reported that a specific PKC isoform is involved in the VEGF signaling pathway. VEGF activated PKC β in NIH3T3 cells overexpressing KDR [50], PKC α and PKC ζ or PKC δ is involved in VEGF-induced ERK activation in human umbilical vein endothelial cells [13, 56].

In the present study, to clarify a PKC isoform involved in the signaling and to investigate its activation mechanism in VEGF-KDR-PLC γ PKC-Raf-ERK pathway, we examined the effect of PKC specific inhibitor or siRNA on ERK phosphorylation induced by VEGF-KDR stimulation in HEK293T cells expressing KDR. We also investigated the spatial activation of PKC by VEGF-KDR stimulation. We demonstrated here that PKC δ mediates the cascade from VEGF-KDR to ERK and PKC δ translocation from the cytoplasm to the plasma membrane through its C1B domain is necessary for VEGF-induced ERK phosphorylation.

Materials and methods

Materials and Chemicals

Human VEGF-A was purchased from PeproTec EC, Ltd. Polyclonal anti-ERK (#9102) and monoclonal anti-phospho-ERK (Thr202/Tyr204) (#9106) antibodies were purchased form Cell Signaling Technology. Polyclonal anti-Flk-1 (sc-505), anti-PKC β I (sc-209), anti-PKC β II (sc-210), anti-PKC γ (sc-211), anti-PKC δ (sc-937), anti-PKC ϵ (sc-214) and anti-PKC η (sc-215) antibodies were purchased from Santa Cruz Biotechnology. Monoclonal anti-PKC α antibody was purchased from Transduction Laboratories. Polyclonal anti-PKC ζ serum (#07-264) was purchased from Upstate. Other materials and chemicals were from commercial sources.

Cell Culture

Human Embryonic Kidney cells (HEK293T) were cultured in Dulbecco's modified Eagle's medium (Nacalai tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum in a humidified atmosphere containing 5% CO₂ at 37°C. All media were supplemented with penicillin (100 units/ml) and streptomycin (100mg/ml)

Preparation of Plasmids

The expression plasmids bearing cDNA of KDR, PKCα, PKCβI, PKCβII, PKCγ, PKCδ, PKCε, PKCη, PKCζ, PKCδ-GFP, PKCδ-(Y311/322F)-mutant-GFP, PKCδmutant deleting C1B domain(ΔC1B mutant)-GFP and myristroylated alanine-rich protein kinase C substrate (MARCKS)-GFP were prepare as described previously [20, 35-38, 41, 42].

Transfection of Plasmids into Cultured Cell

Plasmids were prepared by purification protocol using CsCl density gradient. Transfection of plasmids was performed by lipofection using Polyfect Transfection Reagent (QIAGEN GmbH, Germany) or Lipofectamin 2000 (Invitrogen) according to the manufacture's standard protocol. For co-expression of plasmids, the same amount of each plasmid was mixed and transfected. Experiments were 24-48hr after the transfection

Observation of Translocation of MARCKS-GFP or PKCô-GFP

6hr after transfection, cells were spread onto glass-bottomed culture dish (MatTek, Ashland, MA) and cultured for 18hr before observation. The culture medium was replaced with normal HEPES buffer composed of 5mM HEPES, 135mM NaCl, 5.4mM KCl, 1mM MgCl₂, 1.8mM CaCl₂, and 10mM glucose, pH7.3 at RT. Translocation of fusion proteins was triggered by a direct application of VEGF at a 10 times higher concentration into the buffer to obtain the final concentration at 25ng/ml. The fluorescence of GFP was monitored under a confocal laser scanning fluorescence microscope (model LSM 510 invert, Carl Zweiss, Jena, Germany). GFP-fused protein was monitored at 488nm argon excitation with a 510nm-535nm-band pass barrier filter. Images were collected sequentially every 10 seconds. All experiments were performed at 37°C.

Knockdown of Endogenous PKC8 by siRNA

Knockdown of human PKC8 in HEK293T cells by using siRNA was performed as described previously [16]. Briefly, the siRNA sequences of human PKC8 siRNA corresponded to the coding region 603-621 relative to the first nucleotide to the start codon. 21-nucleotide RNAs with 3' overhangs of 2 deoxythymidine were chemically synthesized by Nihon Bio Service Co. (Asaka, Japan). To generate a duplex of siRNA, 20µM sense and antisense single-strand RNAs were annealed by incubating the mixed siRNA in annealing buffer (30mM HEPES-KOH, 100mM potassium acetate, 2mM magnesium acetate) for 1 minute at 90°C followed by incubation for 1 hour at 37°C. Transfection of duplex siRNA was performed by using Lipofectamin 2000 (Invitrogen) according to the method described by Elbashir *et al.* [7].Annealed siRNA was transfected at final concentration of 250 nM into 90-100% confluent HEK293T cells by lipofection.

