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KR-31372 inhibits KDR/Flk-1 tyrosine phosphorylation via K^+_{ATP} channel opening in its antiangiogenic effect

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Abstract

The aim of this study was to identify the signaling pathway of the antiangiogenesis by (2*R*,3*R*,4*S*)-*N*″-cyano-*N*-(6-nitro-3,4-dihydro-hydroxy-2-methyl-2-dimethoxymethyl 2*H*-1-benzopyran-4yl)-*N*′-benzylguanidine (KR-31372). KR-31372 inhibited the in vitro basal tube formation using Matrigel-coated plate and in vivo neovascularizations in mice induced by Matrigel containing vascular endothelial growth factor ($VEGF_{165}$, 5 ng/ml). $VEGF_{165}$ markedly increased cell proliferation using 5-bromo-2′-deoxyuridine incorporation and chemotactic migration using transwell chamber in human umbilical vein endothelial cells, those of which were significantly suppressed by pretreatment with KR-31372 and levcromakalim concentration dependently. The suppression of all these variables were strongly antagonized by glibenclamide, ATP-sensitive K^+ channel blocker. KR-31372 (10^{-6}–10^{-4} M) and levcromakalim (10^{-5} M) concentration-dependently suppressed the $VEGF_{165}$-induced increases in KDR/Flk-1 tyrosine phosphorylation as well as the extracellular signal-related kinase 1/2 (ERK1/2), p38 MAPK and $p125^{FAK}$ tyrosine phosphorylation. These variables were significantly antagonized by glibenclamide. In conclusion, KR-31372 significantly inhibited the KDR/Flk-1 tyrosine phosphorylation-linked ERK1/2, p38 MAPK and $p125^{FAK}$ tyrosine phosphorylation via mediation of K^+_{ATP} channel opening, thereby resulting in antiangiogenesis.

Keywords: KR-31372; K^+ channel opener, ATP-sensitive; VEGF (vascular endothelial growth factor); $p125^{FAK}$; KDR/Flk-1; Endothelial cell, umbilical vein, human

1. Introduction

Angiogenesis, the growth of new capillaries from the preexisting microvessels (Cao et al., 1996), is often critical in certain pathological conditions, such as rheumatoid arthritis, diabetic retinopathy and solid tumor (Folkman and Shing, 1992). Reportedly, angiogenesis is accomplished via the complex steps, including the proliferation and migration of endothelial cells and degradation of the basement membrane by cellular proteases (Risau, 1997).

In endothelial cells, vascular endothelial growth factor (VEGF) stimulates DNA synthesis preferentially via the KDR/Flk-1-coupled activation of extracellular signal-related kinase 1/2 (ERK1/2) and chemotactic migration in human umbilical vein endothelial cells via tyrosine phosphorylation of focal adhesion kinase (FAK), while Flt-1 regulates cell migration through modulating actin reorganization through p38 MAPK (Millauer et al., 1993; Kanno et al., 2000). Of the various VEGF species, $VEGF_{165}$ is characterized as the most predominant form secreted by various cell types (Ferrara and Davis-Smyth, 1997), and the cell-surface receptors of VE GF_{165} have been demonstrated to be exclusively expressed in the endothelial cells (Neufeld et al., 1999).

In the pilot study, (2*R*,3*R*,4*S*)-*N*″-cyano-*N*-(6-nitro-3,4-dihydro-hydroxy-2-methyl-2-dimethoxymethyl 2*H*-1-benzopyran-4yl)-*N*′-benzylguanidine (KR-31372) showed very weak vasodilator action despite having a benzopyran moiety in its structure, which is a striking contrast to levcromakalim (Hong et al., 1998). The action potential characteristics in cell-attached mode were that KR-31372 showed the opening of K^+_{ATP} channel in isolated rat ventricular myocytes (ED_{50} = 6.1 μM), as did levcromakalim (ED_{50} = 18.2 μM), which were blocked by glibenclamide, a blocker of the K^+_{ATP}

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suggestive of the K^+_{ATP} channel opener. Previously, it has been reported that KR-31372 exerts an inhibitory effect on the oxidized low-density lipoprotein-stimulated syntheses of [^{3}H]thymidine incorporation and migrations of the cultured rat aortic smooth muscle cells (Kim et al., 2000). Recently, the antiangiogenic effect of KR-31372 was identified in the rat sponge implant model (Kim et al., 2001). However, the underlying signaling pathway by which KR-31372 exerts the antiangiogenic effect remains unclear.

In the present study, it was aimed to elucidate whether (1) KR-31372 inhibits the in vitro tube formation of human umbilical vein endothelial cells and the increase in in vivo neovascularization in mice induced by the Matrigel containing $VEGF_{165}$, (2) KR-31372 inhibits the $VEGF_{165}$-induced KDR/Flk-1 tyrosine phosphorylation-linked suppression of ERK1/2, p38 MAPK phosphorylation and $p125^{FAK}$ tyrosine phosphorylation. The effect of KR-31372 was compared with that of levcromakalim under pretreatment with and without glibenclamide, K^+_{ATP} channel blocker.

2. Materials and methods

2.1. Cell cultures

Human umbilical vein endothelial cells (ATCC CRL-1730, endothelial cell line derived from vein of human umbilical cord) were cultured in Kaighn's F12K medium supplemented with 10% heat-inactivated fetal bovine serum, 0.1 mg/ml heparin sodium, 0.03–0.05 mg/ml endothelial cell growth supplement and 1% antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). Cells were grown to confluence at 37 °C in 5% CO_2 on 0.1% gelatin-coated culture dishes and used for experiments at no greater than passage 8.

2.2. Tube formation assays

The assays were performed on 24-well plates that have been coated with 250 μl of growth factor-reduced Matrigel (10 mg protein/ml) per well and polymerized for 30 min at 37 °C. Cells from confluent human umbilical vein endothelial cell cultures were detached with 0.05% trypsin–EDTA solution, suspended in F12K with 1% fetal bovine serum. Cells were plated onto a layer of Matrigel at a density of 1×10^5 cells/well. Inhibitor was given to the cells for 30 min at 37 °C before seeding. After 18 h, the cultures were photographed (200 ×). The picture of the tubules was scanned and network tube area was determined using the GS-710 Calibrated Imaging Densitometer (Bio-Rad Laboratories, Hercules, CA).

