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Inhibition of Tyrosine Phosphorylation of Vascular Endothelial Growth Factor Receptors in Human Umbilical Vein Endothelial Cells: A Potent Anti-Angiogenic Lipid-Rich Extract from Shark

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ABSTRACT We have previously reported that an ethanolic extract of dried shark muscle mixed with olive oil (shark muscle–olive oil [SMO]) has potent anti-angiogenic activity and that this extract appears to inhibit the binding of vascular endothelial growth factor (VEGF) to its receptor(s). In this study, we investigated the effects of SMO on the phosphorylation of VEGF receptor(s) in human umbilical vein endothelial cells (HUVECs). *In vitro* cell proliferation assays showed that SMO significantly reversed the VEGF-promoted increase in HUVEC proliferation. Western blot analysis revealed that SMO treatment markedly inhibited the VEGF-promoted tyrosine phosphorylation of VEGF receptor-2 (KDR) and VEGF receptor-1 (Flt1) in a dose-dependent manner. These results demonstrated that SMO might interfere with or block the binding of VEGF with its receptors, and thereby inhibit the VEGF receptor(s) signal transduction pathway and so inhibit angiogenesis.

KEY WORDS: • ***human umbilical vein endothelial cells • shark lipid • tyrosine phosphorylation • vascular endothelial growth factor receptors***

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INTRODUCTION

ANGIOGENESIS IS THE PROCESS of generating new capillary blood vessels. It depends on the interaction between the angiogenic factors and their receptors.[1] Vascular endothelial growth factor (VEGF) and its receptors play critical roles in the processes of angiogenesis.[2] When VEGF binds to its cell surface receptors (tyrosine kinases) identified as VEGFR-1/Flt-1 and VEGF receptor-2/KDR,[3–5] it induces intrinsic cytoplasmic enzymatic activities, catalyzing the transfer of the *!*-phosphates of ATP to tyrosine residues in protein substrates. This signal transduction promotes vascular cell mitogens, which eventually enhance angiogenesis.[2]

Interfering with the binding of VEGF to its receptors is an important therapeutic strategy for inhibiting angiogenesis. Several strategies have been developed for targeting the VEGF receptor signal pathway as anticancer therapies.[6]

Shark cartilage, which was promoted as an anticancer agent, was said to have anti-angiogenic activity. It was first demonstrated that oral administration of shark cartilage inhibited angiogenesis *in vivo* by our previous study.[7] Subsequent unpublished studies found that an ethanolic extract from shark muscle and spinal cord exerted even higher anti-angiogenic activity than did the cartilage. Recently, we found that an ethanolic extract of dried shark muscle mixed with olive oil (shark muscle–olive oil [SMO]) has potent anti-angiogenic activity and that this inhibitory effect might interfere with the binding of VEGF to its receptor(s) in a cell-free system.[8] However, whether similar effects of SMO can be demonstrated on VEGF receptors in vascular endothelial cells or cancer cells has not been studied. To further investigate the effect of the SMO on the interaction between VEGF and their receptor(s), we used human umbilical vein endothelial cells (HUVECs), which express VEGF receptors,[9] to investigate whether SMO could interfere with VEGF binding to their receptors through alteration of the phosphorylation of tyrosine residues in the VEGF receptors.

MATERIALS AND METHODS

Reagents

Recombinant human VEGF, monoclonal anti-VEGF receptor-1 (Flt-1 receptor) antibody, monoclonal anti-VEGF receptor-2 (KDR) antibody, anti-mouse immunoglobulin G (IgG) (whole molecule)-agarose, endothelial cell growth supplement, Mammalian Cell Lysis Kit, sodium orthovanadate, bovine serum albumin, and MTT reagent (thiazolyl blue tetrazolium bromide) were purchased from Sigma Chemical Co. (St. Louis, MO). Monoclonal anti-phosphotyrosine antibody and horseradish peroxidase-conjugated goat anti-mouse IgG were purchased from R&D Systems (Minneapolis, MN). ECL western blotting detection reagents and Hyperfilm™ ECL were purchased from Amersham Biosciences Pty Ltd. (Sydney, NSW, Australia). The reagents and equipment used for the polyacrylamide gel electrophoresis were purchased from Bio-Rad Laboratories (Hercules, CA). The Immobilon™ membranes were obtained from Millipore Corp. (Bedford, MA). The SMO mixture used in this study was supplied by Aotea Pacific Ltd. (Auckland, New Zealand). This lipid-rich mixture (shark muscle oil extract–olive oil, 9:1 [vol/vol]) was prepared in 20% ethanol at 20 mg/mL as stock concentration and stored at !20°C.