ERK phosphorylation assay

The transfected cells were serum-starved for 2hr and stimulated by final 25ng/ml VEGF for 5min. Then SDS-PAGE sample buffer was added to the cells to stop the signal transduction. Total cell lysates were collected with scrapper, heated to 95°C for 5min, were fractioned by a 8% acrylamide SDS-PEGE gel and immnunoblotted with an antibody that specifically recognized ERK-1 and ERK-2 activated by phosphorylation at Thr202/Tyr204.

<u>Results</u>

Effects of selective PKC inhibitors on VEGF-KDR-induced ERK phosphorylation in transiently KDR-expressed HEK293T cells

25ng/ml of VEGF induced ERK phosphorylation in time dependent manner in the HEK293T cells which transiently express KDR (Fig. 1), although ERK phosphorylation was not observed by the same treatment in wild HEK293T cells (data not shown). The phosphorylation reached maximum between 3⁻ 5 min and the signal slightly decreased in intensity by 30 min after stimulation (Fig. 1). Using the HEK293T cells expressing KDR, we first examined whether ERK phosphorylation by VEGF depends on PKC or not. A broad PKC inhibitor, Gö6983, significantly reduced VEGF-induced ERK phosphorylation, indicating that PKC is involved in the ERK phosphorylation.

Western blotting using isozyme specific antibodies revealed that HEK293T cell mainly expressed PKC β I, β II, δ , ε and ζ (Fig. 2) in addition to faint PKC α . To determine which PKC isozyme is involved in the signaling, we investigated the effect of isozyme specific PKC inhibitors on VEGF-KDR-induced ERK phosphorylation. In addition to Gö6983, GF109203X, an another broad PKC inhibitor, suppressed the ERK phosphorylation. On the other hand, Gö6976, a cPKC specific inhibitor, did not show any effect on ERK phosphorylation, while rottlerin, a PKC δ specific inhibitor significantly suppressed the ERK phosphorylation. These data suggested that PKC δ plays a key role in VEGF-KDR-induced ERK phosphorylation among endogenous PKC isozymes (Fig. 3).

Inhibition of VEGF-KDR-induced ERK phosphorylation by PKC8 siRNA

We have reported successful knock down of PKCS in HEK293T cells using human PKCS-specific siRNA [16]. To ascertain that PKCS is involved in ERK phosphorylation in response to VEGF-KDR stimulation, we knocked down endogenous PKCδ in HEK293T cells using RNAi method. siRNA for human PKCδ significantly decreased PKCδ expression, while it had no effect on the expression of other PKC isozymes (Fig. 4A). Concomitantly siRNA targeting human PKCδ decreased VEGF-KDR-induced ERK phosphorylation in HEK293T cell (Fig. 4B), but control siRNA with scrambled sequence failed.

Translocation of MARCKS-GFP from the plasma membrane to the cytoplasm in response to VEGF-KDR stimulation depends on PKC

It is supposed that activation of PKC δ is necessary for ERK phosphorylation by VEGF treatment, although the targeting site of PKC δ is still unclear. It also remains to be unknown where PKC δ is activated and where the enzyme phosphorylates the substrate leading to ERK phosphorylation. Live imaging studies revealed that PKCô is activated on the plasma membrane [36], in the cytoplasm [36], in the mitochondria [26] and at Golgi complex [19]. We examined where the active PKC δ is localized by using myristoylated alanine-rich C kinase substrate (MARCKS), as a marker of PKC activation. MARCKS, one of physiological PKC substrates, cycles on and off plasma membranes by a mechanism termed the myristoyl-electrostatic switch. While at the membrane, MARCKS binds to and sequesters acidic phospholipids intervening basic effector domain. Activated PKC phosphorylates basic effector domain of MARCKS and then MARCKS translocates from the plasma membrane to the cytosol. Using GFP fused MARCKS (MARCKS-GFP), the translocation of MARCKS can be monitored in living cells by confocal laser scanning microscope [35]. It has enabled us to detect the activation of PKC on the plasma membrane in living cells by monitoring the location of MARCKS. As shown in Fig. 5, VEGF induced MARCKS-GFP translocation from the plasma membrane to the cytoplasm, suggesting that endogenous PKC was activated on the plasma membrane by VEGF-KDR stimulation (Fig. 5A). Pan PKC inhibitors including GF109203X and Gö6983 blocked the MARCKS-GFP translocation (Fig. 5A). Treatment of Gö6976 or BAPTA-AM (data not shown) was no effect on the

MARCKS-GFP translocation, while inhibition of PKCδ with rottlerin blocked MARCKS translocation. These results strongly suggest that VEGF-KDR stimulation induces membrane translocation of PKCδ and its activation on the plasma membrane. In addition to the pharmacological studies, we examined the translocation of MARCKS-GFP in HEK293T cells transfected with siRNA for human PKCδ. In PKCδ-knockdown HEK293T cells, VEGF-KDR stimulation failed to induce the translocation of MARCKS-GFP (Fig. 5B). These findings indicate that activation of PKCδ on the plasma membrane is indispensable for the ERK phosphorylation at downstream of VEGF-KDR signaling.