2.3. Matrigel plug assay in mice

Mice (C57BL/6) were injected subcutaneously with 0.5 ml of Matrigel containing $VEGF_{165}$ (5 ng/ml) with heparin (40 U/ml) under treatment with KR-31372 (10^{-5} M) or KR-31372 plus glibenclamide. The injected Matrigel rapidly formed a single, solid gel plug. After 5 days, mice were sacrificed, and Matrigel plug was recovered, fixed with 10% formaldehyde/phosphate-buffered saline (pH 7.4) and embedded in paraffin and examined with hematoxylin/eosin stain. To quantify the formation of new blood vessel, the amount of hemoglobin was measured using the Total Hemoglobin Kit. The concentration of hemoglobin was calculated from a known amount of hemoglobin assayed in parallel. Four mice were used as one group, and the experiment was repeated twice.

2.4. Cell proliferation assay

Human umbilical vein endothelial cells seeded at a density of 1×10^4 cells/well in 96-well plates were incubated in growth media and allowed to attach for 24 h. Thereafter, cells were incubated for 3 h in Kaighn's F12K medium containing 1% fetal bovine serum. Cells were exposed to KR-31372 (10^{-8}–10^{-4} M) for 5 h and then were stimulated by $VEGF_{165}$ (10 ng/ml) for 48 h. After addition of 5-bromo-2′-deoxyuridine, cells were reincubated for incorporation of 5-bromo-2′-deoxyuridine into the DNA of proliferating cells. The reaction product was quantified by measuring the absorbance at the 450 nm (reference wavelength 690 nm) using the ELISA reader (Bio-Tek Instruments, Winooski, VT).

2.5. Chemotactic migration assay

Chemotactic migration of cultured human umbilical vein endothelial cells was assayed by using Transwell chamber with pores of 8 μm in diameter (Corning Costa, Cambridge, MA). Polycarbonate filters and lower chambers coated with 0.5% gelatin were washed by serum-free medium. Cultured cells were trypsinized and suspended at a concentration of 1×10^6 cells/ml supplemented with 0.4% fetal bovine serum. KR-31372 was given to the cells for 30 min at 37 °C before seeding. Cell suspension (100 μl in volume) was placed on the upper chamber, followed by addition of glibenclamide when necessary. Medium (200 μl) containing $VEGF_{165}$ (25 ng/ml) was placed in the lower chamber. The chamber was incubated at 37 °C for 4 h, and thereafter, the filters were removed, fixed and stained with Wright-Giemsa solution. Nonmigrating cells on the upper surface of the filter were removed by wiping with a cotton swab, and chemotaxis was quantified by counting the cells that migrated to the rear side of the filter with optical microscopy at × 200 magnification. Migration activity was calculated as the ratio of migrated cells to the cells that remained in the front side of the filter and expressed in percentage.

2.6. Western blotting assay for ERK1/ERK2

Human umbilical vein endothelial cells were lysed in lysis buffer containing 50 mM Tris–Cl (pH 8.0), 150 mM

NaCl, 0.02% sodium azide, 100 μg/ml phenylmethylsulflonyl fluoride, 1 μg/ml aprotinin and 1% Triton X-100. Following centrifugation at 12,000 rpm, 50 μg of total protein was loaded into a 12% sodium dodecyl sulfate–polyacrylamide gel, and transferred to nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ). The blocked membranes were then incubated with anti-phospho-ERK antibody specific for Try-204 phosphorylated forms of ERK1 and ERK2 (Santa Cruz Biotechnology, Santa Cruz, CA), and the immunoreactive bands were visualized using the chemiluminescent reagent as recommended by the Supersignal West Dura Extended Duration Substrate Kit (Pierce, Rockford, IL). The signals of the bands were quantified using the calibrated imaging of the densitometer (GS-710, Bio-Rad Laboratories).

2.7. Assay of p38 MAP kinase activity

Using the p38 MAP kinase assay kit (Cell Signaling Technology, Beverly, MA), cells were lysed in cold cell lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerol phosphate, 1 mM Na_3Vo_4, 1 μg/ml leupeptin and 1 mM phenylmethylsulflonyl fluoride). Equal amounts of cell lysates were incubated with immobilized phospho-p38 MAP kinase monoclonal antibody and bead at 4 °C overnight. After centrifugation, pellets were suspended in kinase buffer (25 mM Tris, pH 7.5, 5 mM β-glycerol phosphate, 2 mM dithiothreitol, 0.1 mM Na_3Vo_4, 10 mM $MgCl_2$ and 200 μM ATP) containing activating transcription factor (ATF)-2 as substrates for phospho-p38 MAP kinase. The activity of phospho-p38 MAP kinase was then measured by Western blot using primary antibody (phospho-ATF-2 antibody at 1:1000), followed by horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:2000).

2.8. Immunoblotting for phosphotyrosine in assaying p125FAK and KDR/Flk-1

Human umbilical vein endothelial cells were treated with $VEGF_{165}$ and other drugs for 10 min. The cells were lysed in lysis buffer containing 50 mM Tris–Cl (pH 8.0), 150 mM NaCl, 0.02% sodium azide, 100 μg/ml phenylmethylsulflonyl fluoride, 1 μg/ml aprotinin and 1% Triton X-100. Following centrifugation at 12,000 rpm, 50 μg of total protein was loaded into a 8% sodium dodecyl sulfate–polyacrylamide gel and transferred to nitrocellulose membrane (Amersham Biosciences). The immunoblots were then incubated with buffer (10 mM Tris [pH 7.5], 100 mM NaCl and 0.1% Tween 20) containing 1% bovine serum albumin and the primary antibody of the anti-$p125^{FAK}$ specifically for $p125^{FAK}$ (Santa Cruz Biotechnol-

