Type I Collagen Limits VEGFR-2 Signaling by a SHP2 Protein-Tyrosine Phosphatase–Dependent Mechanism 1

Stefania Mitola, Barbara Brenchio, Marco Piccinini, Leon Tertoolen, Luca Zammataro, Georg Breier, Maria Teresa Rinaudo, Jeroen den Hertog, Marco Arese, Federico Bussolino

Abstract—During angiogenesis, a combined action between newly secreted extracellular matrix proteins and the repertoire of integrins expressed by endothelial cells contributes in the regulation of their biological functions. Extracellular matrix–engaged integrins influence tyrosine kinase receptors, thus promoting a regulatory cross-talk between adhesive and soluble stimuli. For instance, vitronectin has been reported to positively regulate VEGFR-2. Here, we show that collagen I downregulates VEGF-A–mediated VEGFR-2 activation. This activity requires the tyrosine phosphatase SHP2, which is recruited to the activated VEGFR-2 when cells are plated on collagen I, but not on vitronectin. Constitutive expression of SHP21C459S mutant inhibits the negative role of collagen I on VEGFR-2 phosphorylation. VEGFR-2 undergoes internalisation, which is associated with dynamin II phosphorylation. Expression of SHP21C459S impairs receptor internalisation suggesting that SHP2-dependent dephosphorylation regulates this process. These findings demonstrate that collagen I in provisional extracellular matrix surrounding nascent capillaries triggers a signaling pathway that negatively regulates angiogenesis. (Circ Res. 2006;98:45-54.)

Key Words: endothelial cell • extracellular matrix • tyrosine kinase receptor • tyrosine phosphatase

Angiogenesis takes place during development, tissue growth and repair, and aberrantly in several pathological settings. During angiogenesis, endothelial cells (ECs) modify their genetic program. The final consequences are the assumption of a migratory phenotype, cell cycle activation, and the secretion of proteases and proteins of the extracellular matrix (ECM).1 Changes in ECM physico-chemical features are crucial for EC biological functions.2,3 In the resting vasculature, ECM is mainly composed by collagens, laminin, tenasin, proteoglycans, and perlecan;4 such environment favors cell–cell adhesion, survival, and inhibits cell proliferation and migration.2,3 In contrast, provisional ECM surrounding angiogenic ECs include new proteins such as fibronectin, fibrin, vitronectin, collagen I, and thrombospondin. Furthermore, this ECM contains proteolytic cleavage products of laminin, fibronectin, and collagens generated by metalloproteinases released by activated ECs.2,3 A balance between negative and positive cues triggered by provisional ECM is permissive for neovascularization.2,3 For example, collagen I and thrombospondin–1 prevent angiogenesis, whereas fibronectin shows opposite activities.2 Furthermore, the proteolytic of laminin, collagen, and fibronectin generates pro- and antiangiogenic fragments.2,3 Coordinated with these ECM modifications, ECs adapt their repertoire of integrins to allow adhesion to new ECM components and to receive instructive signals from surrounding microenvironment.2,3 In particular, the induction of an angiogenic phenotype is associated with the expression of α,β1, α,β3, and α,β5.8–10 Actually, vascular endothelial growth factor (VEGF)-A and fibroblast growth factor-2 induce angiogenesis that is, respectively, inhibited by blocking α,β1 and α,β3 function.11

VEGF-A represents a rate-limiting step for physiologic and pathologic angiogenesis.12 VEGF-A activates VEGF receptor (R) R-1 and VEGF-2, the latter being the principal target of the ligand in adult life. Different mechanisms are involved in the fine-tuning of VEGF-2 signaling:12,13 (1) VEGFR-2 transcription is regulated by soluble molecules and hypoxia; (2) the catalytic activity of VEGFR-2 may be negatively regulated by VEGFR-1, tissue inhibitor of metalloproteases (TIMP)-2 and dopamine; (3) the association of VEGFR-2 with neuropilin-1 is instrumental for increasing its affinity VEGF-A165; (4) and (5) the localization in specific plasma membrane domains is another mechanism to control VEGFR-2 function. For example, in confluent ECs, VEGF-2 is mainly associated with adherens junctions and mediates survival signals.14 On the contrary, during VEGF–A–mediated EC migration, VEGF-2 forms a complex with α,β3 integrin.15–17 Finally, caveolae depletion results in an increase of VEGF-2 phosphorylation but leads to the inhibition of downstream signals and EC motility.18