C1B domain of PKCS Is Necessary for VEGF-KDR induced ERK phosphorylation

It is reported that PKCS can be activated through several distinct processes [22]. Activation of PKCS by diacylglycerol (DAG) binding to C1B domain is well established and DAG-independent activation of the PKC isozyme was recently shown [14, 15, 19, 20, 23, 24, 47, 49, 58]. Tyrosine phosphorylation of PKCS after hydrogen peroxide or ceramide treatment results in the activation of the PKC isozyme without DAG binding to its C1B domain. We examined whether the knockdown of endogenous PKC8 can be compensated by the overexpression of wild or various PKC8 mutants to investigate how PKCS is activated by VEGF/KDR stimulation. Overexpression of rat-derived wild PKC δ in HEK293T cells transfected with human PKC δ siRNA compensated VEGF-KDR induced ERK phosphorylation (Fig. 6), suggesting that siRNA for human PKCS reduced the amount of endogenous PKCS specifically but not affect other signaling molecules. Phosphorylation of tyrosine 311(Y311) and 332(Y332) of PKC δ has been shown to be responsible for its DAG-independent activation [20, 23, 24]. Double mutant of Y311/332F (phenylalanine) which can not be activated by tyrosine phosphorylation but can be activated by phorbol ester significantly compensated the ERK phosphorylation (Fig.6). Rat PKC δ mutant lacking C1B domain (Δ C1B) failed to induce ERK phosphorylation (Fig. 6).

We also examined the translocation of these mutants in response to VEGF-KDR stimulation. Intense fluorescence of PKC δ (wild type)-GFP was found throughout the cytoplasm and nucleoplasm of the HEK293T cell. Stimulation of VEGF-KDR induced a translocation of PKC δ (wild type)-GFP to plasma membrane. The translocation was observed within 2 min after the stimulation. Thereafter, PKC δ (wild type)-GFP re-translocated to the cytoplasm within 5 min (data not shown). PKC δ (Y311/332F mutant)-GFP also showed a translocation similar to PKC δ (wild type)-GFP in response to VEGF-KDR stimulation, while PKC δ (Δ C1B mutant)-GFP did not translocate from the cytoplasm to the plasma membrane in response to VEGF-KDR stimulation. (Fig.7). These data indicated that C1B domain is responsible for the translocation of PKC δ by VEGF-KDR stimulation.

Discussion

In this report, we have investigated the mechanism of VEGF-KDR induced ERK activation shown by phosphorylation of ERK (Thr202/Tyr204) and identified that δ -isoform of PKC is involved in this signaling. Furthermore, we have shown that VEGF-KDR-induced ERK activation requires the translocation of PKC δ from the cytoplasm to the plasma membrane and the activation of PKC δ at the plasma membrane via C1B domain.

Vascular endothelial cells express several kinds of VEGF receptors. Two structurally similar VEGF-receptors, Flt-1 and KDR/Flk-1, play independent and essential roles in vascular development. KDR-minus homozygous mice die on embryonic day 8.5-9.5 due to the lack of endothelial cell growth and blood vessel formation as well as an extremely poor hematopoiesis [44]. On the other hand, Flt-1-minus homozygous mice also die at the same stage; however, this is due to the overgrowth of endothelial cells and disorganization of blood vessels [11]. These results suggest that at least at the early stage of embryogenesis, KDR is essential for both proliferation and differentiation of endothelial cells, whereas Flt-1 is involved in the assembly of the vascular endothelium as a negative regulator of endothelial cell proliferation. Therefore to investigate VEGF-KDR specific signaling to ERK activation, we have used HEK293T cells transiently expressing KDR, since HEK293T cell do not express any endogenous VEGF receptors.

PKC activation has been shown to be mediated by PLC γ activation through VEGF-KDR leading to ERK activation [50, 51]. PLC γ activation results in the production of DAG and Ca²⁺ mobilization, suggesting that cPKC or nPKC would be involved in the signaling. However, it has been still controversial which isoform of PKC is activated by VEGF-KDR stimulation. Previous reports suggested PKC α , β , δ and ζ as candidates responsible for key enzyme in VEGF-KDR-ERK pathway [13, 42, 50, 56].

In our experiment, treatment of Gö6976, a cPKC specific inhibitor had no effects on the ERK phosphorylation by VEGF-KDR signal, rottlerin inhibited the ERK

phosphorylation (Fig.3), supporting that PKCδ was involved between VEGF-KDR and Raf-MEK-ERK cascade. Although rottlerin has been used in many studies to implicate PKCδ in a variety of cellular events, it also inhibits a number of protein kinases including calmodulin-dependent protein kinase III, p38-regulated/activated kinase and mitogen-activated protein kinase-activated protein kinase 2 [4, 22]. Therefore to confirm the involvement of PKCδ in VEGF-KDR signaling, we performed knockdown of endogenous PKCδ using siRNA method in HEK293T cells. Knockdown of human-derived endogenous PKCδ blocked VEGF-KDR-induced ERK phosphorylation (Fig.4). Rat PKCδ co-transfected with siRNA of human PKCδ recovered VEGF-KDR-induced ERK phosphorylation (Fig.6), but rat PKCδ kinase negative mutant did not (data not shown). These data indicates that PKCδ is indispensable for VEGF-KDR-induced ERK activation and that kinase activity of PKCδ is required to mediate VEGF-KDR signaling to induce ERK phosphorylation.