Cell line

HUVECs were purchased from the American Type Culture Collection (Manassas, VA). These cells were maintained with Ham's F12K medium (Sigma) supplemented with 0.03 mg/mL endothelial cell growth supplement and 10% fetal bovine serum (Sigma).

Cell proliferation assay

Aliquots (100 ″L) of the HUVEC suspensions (1 " 10^5/mL) were placed into wells of a 96-well microplate. SMO was added to appropriate wells to give final concentrations of 0, 10, 50, and 100 ″g/mL. VEGF (50 ng/mL final concentration) was added simultaneously. The cells were incubated for 48 hours at 37°C in a 95% air/5% CO_2 incubator. After incubation, 10 ″L of MTT reagent working solution (thiazolyl blue tetrazolium bromide, dissolved in phosphate-buffered saline [PBS] at 5 mg/mL) was added and incubated for 4 hours at 37°C. One hundred microliters of lysis buffer (10% sodium dodecyl sulfate [SDS]/45% dimethylformamide, pH 4.7) were added to each well and incubated for an additional 4 hours at 37°C. The absorbance in each well was read at 570 nm with the microplate reader.[10]

Cell lysate preparation11

When the HUVECs were grown to 80–90% confluence in 100-mm-diameter tissue culture dishes, the cells were then serum-starved for 24 hours by incubating in a culture medium containing 0.1% fetal bovine serum. After pretreatment with 100 n*M* sodium orthovanadate in PBS for 30 minutes, the cells were incubated with VEGF (50 ng/mL) in the presence of a series of concentrations of SMO for an additional 30 minutes. The cells were then washed with icecold PBS containing 10 m*M* EDTA, 2 m*M* sodium fluoride, and 2 m*M* sodium orthovanadate and lysed in cell lysis buffer (1 " 10^6–5 " 10^6 cells/mL of cold buffer). The extraction mixtures were rocked at 4°C for 30–60 minutes.

Cell lysates were then centrifuged at 3,000 *g* and 4°C for 5 minutes. The protein concentrations of the cell lysates were determined using a protein assay kit (Bio-Rad).

Preparation of immune complex

One milliliter of cell lysate was precleared by incubation with 10 ″L of a 50% suspension of anti-mouse IgG agarose beads for 5 minutes on ice. The beads were pelleted by centrifugation at 3,000 *g* and 4°D for 0.5 minutes, and the supernatant was transferred to a new tube. The preclearing was repeated. The anti-mouse IgG agarose beads were washed three times with Wash Buffer (20 m*M* Tris HCl [pH 8.0], 0.15 *M* NaCl, 2 m*M* EDTA, 1% Nonidet P-40, 10% glycerol, and 1 m*M* sodium orthovanadate) before being added to the lysates.

Immunoprecipitation

Five microliters of mouse anti-phosphotyrosine antibody was added to 500 ″L of cell lysate (approximately 1 mg/mL protein), and the mixture was rocked for 16 hours at 4°C. Twenty microliters of anti-mouse IgG agarose beads (50% suspension) was then added, and the mixture was rocked at 4°C for an additional 2 hours. The anti-mouse IgG-absorbed complexes were centrifuged at 3,000 *g* and 4°C for 0.5 minutes, resuspended in Wash Buffer by trituration with a glass Pasteur pipette, and then repelleted. The complexes were washed a total of three times with Wash Buffer, suspended in 50 ″L of PBS, and transferred to a new tube for the final centrifugation as above. The washed pellet was suspended in 50 ″L of 2" SDS Gel Sample Buffer (250 m*M* Tris [pH 6.8], 6% SDS, 10% glycerol, 10 m*M* sodium fluoride, and bromophenyl blue) by vortex-mixing and then incubated for 3 minutes in a boiling water bath. Anti-mouse IgG agarose was pelleted, and the prepared supernatants were collected and stored at !20°C for western blot analysis.