Original received February 15, 2005; revision received May 31, 2005; final revision received November 22, 2005; accepted November 29, 2005. From the Institute for Cancer Research and Treatment (S.M., B.B., L.Z., M.A., F.B.), Department of Oncological Sciences (S.M., B.B., L.Z., M.A., F.B.), Department of Medicine and Experimental Oncology (M.P., M.T.R.), University of Torino, Torino, Italy; Netherlands Institute for Developmental Biology (L.T., J.d.H.), Utrecht, The Netherlands; Max Planck Institute for Physiological and Clinical Research (G.B.), D-61231 Bad Nauheim, Germany; University Clinic Carl Gustav Carus (G.B.), Dresden, Germany.

Correspondence to Federico Bussolino, MD, IRCC, Strada provinciale 142, Km 3.95. 10060 Cadolmo, Italy. E-mail federico.bussolino@ircc.it

© 2006 American Heart Association, Inc.

Circulation Research is available at http://circres.ahajournals.org

DOI: 10.1161/01.RES.0000199355.32422.7b
Here, we demonstrate that fibrillar collagen I is a negative modulator of VEGFR-2 activation via the recruitment of the cytosolic Src homology-2 domain-containing protein tyrosine phosphatase (SHP2) to the receptor. SHP2 dephosphorylates VEGFR-2 and favors its internalization. These findings extend previous observations on the opposite effects exerted by proteins of ECM on EC functions and provide new insights into how ECM surrounding nascent vessels could generate regulatory signals that are critical for EC response to VEGF-A.

Materials and Methods
For details on reagents and plasmids, cells, cell motility, and immunoprecipitation and immunoblotting, please see the online data supplement available at http://circres.ahajournals.org.

Tyrosine Phosphatase Assay
Tyrosine phosphatase activity was measured on EC immunoprecipitates anti-SHP1 and anti-SHP2 by HitHunter Fluorescence detection tyrosine phosphatase assay kit (Discover, Fremont, Calif). Alternatively, VEGFR-2 was immunoprecipitated as described from 10^7 human ECs stimulated with VEGF-A_165_ (0.23 nmol/L) and beads were washed twice in 50 mmol/L 2-(N-morpholino)ethanesulfonic acid, pH 6.5. VEGF-A_2_ immunoprecipitate was used as substrate and incubated with the aforementioned immunoprecipitates in 2-(N-morpholino)ethanesulfonic acid buffer for 1 hour at 37°C. At the end of incubation, proteins were denatured, separated by SDS-PAGE, and probed with mAb anti-pY or Ab anti-VEGFR-2.

VEGFR-2 Internalization
VEGFR-2 internalization was determined by evaluating the amount of receptor that was resistant to trypsinization or by evaluating the ratio between the amount of [125I-VEGF-A internalized and bound to the cell surface. Further details are described online.

Fluorescence Resonance Energy Transfer Analysis
For fluorescence resonance energy transfer (FRET), PAE cells carrying VEGFR-2-CFP and YFP-SHP2 were observed by a Leitz orthoplan upright microscope (Leitz GMBH, Wetzlar, Germany) equipped with an epifluorescence detection system and a temperature-controlled specimen holder at 33°C. Measurements were made in a DMEM containing 2% fetal calf serum and 10 mmol/L HEPES (pH 7.4) exactly as previously described. Further details are described online.