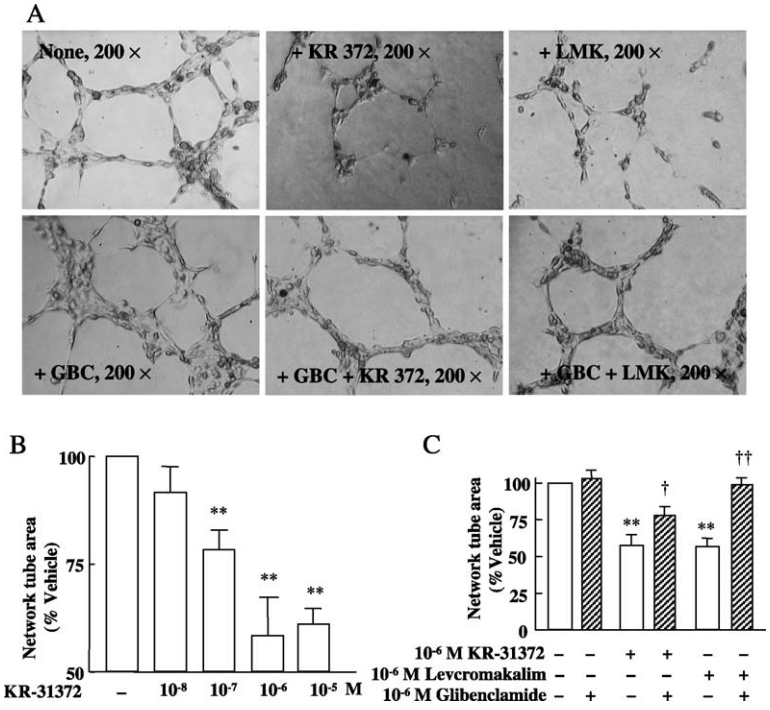

Fig. 1. Effect of KR-31372 (KR 372) and levcromakalim (LMK) on the capillary tube formation of human umbilical vein endothelial cells on the plate with Matrigel. (A) Representative pictures showing the suppression of capillary tube formation of human umbilical vein endothelial cells under KR-31372 (10^{-6} M) and levcromakalim (10^{-6} M) in the absence (top) and the presence of glibenclamide (GBC, 10^{-6} M, bottom) in comparison with vehicle group (200×). (B) Concentration-dependent inhibition of the tube formation by KR-31372 (10^{-8}–10^{-5} M). (C) Antagonizing effect of glibenclamide (10^{-6} M) on the inhibition by either KR-31372 or levcromakalim of tube formation. Results are expressed as means ± S.E.M. of three different preparations with duplicate experiments. $^{**}P<0.01$ vs. None; $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$ vs. KR-31372 or levcromakalim alone.

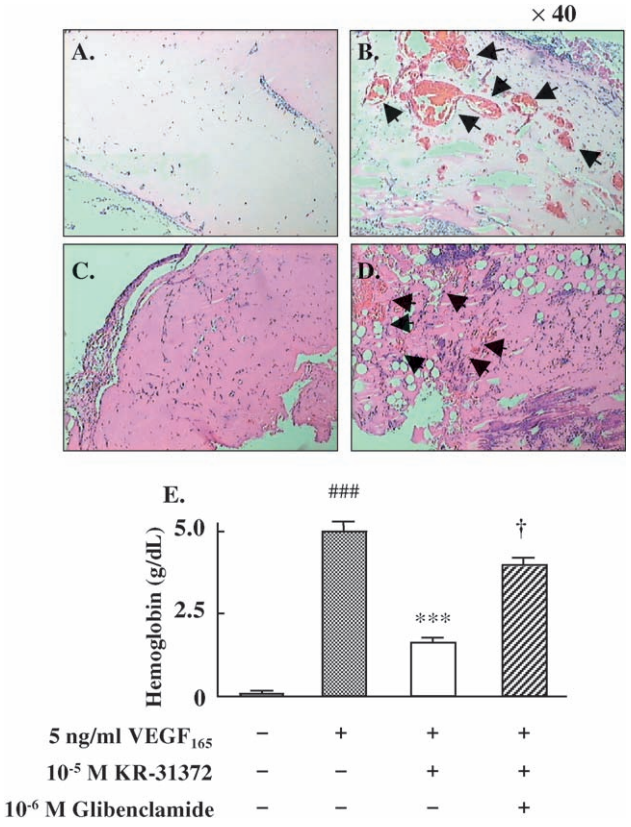

Fig. 2. Inhibitory effect of KR-31372 on the $VEGF_{165}$-induced angiogenesis in vivo by employing Matrigel plug assay in mice. C57BL/6 mice subcutaneously received Matrigel (0.5 ml) containing $VEGF_{165}$ (5 ng/ml) with heparin (40 U/ml) under treatment with KR-31372 (10^{-5} M) or KR-31372 (10^{-5} M) plus glibenclamide (10^{-6} M). (A) Control mice; (B) increased neomicrovessel formation by $VEGF_{165}$ (5 ng/ml); (C) suppression of microvessel formation under treatment with KR-31372 (10^{-5} M); (D) reverse of vessel formation under treatment with KR-31372 plus glibenclamide (10^{-6} M) (40 ×). Arrows indicate the active neomicrovessels containing red blood cells. (E) Analysis of hemoglobin contents within Matrigel plug. The concentration of hemoglobin was calculated from a known amount of hemoglobin assayed in parallel. Four mice were used as a group, and the experiment was repeated twice. Results are expressed as means ± S.E.M. $^{###}P<0.001$ vs. Control; $^{***}P<0.001$ vs. absence of KR-31372; $^{\dagger}P<0.05$ vs. KR-31372 alone.

ogy), or the anti-phospho-VEGFR-2 (Tyr 951) (Cell Signaling Technology) tyrosine phosphorylation for 2 h at room temperature and then incubated with anti-mouse or anti-rabbit secondary antibody conjugated to horseradish peroxidase for 2 h. The immunoreactive bands were visualized using chemiluminescent reagent recommended by the Supersignal West Dura Extended Duration Substrate Kit (Pierce).

2.9. Drugs

Recombinant human $VEGF_{165}$ was purchased from the R&D Systems (Minneapolis, MN). KR-31372 and levcromakalim were donated from The Korea Research Institute of Chemical Technology (Daejon, Korea). Glibenclamide, PD 98059, SB 203580, endothelial cell growth supplement, heparin, gelatin, Wright-Giemsa solution and total hemoglobin kit were from the Sigma (St. Louis, MO). 5-Bromo-2′-deoxyuridine kit was from the Roche Molecular Biochemicals (Mannheim, Germany). Matrigel was from the Becton Dickinson Labware (Bedford, MA). SU 5614 was from the Calbiochem-Novabiochem (San Diego, CA). KR-31372, SU 5614 and glibenclamide were dissolved in dimethyl sulfoxide as a 10^{-2} M stock solution and then diluted with the phosphate-buffered saline.