Western blot analysis

Depending on the different molecular sizes of the markers to be separated, different concentrations of acrylamide in SDS-polyacrylamide gels were prepared: 10% for phosphorylated tyrosine, 8% for VEGF receptor-1, and 6% for VEGF receptor-2. A prestained SDS-polyacrylamide gel electrophoresis broad range standard (catalog number 1610318, Bio-Rad) was used as a molecular marker. Ten microliters of prepared sample was applied, and the gel was run for about 45 minutes at 200 V. The gel was then electrotransferred onto a PVDF membrane (Immobilon transfer membranes) at 5.5 mA/cm^2 for 30 minutes using a transfer apparatus (Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell, Bio-Rad). The membranes were washed with 20 mL of Washing Buffer (0.1% Tween 20 in PBS) for 5 minutes before blocking the membranes with 20 mL of Blocking Buffer (0.1% Tween 20 in PBS with

5% bovine serum albumin) for 60 minutes. The membranes were incubated in the appropriate antibody solution (monoclonal anti-phosphotyrosine antibody [1.0 ″g/mL], anti-VEGF receptor-1 [1:500], and monoclonal anti-VEGF receptor-2 antibody [1:800]) dissolved in 0.1% Tween 20 in PBS containing 5% bovine serum albumin overnight at 4°C with shaking. The membranes were washed with 20 mL of Washing Buffer for 5 minutes. This was repeated five times. The membranes were then incubated at room temperature for an additional 1 hour in the second antibody solution (horseradish peroxidase-conjugated goat anti-mouse IgG) (1:2,000 dilution). The membrane was washed with 20 mL of Washing Buffer for 5 minutes a total of seven times. The complexes were detected by using ECL Reagent (Amersham Biosciences) and exposed to an X-ray film (Hyperfilm ECL, Amersham Biosciences) for 2–10 minutes in an x-ray film cassette.

Statistical analysis

Cell proliferation data for HUVECs were analyzed by PCbased software (Prism version 4.0a, GraphPad Software, San Diego, CA). Each was compared with the VEGF-positive control, with significant differences between the VEGF-positive control and other groups being assessed by analysis of variance. Values in the figures are expressed as means # SD.

RESULTS

Inhibitory effect of SMO on VEGF-enhanced proliferation of HUVECs

To investigate the effect of SMO on the proliferation of HUVECs, the cells were serum-starved for 24 hours and then cultured in medium containing 10% fetal bovine serum in either the absence or the presence of VEGF (50 ng/mL) and with different concentrations of SMO (0, 10, 50, and 100 ″g/mL) for an additional 48 hours.

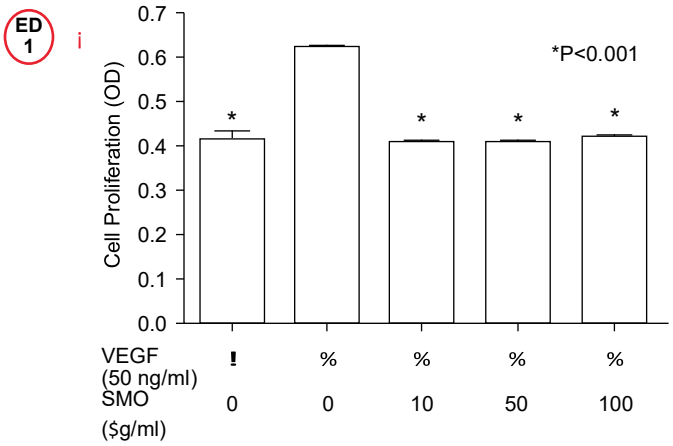

FIG. 1. Effects of SMO on VEGF-induced cell proliferation. HUVECs (1 " 10^5/mL) were incubated in the presence or absence of VEGF and different concentrations of SMO samples (0, 10, 50, and 100 ″g/mL) for 48 hours. The cell proliferation was quantified using the MTT assay. Results are expressed relative to the VEGF positive control (50 ng/mL VEGF, non-SMO treatment) by comparison with non–VEGF- and non–SMO-treated controls and treatment with VEGF (50 ng/mL) and three concentrations of SMO (10, 50, and 100 ″g/mL). Statistical significances were analyzed by analysis of variance. All values shown are means # SD. *P & .001. OD, optical density.