Results
Effect of Type I Collagen and Vitronectin on VEGF-A_165_–Induced EC Chemokinesis
Previous works demonstrated that ECM proteins modulate the autophosphorylation activity of VEGFR-2 and its biological responses. To further support this hypothesis, EC chemokinesis-stimulated by VEGF-A_165_ was evaluated on cells plated on collagen I or vitronectin by time-lapse video microscopy of individual cells (Figure 1). ECs exhibited a baseline mean migration speed of 17.31 ± 0.09 μm/hour on vitronectin and 14.51 ± 0.12 μm/hour on collagen I (P<0.001; t test). In presence of VEGF-A_165_, EC speed increased to 36.09 ± 0.13 μm/hour (P<0.0001 versus unstimulated cells) and to 22.25 ± 0.08 μm/hour (P<0.0002 versus unstimulated cells) on vitronectin and collagen I, respectively. VEGF-A_165_ stimulation resulted in higher directional persistence of cell migration; in contrast unstimulated ECs moved in random directions. The VEGF-A_165_ effect was more evident in cells plated on vitronectin. Unstimulated cells on collagen I and on vitronectin showed a similar directional persistence (vitronectin: 0.12 ± 0.06; collagen I: 0.10 ± 0.02), whereas VEGF-A_165_ increased this parameter in a greater extent in cells on vitronectin (0.43 ± 0.05) than on collagen I (0.19 ± 0.04) (P<0.0001) (Figure 1).

To exclude that the observed effects were caused by different levels of VEGF-2 expression, the high-affinity binding sites of VEGF-A on the surface of EC plated on collagen I or vitronectin were studied. Scatchard analysis indicated that the type of ECM did not influence the number of receptors and their affinity (supplemental Figure I).

Type I Collagen Inhibits VEGFR-2 Phosphorylation by Activating SHP2 Tyrosine Phosphatase
When VEGF-A_165_ stimulated ECs on vitronectin, VEGF-R2 phosphorylation was markedly higher as compared with cells on collagen I, suggesting a modulating role of tyrosine phosphatases. In particular, both SHP1 and SHP2 have been demonstrated to negatively regulate VEGFR-2. Therefore, we measured phosphatase activity in SHP1 and SHP2 immunocomplexes from VEGF-A_165_–stimulated ECs adherent on native ECM. VEGF-A_165_ promoted a rapid activation of SHP2 that peaked at 10 minutes and then declined (Figure 2A). SHP-1 activity increased, but in slight manner, without reaching a well-defined peak within 20
minutes. On the basis of this observation and previous data indicating that SHP2 is involved in GAS6 inhibition of VEGFR-2, we pointed to this phosphatase. Our hypothesis was further confirmed by the ability of SHP2 immunocomplexes isolated from ECs stimulated with VEGF-A165 to dephosphorylate in vitro phosphorylated VEGFR-2 (Figure 2B; supplemental Figure II). VEGF-A165 rapidly phosphorylated SHP2 in tyrosine residues, an event known to be associated with SHP2 catalytic activity (Figure 2C; supplemental Figure II). Both catalytic activity (Figure 2D) and tyrosine phosphorylation (Figure 2E) were more pronounced in cells on collagen I than on vitronectin.

**SHP2 Binds VEGFR-2**

Figure 3A shows that SHP2 associated with activated VEGF-A165 immunoprecipitated from ECs on native ECM. To further analyze this interaction, we performed a far Western blot analysis using SHP2GST fusion protein. Figure 3B and supplemental Figure III show that phosphorylated VEGFR-2.
bound SHP2-GST in a time dependent-manner. To follow this interaction in living cells, we analyzed FRET of VEGFR-2-CFP and YFP-SHP2. Figure 3C and 3D shows that FRET occurred at the plasma membrane level and disappeared when VEGF-A₁₆₅ was removed from medium.

**Type I Collagen Elicits SHP2 Interaction With VEGFR-2**

Next, we investigated whether ECM could modulate the association of SHP2 with VEGFR-2. VEGFR-2 was immunoprecipitated from ECs on collagen I or on vitronectin and the association with SHP2 was analyzed. The amount of
associated SHP2 with VEGFR-2 in cells on vitronectin was much reduced compared with cells on collagen I (Figure 3E; supplemental Figure III). In these experiments EC adhesion to ECM substrates was similar, thus excluding the possibility that the observed effect could be caused by differences in cells adhesion (not shown).