2.10. Statistics

The results are expressed as means ± S.E.M. Two-way repeated measures analysis of variance test was used for the comparison of concentration-dependent changes in cell proliferation and chemotactic migration between inhibitor-treated and untreated groups. Statistical differences between groups were determined by paired or unpaired Student's *t* test or analysis of variance. $P<0.05$ was considered to be significant.

3. Results

3.1. Tube formation

Matrigel-plated human umbilical vein endothelial cells elongated and migrated, forming a tubular network as evidenced by morphological changes. Tube formation was

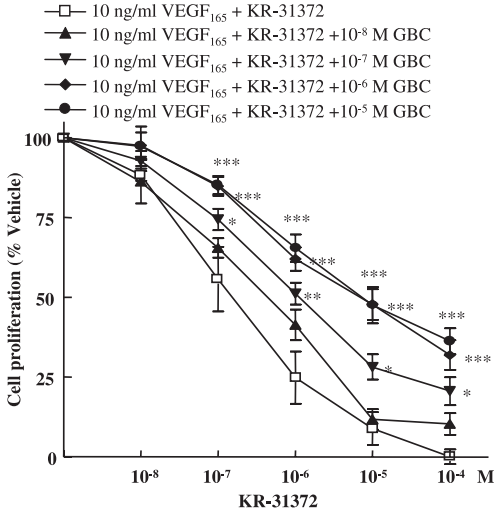

Fig. 3. Effect of KR-31372 on the cell proliferation of human umbilical vein endothelial cells by using 5-bromo-2′-deoxyuridine. KR-31372 (10^{-8}–10^{-4} M) was added to human umbilical vein endothelial cells for 5 h and then incubated in the medium containing 10 ng/ml $VEGF_{165}$ in the absence and the presence of glibenclamide (10^{-8}–10^{-5} M) for 48 h. Results are expressed as means ± S.E.M. of three different preparations with quadruplicate experiments. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$ vs. absence of glibenclamide. Significant differences were identified between the cell proliferation inhibition relation of KR-31372 and KR-31372 + glibenclamide (10^{-7}–10^{-5} M) by two-way repeated measures analysis of variance ($P<0.0001$).

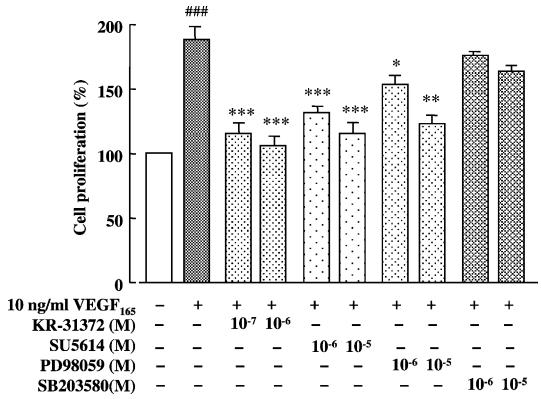

Fig. 4. Inhibitory effect of SU 5614 (a specific KDR/Flk-1 receptor tyrosine kinase inhibitor), GF 109203X (a protein kinase C inhibitor) and PD 98059 (a specific inhibitor of ERK phosphorylation) and SB 203580 (a specific p38 MAP kinase inhibitor) on the $VEGF_{165}$ (10 ng/ml)-induced DNA synthesis in human umbilical vein endothelial cells in comparison with that of KR31372. Results are expressed as means ± S.E.M. of three different preparations with quadruplicate experiments. $^{###}P<0.001$ vs. None; $*P<0.05$, $**P<0.01$, $***P<0.001$ vs. $VEGF_{165}$ alone.

quantitatively estimated by measuring the length covered by the tubular network using the image analysis program. KR-31372 (10^{-8}–10^{-5} M) concentration-dependently suppressed the tube formation. Both KR-31372 and levcromakalim markedly suppressed the tube formation by 58.3 ± 8.9% and 56.7 ± 6.5% at 10^{-6} M of each drug, respectively (Fig. 1). The suppression of tube formation by KR-31372 (10^{-6} M) was concentration-dependently reversed by glibenclamide (10^{-8}–10^{-5} M) (data not shown). Glibenclamide (10^{-6} M) reversed the suppressed tube formation by KR-31372 to 76.9 ± 7.3% ($P<0.05$) and by levcromakalim to 99.2 ± 5.9% ($P<0.01$) (Fig. 1C).

3.2. Matrigel plug assay in mice

Five days after implantation, histological examination and hemoglobin content were estimated. The microvessel formation significantly increased in the Matrigel containing $VEGF_{165}$ (5 ng/ml) (Fig. 2B), whereas it was not evident in the Matrigel without $VEGF_{165}$ (Fig. 2A). KR-31372 (10^{-5} M) significantly reduced the formation of $VEGF_{165}$-stimulated neomicrovessels (Fig. 2C), which was antagonized by glibenclamide (10^{-6} M) (Fig. 2D). The formation of new blood vessels was further confirmed by measurement of hemoglobin in Matrigel. As shown in Fig. 2E, the hemoglobin content, which was negligible in the control group, was markedly elevated in the Matrigel containing 5 ng/ml $VEGF_{165}$ to 5.1 ± 0.3 g/dl ($P<0.001$), which was significantly reduced by treatment with 10^{-5} M KR-31372 (1.6 ± 0.1 g/dl, $P<0.001$). Pretreatment with glibenclamide (10^{-6} M) markedly reversed the KR-31372-induced reduced hemoglobin content, indicating that KR-31372 elicits a strong antiangiogenic activity in in vivo experiment.