MARCKS was used for the spatio-temporal analysis of PKC activation. We have visualized the phosphorylation and translocation of MARCKS from the plasma membrane to the cytoplasm, when PKC was activated on the plasma membrane. In contrast, MARCK was not phosphorylated by PKC when PKC was activated in the cytosol [35]. In HEK293T cells transiently expressing KDR, VEGF stimulation induced the translocation of MARCKS-GFP from the plasma membrane to the cytoplasm. Pharmacological experiments using PKC inhibitors including rottlerin and siRNA treatment suggested that VEGF-KDR signal translocated and activated PKC8 on the plasma membrane and induce the phosphorylation of MARCKS. Although it is unknown why PKC8 is specifically activated by VEGF-KDR signal, it is possible that VEGF-KDR signal produces a lipid mediator specific for PKC8 activation or that receptor for activated C-kinase (RACK) for PKC8 is involved in this pathway.

Several activation mechanisms of PKC δ have been reported. It is activated by the binding with DAG or phorbol ester to C1 domain and by DAG-independent mechanisms such as tyrosine phosphorylation and proteolytic reaction [22]. But it is still unknown how PKC8 is activated by VEGF-KDR signal. Previously, we have shown that ceramide induces the translocation of PKC δ to the Golgi complex and activates PKCδ via phosphorylation of Tyr311 and Tyr332 [19, 20]. Unlike ceramide stimulation, unphosphorylatable PKC8 (Y311/332F mutant) could induce phosphorylation of ERK in response to VEGF, suggesting that tyrosine phosphorylation is not involved VEGF-KDR induced PKCS activation (Fig.6). PKCS consists of C2-like domain, pseudosubstrate-region, C1-domain including C1A and C1B, kinase domain and several variable reigions. Tandem C1 domains are not equivalent, DAG and phorbol ester mainly binds C1B domain of PKCS [14, 47, 49]. Takahashi et al. reported that VEGF-KDR activates PLCy leading ERK activation and that activated PLCy hydrolyses PIP2 to diacylglycerol (DAG) and IP3, inducing Ca2+ influx. Therefore it is predicted that PKCô translocated from the cytoplasm to the plasma membrane, where this kinase is activated via DAG binding with C1B domain. In agreement with this hypothesis, rat PKC8 lacking the C1B domain could not recover VEGF-KDR-induced ERK activation in human PKC8 knocked-down HEK293T cells. Furthermore PKC8 lacking C1B domain could not translocate from the cytoplasm to the plasma membrane by VEGF in HEK293T expressing KDR (Fig.6 and 7).

VEGF-KDR signal has the important role in normal and pathological angiogenesis [9, 10, 31]. As the action induced by VEGF-KDR signal via ERK is necessary for angiogenesis, VEGF-KDR pathway has attracted a great attention in the context of potential therapy for angiogenesis. Until now two categories of angiogenesis inhibitors are clinically developed [27]. One is nucleotide- and peptide-based anti-VEGF reagents including Bevacizumab which is recombinant humanised anti-VEGF monoclonal antibody and AngiozymeTM which is a stabilized ribozyme directed against the pre-mRNA of the KDR gene. Another is KDR kinase chemical inhibitors including SU5416, SU6668, SU11248, ZD6474 and Vatalanib.

In this report we have revealed an important role of PKC δ and its activation mechanism in VEGF-KDR-induced ERK activation. These results suggested that in addition to drugs acting on VEGF or KDR, the blocker of PKCô translocation by VEGF-KDR is also a candidate for pathological angiogenesis. However, the mechanism of PKCô specific activation after VEGF treatment should be clarified.

Acknowledgements

This work was supported by a 21st Century COE Program of the Ministry of Education, Culture, Sports, Science and Technology of Japan, a grant from the Ministry of Education, Culture, Sports, Science and Technology of Japan, the Uehara Memorial Foundation, the Sankyo Foundation of Life Science and Research Fellowship of the Japan Society for the Promotion of Science for Young Scientists.