**Effect of SHP2<sup>C459S</sup> Overexpression on VEGFR-2 Activation**

To investigate the involvement of SHP2 in VEGFR-2 signaling, ECs were infected with a retroviral vector encoding SHP2<sup>C459S</sup> and the expression of the proteins was analyzed on cell lysate by immunoblot. B, Infected cells (2×10⁴) plated on collagen I were stimulated for the indicated times with VEGF-A<sub>165</sub> (0.23 nmol/L). Cell lysates were immunoprecipitated as indicated. C, Densitometric analysis of the experiment shown in (B). Mean±SD of 4 experiments. ANOVA was F= 77.55. §P<0.05 vs unstimulated cells. *P<0.05 vs cells carrying vector alone. White and black bars, respectively, indicate EC-PINCO and EC-SHP2<sup>C459S</sup>. D, Cells plated on collagen I or on vitronectin were pre-treated with orthovanadate (1 mmol/L, 15 minutes) and then treated VEGF-A<sub>165</sub> (0.23 nmol/L) for 10 minutes. After immunoprecipitation, samples were divided in 2 aliquots, run in two gels, and immunoblotted as indicated. E, Densitometric analysis of the experiment shown in (D). Mean±SD of 5 experiments. Before ANOVA (F=31.04) data were normalized according to z score §P<0.05 vs unstimulated cells plated on collagen I or vitronectin. *P<0.05 vs cells plated on collagen I and stimulated with VEGF-A<sub>165</sub>. White and black bars, respectively, indicate EC-PINCO and EC-SHP2<sup>C459S</sup>.

**Figure 4. Effect of overexpression of SHP2<sup>C459S</sup> on VEGFR-2 activation.** A, ECs were infected with a retroviral vector encoding SHP2<sup>C459S</sup> and the expression of the proteins was analyzed on cell lysate by immunoblot. B, Infected cells (2×10⁴) plated on collagen I were stimulated for the indicated times with VEGF-A<sub>165</sub> (0.23 nmol/L). Cell lysates were immunoprecipitated as indicated. C, Densitometric analysis of the experiment shown in (B). Mean±SD of 4 experiments. ANOVA was F= 77.55. §P<0.05 vs unstimulated cells. *P<0.05 vs cells carrying vector alone. White and black bars, respectively, indicate EC-PINCO and EC-SHP2<sup>C459S</sup>. D, Cells plated on collagen I or on vitronectin were pre-treated with orthovanadate (1 mmol/L, 15 minutes) and then treated VEGF-A<sub>165</sub> (0.23 nmol/L) for 10 minutes. After immunoprecipitation, samples were divided in 2 aliquots, run in two gels, and immunoblotted as indicated. E, Densitometric analysis of the experiment shown in (D). Mean±SD of 5 experiments. Before ANOVA (F=31.04) data were normalized according to z score §P<0.05 vs unstimulated cells plated on collagen I or vitronectin. *P<0.05 vs cells plated on collagen I and stimulated with VEGF-A<sub>165</sub>. White and black bars, respectively, indicate EC-PINCO and EC-SHP2<sup>C459S</sup>.
phosphorylation caused by orthovanadate. When EC-SHP2C459S were plated on vitronectin, VEGFR-2 phosphorylation was completely unaffected by orthovanadate treatment (Figure 4D and 4E). These data support the concept type I VEGFR-2 endocytosis was detectable 10 minutes after stimulation and reached the peak at 15 minutes (Figure 6A and 6B). Very little amount of internalized VEGFR-2 was detected in ECs overexpressing SHP2C459S. These results were confirmed by internalisation analysis of [125I]VEGF-A. Expression of SHP2C459S reduced the internalisation of the ligand (Figure 6C).

The GTPase dynamin II is a regulator of both clathrin- and caveolae-dependent endocytosis and its activity is positively regulated by tyrosine phosphorylation.37,38 In ECs dynamin II is a substrate of Src kinase,39 which is activated by VEGF-A.33,40 Furthermore, SHP2 regulates Src by controlling phosphorylation of its inhibitory tyrosine 529.41 If SHP2 contributes to the VEGFR-2 endocytosis, Src and dynamin II should be inhibited in cells carrying SHP2C459S. Indeed, VEGF-A165-evoked tyrosyl phosphorylation of dynamin II and phosphorylation of Src Tyr-416 were impaired in these cells (Figure 6D and 6E; supplemental Figure V). These data demonstrate that SHP2 stimulation by VEGFR-2 modulates dynamin II activation, probably through Src kinase activation.