3.3. Cell proliferation

$VEGF_{165}$ (1–20 ng/ml) concentration-dependently increased DNA synthesis. When human umbilical vein

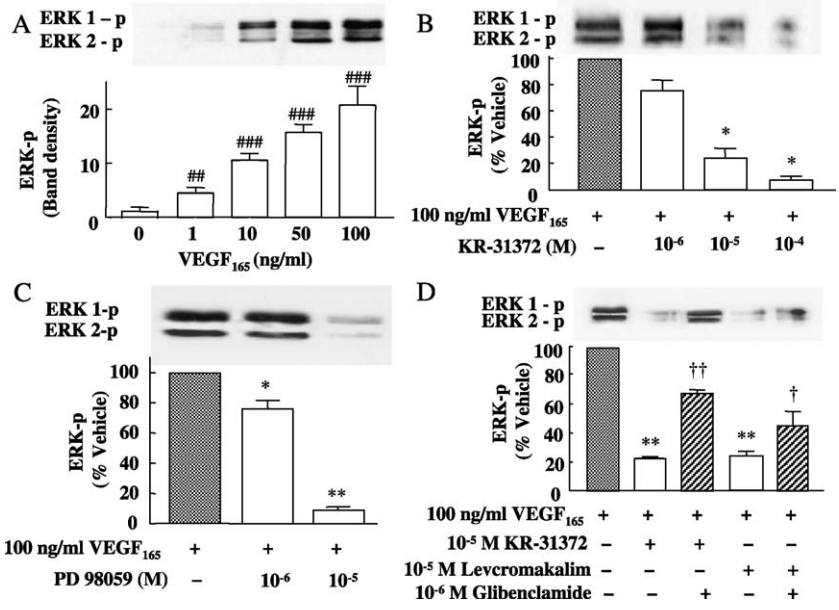

Fig. 5. Representative immunoblotting for ERK1/ERK2 phosphorylation in human umbilical vein endothelial cells and densitometric analysis. (A) Concentration-dependent increases in ERK1/ERK2 phosphorylation stimulated by $VEGF_{165}$ (1–100 ng/ml). (B) Concentration-dependent suppression of ERK1/2 phosphorylation by KR-31372 (10^{-6}–10^{-4} M) using anti-phospho-specific ERK antibody. (C) Suppression of ERK1/2 phosphorylation by PD 98059 (ERK phosphorylation inhibitor, 10^{-6} and 10^{-5} M). (D) Reversal by glibenclamide (10^{-6} M) of the KR-31372 (10^{-5} M) and levcromakalim (10^{-5} M)-induced inhibition of ERK1/2 phosphorylation. Results are expressed as means ± S.E.M. of three different experiments. $^{##}P<0.01$, $^{###}P<0.001$ vs. zero $VEGF_{165}$; $*P<0.05$, $**P<0.01$ vs. $VEGF_{165}$ alone; $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$ vs. absence of glibenclamide.

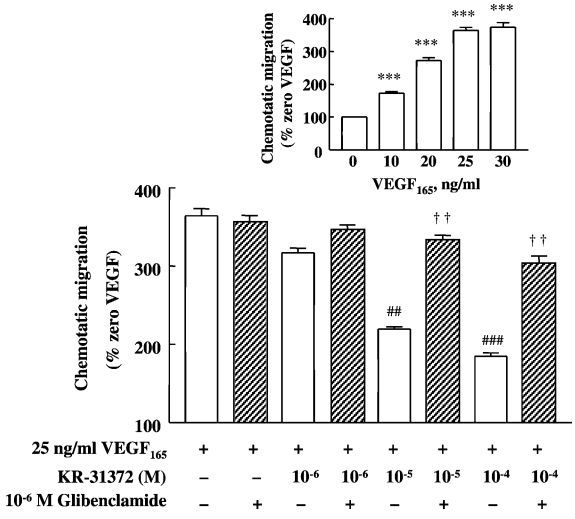

Fig. 6. Effect of KR-31372 on the chemotactic migration of human umbilical vein endothelial cells. KR-31372 (10^{-6}–10^{-4} M) was placed in the lower wells of chemotaxis chamber in the absence and the presence of 10^{-6} M glibenclamide. The baseline migration in the absence of KR-31372 was 53.9 ± 4.2 cells/field. Top, concentration-dependent increases in chemotactic migration by $VEGF_{165}$. Results are expressed as means ± S.E.M. of three different assays with triplicate experiments. ***$P<0.001$ vs. zero $VEGF_{165}$; $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ vs. $VEGF_{165}$ alone; $^{\dagger\dagger}P<0.01$ vs. absence of glibenclamide. Significant difference by two-way repeated measures analysis of variance ($P<0.0001$) was observed between the inhibition of cell chemotatic migration by KR-31372 in the absence and presence of glibenclamide.

endothelial cells were incubated in the medium containing 10 ng/ml $VEGF_{165}$ for 48 h, DNA synthesis increased to 180.1 ± 5.5% (data not shown). KR-31372 (10^{-8}–10^{-4} M) significantly suppressed the cell proliferation induced by $VEGF_{165}$ (10 ng/ml) in a concentration-dependent manner, which was reversed by glibenclamide (10^{-6} and 10^{-5} M) (Fig. 3). Further, $VEGF_{165}$-induced DNA synthesis in human umbilical vein endothelial cells was significantly suppressed by SU 5614 (a specific KDR/Flk-1 receptor tyrosine kinase inhibitor) and PD 98059 (a specific inhibitor of ERK phosphorylation), but not by SB 203580 (a specific p38 MAP kinase inhibitor) (Fig. 4).

3.4. ERK phosphorylation

$VEGF_{165}$ (1–100 ng/ml) increased the ERK1/2 (p44 ERK1 and p42 ERK2) phosphorylation (Fig. 5A). KR-31372 (10^{-6}–10^{-4} M) concentration-dependently suppressed the phosphorylation of ERK1/2 stimulated by 100 ng/ml of $VEGF_{165}$, as did PD 98059 (10^{-6} and 10^{-5} M), a selective antagonist of ERK phosphorylation (Fig. 5B and C). Levcromakalim (10^{-5} M), a K^{+}_{ATP} channel opener, also showed a strong suppression of ERK phosphorylation, as did KR-31372. Glibenclamide (10^{-6} M) significantly reversed both KR-31372- and levcromakalim-induced inhibition of ERK phosphorylation (Fig. 5D).