Reference

- B. Barleon, S. Sozzani, D. Zhou, H.A. Weich, A. Mantovani, D. Marm Migration of human monocytes in response to vascular endothelial growth factor (VEGF) is mediated via the VEGF receptor flt-1, Blood 87 (1996) 3336-3343.
- P.N. Bernatchez, S. Soker, M.G. Sirois, Vascular endothelial growth factor effect on endothelial cell proliferation, migration, and platelet-activating factor synthesis is Flk-1-dependent, J. Biol. Chem. 274 (1999) 31047-31054.
- D.T. Connolly, D.M. Heuvelman, R. Nelson, J.V. Olander, B.L. Eppley, J.J. Delfino, N.R. Siegel, R.M. Leimgruber, J. Feder, Tumor vascular permeability factor stimulates endothelial cell growth and angiogenesis, J. Clin. Invest. 84 (1989) 1470-1478.
- S.P. Davies, H. Reddy, M. Caivano, P. Cohen, Specificity and mechanism of action of some commonly used protein kinase inhibitors, Biochem. J. 351 (2000) 95-105.
- C. de Vries, J.A. Escobedo, H. Ueno, K. Houck, N. Ferrara, L.T. Williams, The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor, Science 255 (1992) 989-991.
- A. Eichmann, C. Marcelle, C. Breant, N.M. Le Douarin, Two molecules related to the VEGF receptor are expressed in early endothelial cells during avian embryonic development, Mech. Dev. 42 (1993) 33-48.
- S.M. Elbashir, J. Harborth, W. Lendeckel, A. Yalcin, K. Weber, T. Tuschl, Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells, Nature 411 (2001) 494-498.
- N. Ferrara, W.J. Henzel, Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells, Biochem. Biophys. Res. Commun. 161 (1989) 851-858.
- 9. N. Ferrara, K.A. Houck, L.B. Jakeman, J. Winer, D.W. Leung, The vascular

endothelial growth factor family of polypeptides, J. Cell. Biochem. 47 (1991) 211-218.

- J. Folkman, What is the evidence that tumors are angiogenesis dependent?, J. Natl. Cancer Inst. 82 (1990) 4-6.
- G.H. Fong, J. Rossant, M. Gertsenstein, M.L. Breitman, Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium, Nature 376 (1995) 66-70.
- H.P. Gerber, A. McMurtrey, J. Kowalski, M. Yan, B.A. Keyt, V. Dixit, N. Ferrara, Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3'-kinase/Akt signal transduction pathway. Requirement for Flk-1/KDR activation, J. Biol. Chem. 273 (1998) 30336-30343.
- 13. G. Gliki, R. Abu-Ghazaleh, S. Jezequel, C. Wheeler-Jones, I. Zachary, Vascular endothelial growth factor-induced prostacyclin production is mediated by a protein kinase C (PKC)-dependent activation of extracellular signal-regulated protein kinases 1 and 2 involving PKC-delta and by mobilization of intracellular Ca2+, Biochem. J. 353 (2001) 503-512.
- 14. M. Hunn, A.F. Quest, Cysteine-rich regions of protein kinase Cdelta are functionally non-equivalent. Differences between cysteine-rich regions of non-calcium-dependent protein kinase Cdelta and calcium-dependent protein kinase Cgamma, FEBS Lett. 400 (1997) 226-232.
- J.H. Hurley, A.C. Newton, P.J. Parker, P.M. Blumberg, Y. Nishizuka, Taxonomy and function of C1 protein kinase C homology domains, Protein Sci. 6 (1997) 477-480.
- N. Irie, N. Sakai, T. Ueyama, T. Kajimoto, Y. Shirai, N. Saito, Subtype- and species-specific knockdown of PKC using short interfering RNA, Biochem. Biophys. Res. Commun. 298 (2002) 738-743.
- 17. L.B. Jakeman, J. Winer, G.L. Bennett, C.A. Altar, N. Ferrara, Binding sites for vascular endothelial growth factor are localized on endothelial cells in adult rat

tissues, J. Clin. Invest. 89 (1992) 244-253.

- A. Kaipainen, J. Korhonen, K. Pajusola, O. Aprelikova, M.G. Persico, B.I. Terman, K. Alitalo, The related FLT4, FLT1, and KDR receptor tyrosine kinases show distinct expression patterns in human fetal endothelial cells, J. Exp. Med. 178 (1993) 2077-2088.
- T. Kajimoto, S. Ohmori, Y. Shirai, N. Sakai, N. Saito, Subtype-specific translocation of the delta subtype of protein kinase C and its activation by tyrosine phosphorylation induced by ceramide in HeLa cells, Mol. Cell. Biol. 21 (2001) 1769-1783.
- T. Kajimoto, Y. Shirai, N. Sakai, T. Yamamoto, H. Matsuzaki, U. Kikkawa, N. Saito, Ceramide-induced apoptosis by translocation, phosphorylation, and activation of protein kinase Cdelta in the Golgi complex, J. Biol. Chem. 279 (2004) 12668-12676.
- P.J. Keck, S.D. Hauser, G. Krivi, K. Sanzo, T. Warren, J. Feder, D.T. Connolly, Vascular permeability factor, an endothelial cell mitogen related to PDGF, Science 246 (1989) 1309-1312.
- U. Kikkawa, H. Matsuzaki, T. Yamamoto, Protein kinase C delta (PKC delta): activation mechanisms and functions, J. Biochem. (Tokyo) 132 (2002) 831-839.
- H. Konishi, M. Tanaka, Y. Takemura, H. Matsuzaki, Y. Ono, U. Kikkawa, Y. Nishizuka, Activation of protein kinase C by tyrosine phosphorylation in response to H2O2, Proc. Natl. Acad. Sci. U. S. A. 94 (1997) 11233-11237.
- 24. H. Konishi, E. Yamauchi, H. Taniguchi, T. Yamamoto, H. Matsuzaki, Y. Takemura, K. Ohmae, U. Kikkawa, Y. Nishizuka, Phosphorylation sites of protein kinase C delta in H2O2-treated cells and its activation by tyrosine kinase in vitro, Proc. Natl. Acad. Sci. U. S. A. 98 (2001) 6587-6592.
- D.W. Leung, G. Cachianes, W.J. Kuang, D.V. Goeddel, N. Ferrara, Vascular endothelial growth factor is a secreted angiogenic mitogen, Science 246 (1989) 1306-1309.