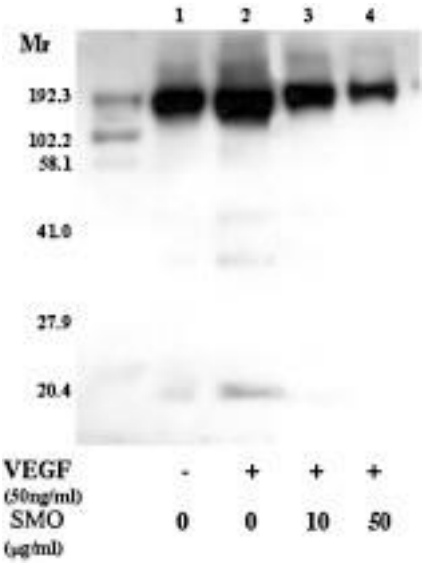

FIG. 2. Effects of SMO on VEGF-induced tyrosine phosphorylation. HUVECs were incubated with or without 50 ng/mL VEGF in the presence and absence of different concentrations of SMO for an additional 30 minutes. The cell lysates were prepared as described in Materials and Methods. The phosphorylated tyrosine kinase proteins were immunoprecipitated with anti-phosphotyrosine antibody and detected with anti-phosphotyrosine antibody by western blot analysis.

As shown in Figure 1, in the VEGF positive control, VEGF treatment enhanced HUVEC proliferation compared with untreated cells. SMO treatment reversed this enhancement in HUVECs at all concentrations (10, 50, and 100 ″g/mL) (Fig. 1).

Inhibitory effect of SMO on VEGF-induced tyrosine phosphorylation in HUVECs

To determine whether SMO inhibited tyrosine kinase activation, phosphoproteins from control and VEGF-stimulated HUVEC lysates were immunoprecipitated with antibody to phosphotyrosine and analyzed by western blotting using anti-phosphotyrosine antibody.

In the lysates (Fig. 2) one phosphotyrosine protein of about 200 kDa was identified in the olive oil-treated control without added VEGF (lane 1), while in the control with VEGF (lane 2), this band markedly increased in intensity, and another three bands (about 45, 35, and 15 kDa) were detected. When the HUVECs were treated with SMO (lanes 3 and 4), the 200-kDa protein was significantly reduced in a dose-dependent manner, and the lower-molecular-size bands disappeared as well.

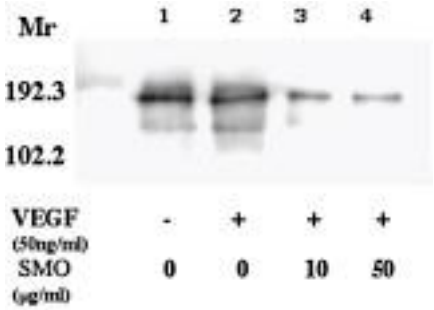

FIG. 3. Effects of SMO on VEGF-induced VEGF receptor-2 (KDR) tyrosine phosphorylation. HUVECs were incubated with or without 50 ng/mL VEGF in the presence and absence of different concentrations of SMO for an additional 30 minutes. The cell lysates were prepared as described in Materials and Methods. The phosphorylated tyrosine kinase proteins were immunoprecipitated with anti-phosphotyrosine antibody and detected with anti-KDR antibody by western blot analysis.

Inhibitory effects of SMO on VEGF receptor-2 tyrosine phosphorylation in HUVECs

When the immunoprecipitated phosphoproteins from HUVECs were immunoblotted with anti-VEGF receptor-2 antibody (anti-KDR), it was noted that VEGF treatment induced significant phosphorylation of the VEGF receptor-2

when compared with non–VEGF-treated control immunoprecipitates (Fig. 3). In this study only the 150-kDa and 200kDa forms of the receptor were detected. This VEGF receptor-2 phosphorylation was inhibited markedly in a dosedependent manner by treating the cells with SMO (Fig. 3).