3.5. Chemotactic migration

$VEGF_{165}$ (10–30 ng/ml) concentration-dependently stimulated the chemotactic motility of human umbilical vein endothelial cells with near maximal activity (374.2 ± 13.8%) observed at 25 ng/ml (Fig. 6, Top). Pretreatment with KR-31372 (10^{-5} and 10^{-4} M) suppressed the $VEGF_{165}$ (25 ng/ml)-stimulated chemotactic motility by 56.4 ± 6.53% ($P<0.01$) and by 69.7 ± 5.1% ($P<0.001$), respectively.

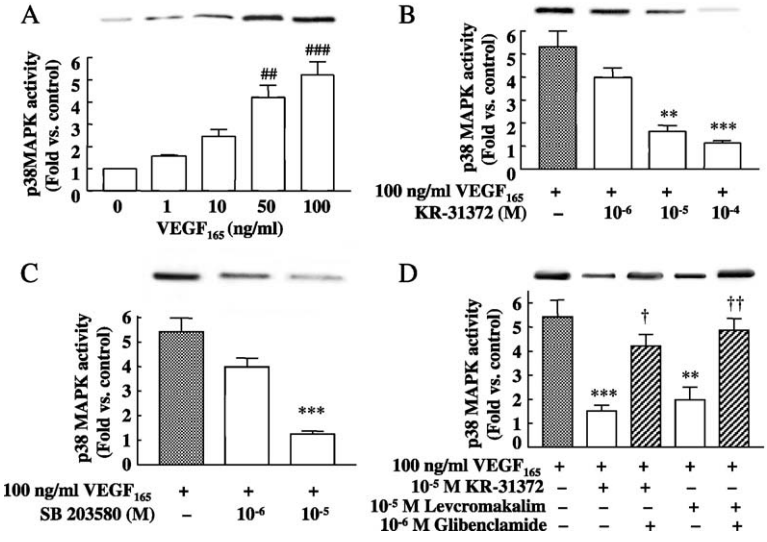

Fig. 7. Representative immunoblotting for p38 MAPK phosphorylation in human umbilical vein endothelial cells and densitometric analysis. (A) Concentration-dependent increases in p38 MAPK phosphorylation stimulated by $VEGF_{165}$ (1–100 ng/ml). (B) Concentration-dependent suppression of p38 MAPK phosphorylation by KR-31372 (10^{-6}–10^{-4} M). (C) Suppression of p38 MAPK phosphorylation by SB 203580 (p38 MAPK phosphorylation inhibitor, 10^{-6} and 10^{-5} M. (D) Reversal by glibenclamide (10^{-6} M) of the KR-31372 (10^{-5} M) and levcromakalim (10^{-5} M)-induced inhibition of p38 MAPK phosphorylation. Results are expressed as means ± S.E.M. of four different experiments. $^{\#}P<0.05$, $^{\#\#\#}P<0.001$ vs. zero $VEGF_{165}$; **$P<0.01$, ***$P<0.001$ vs. $VEGF_{165}$ alone; $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$ vs. absence of glibenclamide.

The suppressed cell migration was significantly reversed by pretreatment with 10^{-6} M glibenclamide (Fig. 6).

3.6. p38 MAPK activity

$VEGF_{165}$(1–100 ng/ml) increased the p38 MAPK phosphorylation (Fig. 7A). KR-31372 (10^{-6}–10^{-4} M) significantly suppressed the p38 MAPK phosphorylation stimulated by 100 ng/ml $VEGF_{165}$ in a concentration-dependent manner, as did SB 203580 (10^{-6} and 10^{-5} M, a specific p38 MAPK inhibitor) (Fig. 7B and C). Glibenclamide (10^{-6} M) significantly reversed both KR-31372 (10^{-5} M, $P<0.05$)- and levcromakalim (10^{-5} M, $P<0.01$)-induced inhibition of p38 MAPK phosphorylation (Fig. 7D).

3.7. $p125^{FAK}$ tyrosine phosphorylation

$VEGF_{165}$ (1–50 ng/ml) concentration-dependently increased the $p125^{FAK}$ tyrosine phosphorylation, which was maximized at 25 ng/ml $VEGF_{165}$. As shown in the densitometric analysis, $VEGF_{165}$-induced $p125^{FAK}$ tyrosine phosphorylation reached a maximum by 25 ng/ml $VEGF_{165}$ to 35.4 ± 4.5-fold ($P<0.001$) (Fig. 8A), which was concentration-dependently suppressed by KR-31372 (10^{-6}–10^{-4} M) (Fig. 8B). KR-31372 (10^{-5} M)-induced suppression of the $p125^{FAK}$ tyrosine phosphorylation was significantly reversed by glibenclamide (10^{-6} M, $P<0.001$) (Fig. 8C).

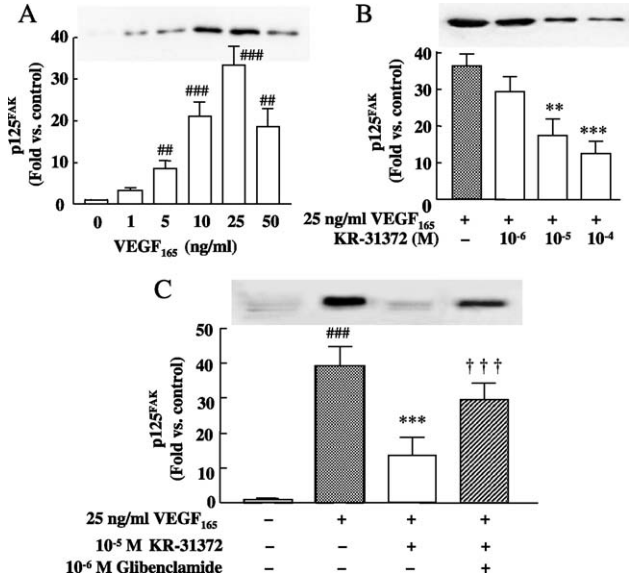

Fig. 8. Representative immunoblotting for $p125^{FAK}$ tyrosine phosphorylation in human umbilical vein endothelial cells assayed by using anti-$p125^{FAK}$ antibody and densitometric analysis. (A) Concentration-dependent increases in $p125^{FAK}$ tyrosine phosphorylation by $VEGF_{165}$ (1–50 ng/ml). (B) Concentration-dependent suppression of $p125^{FAK}$ tyrosine phosphorylation by KR-31372 (10^{-6}–10^{-4} M). (C) Reversal by glibenclamide (10^{-6} M) of the KR-31372 (10^{-5} M)-induced inhibition of $p125^{FAK}$ tyrosine phosphorylation. Results are expressed as means ± S.E.M. of three different experiments. $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ vs. zero $VEGF_{165}$; $^{**}P<0.01$, $^{***}P<0.001$ vs. $VEGF_{165}$ alone; $^{\dagger\dagger\dagger}P<0.001$ vs. absence of glibenclamide.