- P.K. Majumder, P. Pandey, X. Sun, K. Cheng, R. Datta, S. Saxena, S. Kharbanda, D. Kufe, Mitochondrial translocation of protein kinase C delta in phorbol ester-induced cytochrome c release and apoptosis, J. Biol. Chem. 275 (2000) 21793-21796.
- P.W. Manley, G. Bold, J. Bruggen, G. Fendrich, P. Furet, J. Mestan, C. Schnell,
 B. Stolz, T. Meyer, B. Meyhack, W. Stark, A. Strauss, J. Wood, Advances in the structural biology, design and clinical development of VEGF-R kinase inhibitors for the treatment of angiogenesis, Biochim. Biophys. Acta 1697 (2004) 17-27.
- 28. W. Matthews, C.T. Jordan, M. Gavin, N.A. Jenkins, N.G. Copeland, I.R. Lemischka, A receptor tyrosine kinase cDNA isolated from a population of enriched primitive hematopoietic cells and exhibiting close genetic linkage to c-kit, Proc. Natl. Acad. Sci. U. S. A. 88 (1991) 9026-9030.
- B. Millauer, S. Wizigmann-Voos, H. Schnurch, R. Martinez, N.P. Moller, W. Risau, A. Ullrich, High affinity VEGF binding and developmental expression suggest Flk-1 as a major regulator of vasculogenesis and angiogenesis, Saibo 72 (1993) 835-846.
- T. Murohara, J.R. Horowitz, M. Silver, Y. Tsurumi, D. Chen, A. Sullivan, J.M. Isner, Vascular endothelial growth factor/vascular permeability factor enhances vascular permeability via nitric oxide and prostacyclin, Circulation 97 (1998) 99-107.
- T. Mustonen, K. Alitalo, Endothelial receptor tyrosine kinases involved in angiogenesis, J. Cell Biol. 129 (1995) 895-898.
- 32. Y. Nishizuka, The molecular heterogeneity of protein kinase C and its implications for cellular regulation, Nature 334 (1988) 661-665.
- Y. Nishizuka, Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C, Science 258 (1992) 607-614.
- 34. Y. Nishizuka, Protein kinase C and lipid signaling for sustained cellular

responses, FASEB J. 9 (1995) 484-496.

- S. Ohmori, N. Sakai, Y. Shirai, H. Yamamoto, E. Miyamoto, N. Shimizu, N. Saito, Importance of protein kinase C targeting for the phosphorylation of its substrate, myristoylated alanine-rich C-kinase substrate, J. Biol. Chem. 275 (2000) 26449-26457.
- 36. S. Ohmori, Y. Shirai, N. Sakai, M. Fujii, H. Konishi, U. Kikkawa, N. Saito, Three distinct mechanisms for translocation and activation of the delta subspecies of protein kinase C, Mol. Cell. Biol. 18 (1998) 5263-5271.
- 37. Y. Ono, T. Fujii, K. Ogita, U. Kikkawa, K. Igarashi, Y. Nishizuka, The structure, expression, and properties of additional members of the protein kinase C family, J. Biol. Chem. 263 (1988) 6927-6932.
- 38. Y. Ono, T. Fujii, K. Ogita, U. Kikkawa, K. Igarashi, Y. Nishizuka, Protein kinase C zeta subspecies from rat brain: its structure, expression, and properties, Proc. Natl. Acad. Sci. U. S. A. 86 (1989) 3099-3103.
- M.S. Pepper, N. Ferrara, L. Orci, R. Montesano, Vascular endothelial growth factor (VEGF) induces plasminogen activators and plasminogen activator inhibitor-1 in microvascular endothelial cells, Biochem. Biophys. Res. Commun. 181 (1991) 902-906.
- 40. T.P. Quinn, K.G. Peters, C. De Vries, N. Ferrara, L.T. Williams, Fetal liver kinase 1 is a receptor for vascular endothelial growth factor and is selectively expressed in vascular endothelium, Proc. Natl. Acad. Sci. U. S. A. 90 (1993) 7533-7537.
- N. Sakai, K. Sasaki, N. Ikegaki, Y. Shirai, Y. Ono, N. Saito, Direct visualization of the translocation of the gamma-subspecies of protein kinase C in living cells using fusion proteins with green fluorescent protein, J. Cell Biol. 139 (1997) 1465-1476.
- A. Sasaki, T. Taketomi, R. Kato, K. Saeki, A. Nonami, M. Sasaki, M. Kuriyama,
 N. Saito, M. Shibuya, A. Yoshimura, Mammalian Sprouty4 suppresses

Ras-independent ERK activation by binding to Raf1, Nat. Cell Biol. 5 (2003) 427-432.