Inhibitory effects of SMO on VEGF receptor-1 tyrosine phosphorylation in HUVECs

When the immunoprecipitated phosphoproteins from HUVECs were immunoblotted with anti-VEGF receptor-1 antibody (anti-Flt1), it was noted that VEGF treatment induced significant phosphorylation of VEGF receptor-1

when compared with non–VEGF-treated control immunoprecipitates (Fig. 4). This VEGF receptor-1 phosphorylation was inhibited markedly by treating the cells with SMO in a dose-dependent manner (Fig. 4).

DISCUSSION

Our previous study demonstrated that SMO inhibits the pro-angiogenic activity induced by a number of cytokines and growth factors, including fibroblast growth factor-2 and VEGF.[8] With VEGF this is mediated through at least two of the cell surface receptors for this growth factor, VEGF receptor-1 and VEGF receptor-2.[8] In this study, we have investigated the possible effects of SMO on VEGF receptor tyrosine phosphorylation by using the VEGF receptor(s)-expressing cell line HUVEC.

The results revealed that SMO blocked or inhibited VEGF-induced receptor tyrosine phosphorylations in this cell line. VEGF is a strong activator of extracellular signalregulated protein kinases via VEGF receptor-2.[3] Activation of VEGF receptor-2 occurs through ligand-induced dimerization and receptor autophosphorylation at multiple tyrosine residues in the intracellular domain.[12] Our data indicated that VEGF enhanced cell proliferation in HUVECs. This increased growth was reversed to the initial nonstimulated level when the cells were treated with SMO (Fig. 1). VEGF activates a p42/p44 mitogen-activated protein kinase activity, and this pathway plays a central role in angiogenesis.[13] When VEGF binds to its receptors, it stimulates the tyrosine phosphorylation of multiple components including protein with apparent molecular sizes of M_r 205, 145, 120, 97, and 65–70 kDa in HUVECs. Of these, the protein M_r 205 kDa corresponded to both VEGF receptor-1 (Flt-1) and receptor-2 (KDR).[14] VEGF is also known to stimulate a 145kDa phospholipase C-*!*, which is a major mitogenic signaling mechanism for VEGF.[15] VEGF-dependent endothelial cell survival is mediated in part via phosphatidylinositol 3kinase.[12,14] Our data showed that VEGF strongly promoted tyrosine phosphorylation in HUVECs and that SMO treatment significantly blocked or reduced this elevation in tyrosine phosphorylation (Fig. 2).

HUVECs express high levels of VEGF receptor-2.[16,17] Our data demonstrated that SMO treatment markedly inhibited the tyrosine phosphorylation of VEGF receptor-2 in HUVECs (Fig. 3). We also demonstrated that SMO treatment inhibited VEGF receptor-1 (Flt-1) tyrosine phosphorylation in these cells (Fig. 4). VEGF receptor-1 (Flt-1) is also a transmembrane receptor from the tyrosine kinase family and is expressed in HUVECs. The receptor-2-expressing cells showed striking changes in cell morphology, actin reorganization, and membrane ruffling, chemotaxis, and mitogenicity upon VEGF stimulation, whereas VEGF receptor-1-expressing cells lacked such responses.[3,5,18] VEGF receptor-2 is known to undergo ligand-induced autophos-

FIG. 4. Effects of SMO on VEGF-induced VEGF receptor-1 (Flt1) tyrosine phosphorylation. HUVECs were incubated with or without 50 ng/mL VEGF in the presence or absence of different concentrations of SMO for an additional 30 minutes. The cell lysates were prepared as described in Materials and Methods. The phosphorylated tyrosine kinase proteins were immunoprecipitated with anti-phosphotyrosine antibody and detected with anti-Flt1 antibody by western blot analysis.