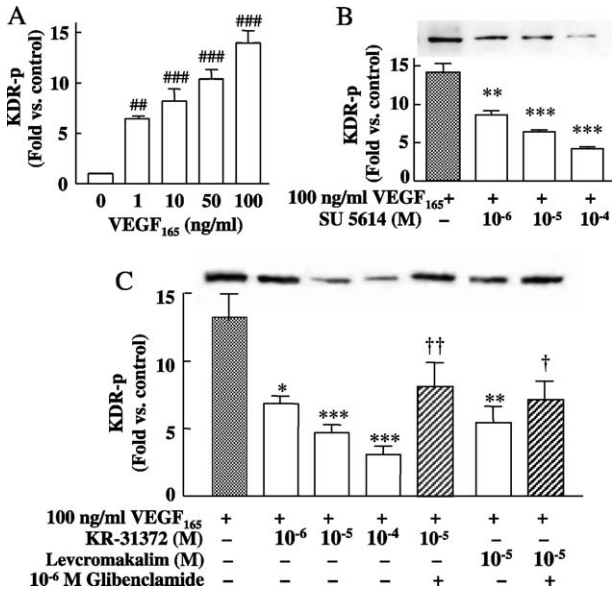

Fig. 9. Representative immunoblotting for KDR tyrosine phosphorylation in human umbilical vein endothelial cells assayed by using anti-phospho-VEGF receptor-2 antibody (Tyr951). (A) $VEGF_{165}$-stimulated increases in KDR tyrosine phosphorylation. (B) Concentration-dependent suppression of KDR tyrosine phosphorylation by SU 5614 (antagonist of VEGF receptor; 10^{-6}–10^{-4} M). (C) Suppression of KDR tyrosine phosphorylation by KR-31372 (10^{-6}–10^{-4} M) and levcromakalim (10^{-5} M) and their reversal by glibenclamide (10^{-6} M). Results are expressed as means ± S.E.M. of four different experiments. $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ vs. zero $VEGF_{165}$; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs. $VEGF_{165}$ alone; $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$ vs. corresponding results in absence of glibenclamide.

3.8. KDR tyrosine phosphorylation

$VEGF_{165}$ (1–100 ng/ml) stimulated the KDR tyrosine phosphorylation of human umbilical vein endothelial cells in a concentration-dependent manner (Fig. 9A). Pretreatment with SU 5614 (10^{-6}–10^{-4} M) significantly suppressed the KDR tyrosine phosphorylation stimulated by $VEGF_{165}$ (100 ng/ml) (Fig. 9B). KR-31372 (10^{-6}–10^{-4} M) as well as levcromakalim (10^{-5} M) suppressed the KDR tyrosine phosphorylation (at 100 ng/ml of $VEGF_{165}$, 13.9 ± 1.3 relative density). The KDR tyrosine phosphorylation suppressed by either KR-31372 (10^{-5} M) or levcromakalim (10^{-5} M) was significantly reversed by pretreatment with glibenclamide (10^{-6} M) (Fig. 9C).

4. Discussion

The major findings of the present study were that $VEGF_{165}$ markedly enhanced both in vitro tube formation in human umbilical vein endothelial cells and in vivo Matrigel-induced neovascularization in mice in association with increased cell proliferation and chemotactic migration via

increased KDR/Flk-1 tyrosine phosphorylation-linked activation of ERK1/2 and increased $p125^{FAK}$ tyrosine phosphorylation. KR-31372 and levcromakalim concentration-dependently suppressed these variables, which were inhibited by glibenclamide, ATP-sensitive K^+ channel blocker.

VEGF, a central regulator of vasculogenesis and angiogenesis, binds to the two tyrosine kinase receptors for VEGF: Flt-1 (VEGFR-1) and KDR/Flk-1 (VEGFR-2) (Quinn et al., 1993; Ferrara and Davis-Smyth, 1997; Neufeld et al., 1999). In the transfection study by Kroll and Waltenberger (1997), stimulation of the porcine aortic endothelial cells overexpressing KDR/Flk-1 with VEGF elicited the MAPK activation and increase in actin reorganization with chemotaxis and proliferation responses, while none of these events were detected in cells transfected with Flt-1. A growing number of evidence has accumulated that ERK1/2 and $p125^{FAK}$ (a member of non-receptor protein tyrosine kinases) are the downstream targets of KDR/Flk-1-coupled protein kinase C (Kanno et al., 2000), and endothelial cell proliferation is stimulated by the ERK1/2 signaling pathway (Seetharam et al., 1995; Takahashi and Shibuya, 1997), while migration is by $p125^{FAK}$ pathway (Abedi and Zachary, 1995). The findings that $VEGF_{165}$-induced DNA synthesis in human umbilical vein endothelial cells was significantly suppressed by SU 5614 (a specific KDR/Flk-1 receptor tyrosine kinase inhibitor, Fong et al., 1999) and PD 98059 (a specific inhibitor of ERK phosphorylation, Alessi et al., 1995), but not by SB 203580 (a specific p38 MAP kinase inhibitor), suggest that cell proliferation stimulated by VEGF is mediated via activation of KDR/Flk-1 receptor-linked protein kinase C and ERK1/2 phosphorylation (Yu and Sato, 1999). In line with these results, our result showing that KR-31372 strongly blocked the VEGF-induced activation of ERK1/2 phosphorylation in human umbilical vein endothelial cells as did PD 98059, a specific inhibitor of ERK phosphorylation (Alessi et al., 1995), and that it suppressed not only the ERK1/2 phosphorylation but also KDR tyrosine phosphorylation as did SU 5614, a specific inhibitor of KDR, strongly indicating that KR-31372 inhibits the upstream target, KDR/Flk-1, for the antiangiogenesis. When we observed the effect of KR-31372 on the binding of $VEGF_{165}$ to the VEGF receptor (KDR/Flk-1) as an extracellular binding assay in the human umbilical vein endothelial cells, % inhibition was, however, low ($12.5 \pm 0.1\%$ at 10 μM and $16.2 \pm 4.3\%$ at 100 μM), strongly indicating that inhibition of KDR tyrosine phosphorylation by KR-31372 was not ascribed to the direct antagonism, but by the indirect mechanism coupled to K^+_{ATP} channel opening. Further study to elucidate the indirect mechanism remains to be clarified.