- D.R. Senger, S.J. Galli, A.M. Dvorak, C.A. Perruzzi, V.S. Harvey, H.F. Dvorak, Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid, Science 219 (1983) 983-985.
- F. Shalaby, J. Rossant, T.P. Yamaguchi, M. Gertsenstein, X.F. Wu, M.L. Breitman, A.C. Schuh, Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice, Nature 376 (1995) 62-66.
- H. Shen, M. Clauss, J. Ryan, A.M. Schmidt, P. Tijburg, L. Borden, D. Connolly,
 D. Stern, J. Kao, Characterization of vascular permeability factor/vascular endothelial growth factor receptors on mononuclear phagocytes, Blood 81 (1993) 2767-2773.
- 46. M. Shibuya, S. Yamaguchi, A. Yamane, T. Ikeda, A. Tojo, H. Matsushime, M. Sato, Nucleotide sequence and expression of a novel human receptor-type tyrosine kinase gene (flt) closely related to the fms family, Oncogene 5 (1990) 519-524.
- 47. M. Shindo, K. Irie, A. Nakahara, H. Ohigashi, H. Konishi, U. Kikkawa, H. Fukuda, P.A. Wender, Toward the identification of selective modulators of protein kinase C (PKC) isozymes: establishment of a binding assay for PKC isozymes using synthetic C1 peptide receptors and identification of the critical residues involved in the phorbol ester binding, Bioorg. Med. Chem. 9 (2001) 2073-2081.
- Y. Shirai, K. Kashiwagi, K. Yagi, N. Sakai, N. Saito, Distinct effects of fatty acids on translocation of gamma- and epsilon-subspecies of protein kinase C, J. Cell Biol. 143 (1998) 511-521.
- 49. Z. Szallasi, K. Bogi, S. Gohari, T. Biro, P. Acs, P.M. Blumberg, Non-equivalent roles for the first and second zinc fingers of protein kinase Cdelta. Effect of their mutation on phorbol ester-induced translocation in NIH 3T3 cells, J. Biol.

Chem. 271 (1996) 18299-18301.

- 50. T. Takahashi, H. Ueno, M. Shibuya, VEGF activates protein kinase C-dependent, but Ras-independent Raf-MEK-MAP kinase pathway for DNA synthesis in primary endothelial cells, Oncogene 18 (1999) 2221-2230.
- 51. T. Takahashi, S. Yamaguchi, K. Chida, M. Shibuya, A single autophosphorylation site on KDR/Flk-1 is essential for VEGF-A-dependent activation of PLC-gamma and DNA synthesis in vascular endothelial cells, EMBO J. 20 (2001) 2768-2778.
- B.I. Terman, M. Dougher-Vermazen, M.E. Carrion, D. Dimitrov, D.C. Armellino,
 D. Gospodarowicz, P. Bohlen, Identification of the KDR tyrosine kinase as a receptor for vascular endothelial cell growth factor, Biochem. Biophys. Res.
 Commun. 187 (1992) 1579-1586.
- Y. Tsurumi, T. Murohara, K. Krasinski, D. Chen, B. Witzenbichler, M. Kearney,
 T. Couffinhal, J.M. Isner, Reciprocal relation between VEGF and NO in the regulation of endothelial integrity, Nat. Med. 3 (1997) 879-886.
- E.N. Unemori, N. Ferrara, E.A. Bauer, E.P. Amento, Vascular endothelial growth factor induces interstitial collagenase expression in human endothelial cells, J. Cell. Physiol. 153 (1992) 557-562.
- 55. R. van der Zee, T. Murohara, Z. Luo, F. Zollmann, J. Passeri, C. Lekutat, J.M. Isner, Vascular endothelial growth factor/vascular permeability factor augments nitric oxide release from quiescent rabbit and human vascular endothelium, Circulation 95 (1997) 1030-1037.
- 56. M. Wellner, C. Maasch, C. Kupprion, C. Lindschau, F.C. Luft, H. Haller, The proliferative effect of vascular endothelial growth factor requires protein kinase C-alpha and protein kinase C-zeta, Arterioscler. Thromb. Vasc. Biol. 19 (1999) 178-185.
- 57. A. Yamane, L. Seetharam, S. Yamaguchi, N. Gotoh, T. Takahashi, G. Neufeld,M. Shibuya, A new communication system between hepatocytes and sinusoidal

endothelial cells in liver through vascular endothelial growth factor and Flt tyrosine kinase receptor family (Flt-1 and KDR/Flk-1), Oncogene 9 (1994) 2683-2690.