phorylation in intact cells, and both VEGF receptors were phosphorylated *in vitro* in response to VEGF, with VEGF

receptor-2 being much more efficiently modified than VEGF receptor-1.[5]

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REFERENCES

1. Risau W: Mechanisms of angiogenesis. *Nature* 1997;386: 671–674.
2. Ferrara N: Role of vascular endothelial growth factor in the regulation of angiogenesis. *Kidney Int* 1999;56:794–814.
3. de Vries C, Escobedo JA, Ueno H, Houck K, Ferrara N, Williams LT: The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor. *Science* 1992;255:989–991.
4. Terman BI, Dougher-Vermazen M, Carrion ME, Dimitrov D, Armellino DC, Gospodarowicz D, Bohlen P: Identification of the KDR tyrosine kinase as a receptor for vascular endothelial cell growth factor. *Biochem Biophys Res Commun* 1992;187: 1579–1586.
5. Waltenberger J, Claesson-Welsh L, Siegbahn A, Shibuya M, Heldin CH: Different signal transduction properties of KDR and Flt1, two receptors for vascular endothelial growth factor. *J Biol Chem* 1994;269:26988–26995.
6. McMahon G: VEGF receptor signalling in tumor angiogenesis. *Oncologist* 2000;5(Suppl 1):3–10.
7. Davis PF, Yi H, Furneaux RH, Johnston PS, Rüger BM, Slim GC: Inhibition of angiogenesis by oral ingestion of powdered shark cartilage in a rat model. *Microvasc Res* 1997;54:178–182.
8. Yuan L, Yoshida M, Davis PF: Inhibition of pro-angiogenic factors by a lipid-rich shark extract. *J Med Food* 2006;9:300–306.
9. Soker S, Gollamudi-Payne S, Fidder H, Charmahelli H, Klagsburn M: Inhibition of vascular endothelial growth factor (VEGF)induced endothelial cell proliferation by a peptide corresponding the exon 7-encoding domain of VEGF 16S. *J Biol Chem* 1997; 272:31582–31588.
10. Mosmann T: Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 1983;65:55–63.
11. Brekken RA, Overholser JP, Stastny VA, Waltenberger J, Minna JD, Thorpe PE: Selective inhibition of vascular endothelial growth factor (VEGF) receptor 2 (KDR/Flk-1) activity by a monoclonal anti-VEGF antibody blocks tumor growth in mice. *Cancer Res* 2000;60:5117–5124.
12. Zachary I: VEGF signalling: integration and multi-tasking in endothelial cell biology. *Biochem Soc Trans* 2003;31:1171–1177.
13. Pages G, Milanini J, Richard DE, Berra E, Gothie E, Vinals F, Pouyssegur J: Signaling angiogenesis via p42/p44 MAP kinase cascade. *Ann N Y Acad Sci* 2000;902:187–200.
14. Abedi H, Zachary I: Vascular endothelial growth factor stimulates tyrosine phosphorylation and recruitment to new focal adhesions of focal adhesion kinase and paxillin in endothelial cells. *J Biol Chem* 1997;272:15442–15451.
15. Takahashi T, Shibuya M: The 230 kDa mature form of KDR/Flk1 (VEGF receptor-2) activates the PLC-gamma pathway and partially induces mitotic signals in NIH3T3 fibroblasts. *Oncogene* 1997;14:2079–2089.
16. Esser S, Lampugnani MG, Corada M, Dejana E, Risau W: Vascular endothelial growth factor induces VE-cadherin tyrosine phosphorylation in endothelial cells. *J Cell Sci* 1998;111: 1853–1865.
17. Barr MP, Byrne AM, Duffy AM, Condron CM, Devocelle M, Harriott P, Bouchier-Hayes DJ, Harmey JH: A peptide corresponding to the neuropilin-1-binding site on VEGF165 induces apoptosis of neuropilin-1-expressing breast tumour cells. *Br J Cancer* 2005;92:328–333.
18. Yoshiji H, Gomez DE, Shibuya M, Thorgeirsson UP: Expression of vascular endothelial growth factor, its receptor, and other angiogenic factors in human breast cancer. *Cancer Res* 1996;56: 2013–2016.