On the other hand, VEGF acts as a potent chemotactic agent for endothelial cells. VEGF increases cell migration and induces a marked reorganization of the microfilament network by the formation of stress fibers. Rousseau et al. (1997) showed that the p38 MAPK pathway conveys the VEGF signals to microfilaments inducing rearrangements of the actin cytoskeleton that regulate cell migration. The p38 MAPK phosphorylation stimulated by $VEGF_{165}$ was significantly suppressed by KR-31372, as did SB 203580, a specific p38 MAPK inhibitor. Abedy and Zachary (1997) documented that VEGF stimulated migration and actin stress fiber formation in confluent human umbilical vein endothelial cells, and they postulated that $p125^{FAK}$/paxillin plays a role of components in the VEGF-stimulated signaling pathway as a novel mechanism for VEGF regulation of endothelial cell functions. KR-31372 strongly inhibited the $p125^{FAK}$ tyrosine kinase phosphorylation stimulated by $VEGF_{165}$, which was antagonized by glibenclamide. Although the effect of KR-31372 on the VEGF-induced actin cytoskeleton was undetermined in the present study, it was speculated that KR-31372-induced inhibition of KDR tyrosine phosphorylation might lead to inhibition of VEGF-stimulated $p125^{FAK}$ tyrosine phosphorylation in human umbilical vein endothelial cells, thereby contributing to the antiangiogenesis. These facts lead to the suggestion that the K^+ channel openers, including KR-31372 and levcromakalim, might elicit the inhibitory effect on the VEGFR-2 (KDR/Flk1) receptor tyrosine kinase, which is inhibitable by glibenclamide.

Recently, the importance of endothelial nitric oxide synthase (eNOS)/nitric oxide (NO) in VEGF-induced endothelial proliferation and vessel organization has been emphasized (Papapetropoulos et al., 1997). Shen et al. (1999) documented that VEGF increased eNOS expression via activation of the KDR receptor tyrosine kinase in bovine adrenal cortex endothelial cells. In accordance with these reports, we observed that in human umbilical vein endothelial cells VEGF significantly increased eNOS protein expression in Western blotting, and KR-31372 significantly suppressed the increased eNOS expression, which was glibenclamide-inhibitable (data not shown). These findings provide further evidence to support the hypothesis that KR-31372 inhibits the KDR/flk-1 receptor tyrosine kinase via mediation of glibenclamide-inhibitable mechanism.

As a mechanistic evidence, K^+ channel openers exert an increase in K^+conductance via activation of K^+ channels, leading to hyperpolarization of the plasma membrane and consequently inhibiting activation of voltage-sensitive Ca^{2+} channels (Hamilton et al., 1986). In the previous results, treatment with glibenclamide also reversed the clearance of ^{99m}Tc that was suppressed by KR-31372 (Kim et al., 2001). Reportedly, glibenclamide, a sulfonylurea family, stimulates insulin secretion from pancreatic β-cells by triggering the opening of voltage-gated Ca^{2+} channels following interacting directly with the K^+_{ATP} channel, and used in the treatment of type 2 diabetes mellitus (Ashcroft and Gribble, 2000). Additionally, glibenclamide has been demonstrated to inhibit the mitochondrial ATP-regulated K^+ channels ($mitoK^+_{ATP}$ channel) in heart and brain, even though the molecular identity of the $mitoK^+_{ATP}$ channel is unknown (Debska et al., 2002). In the present experiment, we did not confirm whether the antagonizing effect of glibenclamide was ascribed to the inhibition of $mitoK^+_{ATP}$ channel.

Kohn et al. (1995) emphasized a role for Ca^{2+}-mediated signal transduction in FGF2-stimulated proliferation and invasion of the endothelial cells. They addressed Ca^{2+} dependency of endothelial cell proliferation and invasion by using an inhibitor of ligand-stimulated Ca^{2+} influx, carboxyamidotriazole that inhibited the tubular formation on Matrigel and FGF2-induced tyrosine phosphorylation. Considering these reports, the plausible explanation can be that KR-31372 suppresses the angiogenesis by inhibition of the Ca^{2+} influx through voltage-sensitive Ca^{2+} channels. Stepien et al. (1998) have postulated that the inhibitory effect of amlodipine, a Ca^{2+} channel blocker, on the cell proliferation is not related to blockade of voltage-sensitive Ca^{2+} channels, but to interference with mitogenic signaling system. Recently, Kawasaki et al. (2000) demonstrated dissociation between the Ca^{2+} signal and tubular formation induced by VEGF in bovine aortic endothelial cells. Given that KR-31372 exerts a potent inhibitory action on the proliferation and migration of endothelial cells without exerting the hemodynamic alterations, this agent may prove useful in the suppression of angiogenesis.

Taken together, KR-31372 significantly suppressed the in vitro tube formation in human umbilical vein endothelial cells and in vivo Matrigel-induced neovascularization in mice in association with inhibition of the triad of angiogenesis: DNA synthesis and migration in human umbilical vein endothelial cells via inhibition of KDR tyrosine phosphorylation-coupled suppression of ERK1/2, p38 MAPK and $p125^{FAK}$ tyrosine phosphorylation, those of which were mediated by glibenclamide-inhibitable K^{+}_{ATP} channel activation. It remains, however, unidentified whether KR-31372 inhibits the tube formation in association with migration and proliferation of endothelial cells through Ca^{2+} channel-dependent pathway or not.

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