58. G. Zhang, M.G. Kazanietz, P.M. Blumberg, J.H. Hurley, Crystal structure of the cys2 activator-binding domain of protein kinase C delta in complex with phorbol ester, Saibo 81 (1995) 917-924.

Figure legends

Fig. 1. PKC dependent ERK phosphorylation induced by VEGF in HEK293T cells expressing KDR

48hr after the transfection with KDR, cells were pretreated without (DMSO) or with 2µM Gö6983 for 1hr and stimulated by 25ng/ml VEGF for the indicated times. The signaling of the cells was stopped by adding SDS-sample buffer and the extracts were loading to SDS-PAGE and transferred a membrane. Phosphorylation of ERK1, 2 was detected with anti phospho-Thr202/Tyr204 ERK antibody.

Fig. 2. Expression of multiple PKC isozymes in HEK293T cell

Total cell lysate (25mg) extracted from HEK293T cells (HEK293T) were fractioned by SDS-PAGE and western blot analyses were performed with anti PKC isozyme specific antibodies. Cells expressing PKC α , β I, β II, γ , δ , ε , and ζ were used as a positive control (Control).

Fig. 3. Effects of PKC inhibitors on VEGF-induced ERK phosphorylation in HEK293T cells expressing KDR

HEK293T cells transfected with KDR were incubated for 1hr in absence (DMSO) or presence of 1 μ M GF109203X, 1 μ M Gö6983, 1 μ M Gö6976 or 5 μ M Rottlerin. The results shown are representative of two. Then these cells were treated with 25ng/ml VEGF for 5min. The signaling of the cells was stopped by adding SDS-sample buffer and the extracts were loading to SDS-PAGE and transferred a membrane. Phosphorylation of ERK1, 2 was detected with anti phospho-Thr202/Tyr204 ERK antibody.

Fig. 4. Effect of endogenous PKC8 knockdown by siRNA on VEGF-induced ERK phosphorylation in transiently KDR-expressed HEK293T cell

HEK293T cells were co-transfected with KDR and either siRNA for human PKC δ (hPKC δ siRNA) or scrambled siRNA (random siRNA). (A) 48hr after the transfection, total cell lysate (25µg) were subjected to SDS-PAGE and western blot analyses were performed with different PKC isoforms as indicated. (B) 48hr after the transfection, cells were stimulated by 25ng/ml VEGF for the indicated times. Phosphorylation of ERK1, 2 was analysed by western blotting with anti phospho-Thr202/Tyr204 ERK antibody.

Fig. 5. Inhibition of VEGF-induced MARCKS-GFP translocation by PKC inhibitor and siRNA

(A) HEK293T cells were co-transfected with KDR and MARCKS-GFP. 6hr after the transfection, the cells were seeded to 35mm Glass bottom plate. 18hr after the seeding, the cells were pretreated without (control) or with 1 μ M GF109203X, 1 μ M Gö6983, 1 μ M Gö6976, or 5 μ M Rottlerin for 1hr. Then the cells were treated with 25ng/ml VEGF and translocation of MARCKS-GFP was analysed by confocal laser scanning microscope. (B) HEK293T cells were co-transfected with KDR, MARCKS-GFP and either scrambled siRNA (Control siRNA) or siRNA for human PKC8 (hPKC8 siRNA). 6hr after the transfection, the cells were seeded to 35mm Glass bottom plate. 19hr after the seeding, the cells were treated with 25ng/ml VEGF and translocation of MARCKS-GFP was analysed by confocal laser scanning microscope. The results shown were representative of twelve experiments

Fig. 6. Recovery of ERK phosphorylation by rat PKC&-GFP in human PKC& siRNA treated HEK293T cell.

HEK293T cells were co-transfected with KDR, siRNA for human PKCδ and either one of GFP-tagged rat PKC (rPKCδ-GFP) or its mutants indicated or GFP alone. 48hr after the transfection, cells were treated without (-) or with 25ng/ml VEGF (+) for 5min. (A) The cell lysates were analyzed by western blot analysis with the antibodies indicated. (B) The intensity of phosphorylation of ERK was analyzed by NIH image software. Results are presented as mean \pm S.D. (*n*=3). *; P < 0.05 comparing ERK phosphorylation in the cells transfected with human PKC δ siRNA and GFP alone.

Fig. 7. Translocation of rat PKCS-GFP or its mutants induced by VEGF-KDR stimulation

HEK293T cells were co-transfected with KDR and either one of GFP-tagged rat PKC or its mutants indicated. 6hr after the transfection, the cells were seeded to 35mm Glass bottom plate. 18hr after the seeding, the cells were treated with 25ng/ml VEGF. Translocation of MARCKS-GFP is examined by confocal laser scanning microscope. The results shown were representative of six experiments. Figure











A



B