**SHP2 Binds Directly to Tyrosine 1173 in the VEGFR-2**

C-terminal Tyr-1212 and -1173 are the two major VEGF-A-dependent autophosphorylation sites42 and their removal impairs VEGFR-2 downregulation,43 suggesting that one of these Tyr residues could bind SHP2. VEGFR-2 and its mutants VEGFR-2Y1173F and VEGFR-2Y1212F were immunoprecipitated from transfected PAE cells and the association with SHP2 was analyzed by immunoblot. The VEGF-A165-dependent tyrosine phosphorylation of the receptor was lower in PAE carrying VEGFR-2Y1173F and VEGFR-2Y1212F mutants than in cells transfected with wild type receptor accordingly to the relevance of these residues in the autophosphorylation event (Figure 7A). Mutation in tyrosine 1173 abrogated the interaction with SHP2, suggesting that this reside is crucial for SHP2 binding to VEGFR-2 (Figure 7B).

**Discussion**

Our study establishes a role for collagen I, a protein released within the provisional ECM by angiogenic ECs, as a negative regulator of VEGFR-2 through activation of tyrosine phosphatase SHP2. This concept parallels the observation that αβ integrin engaged by collagen activates the T-cell protein tyrosine phosphatase function that inhibits epidermal growth factor receptor signaling.44

We demonstrated that EC adhesion to collagen I reduces VEGF-A165–induced VEGFR-2 autophosphorylation by recruiting SHP2 to the phosphorylated tyrosine 1173. This
observation explains our previous data showing that EC proliferation stimulated by VEGF-A165 is lower on collagen I than on vitronectin. Moreover, we provide evidences that: (1) EC motility induced by VEGF-A165 is enhanced on vitronectin as compared with collagen I; (2) SHP2 activity and tyrosine residue phosphorylation are activated by VEGF-A when ECs adhere on native ECM or on vitronectin; however, these effects are greatly increased when EC adhere

Figure 6. Effect of overexpression of SHP2C459S on VEGFR-2 internalization, dynamin II, and Src phosphorylation. ECs (2×10⁶) overexpressing SHP2C459S were plated on collagen I and stimulated for the indicated times with VEGF-A₁₆₅ (0.23 nmol/L). A, Cells were submitted to trypsin treatment as described in Methods, and cell lysates were immunoprecipitated with an Ab anti the C-terminus of VEGFR-2 and immunoblotted with an Ab anti-VEGFR-2. B, Densitometric analysis of the experiment shown in (A). Mean±SD of 4 experiments. ANOVA was F=21.50. §P<0.05 vs unstimulated cells. *P<0.05 vs SHP2C459S cells stimulated for 15 minutes with VEGF-A₁₆₅. White, black, and hatched bars, respectively, indicate EC, EC-Pinco, and EC-SHP2 C459S. C, EC overexpressing SHP2 C459S (Œ) or vector alone (○) were incubated, then incubated at 37°C with [¹²⁵I]VEGF-A. Internalization was monitored as outlined in Methods. Mean±SD of three samples in one experiment of two. D, Cell lysates were immunoprecipitated with Ab anti-dynamin II, divided in 2 aliquots, separated by SDS-PAGE and blotted as indicated. E, Total amount of Src and pY416 Src was detected by immunoblotting on cell lysates. Results are representative of 7 (C) and 4 (D) experiments, respectively. Densitometric analysis of (C) and (D) is reported in supplemental Figure VI.

Figure 7. SHP2 interacts with Y1173 of VEGFR2. Wild-type and mutant VEGFR-2 cDNAs were transiently transfected in PAE cells that were stimulated with VEGF-A₁₆₅. Lysates were immunoprecipitated with anti-VEGFR-2 Ab and immunoblotted with anti-pY (A) or anti-SHP2 (B). Results are representative of at least 3 experiments.
on collagen I; (3) constitutive expression of SHP2C459S blocks the inhibition of VEGFR-2 phosphorylation observed in cells adhering to collagen I; (4) VEGFR-2 is a substrate of SHP2; (5) in living cells VEGFR-2 closely interacts with SHP2 in proximity of plasma membrane of ECs; and (6) VEGF-A165 dependent association of VEGFR-2 with SHP2 occurs when ECs are plated on collagen I, but not on vitronectin.

Downregulation of tyrosine kinase receptors by phosphory-tyrosine phosphatases could be considered a simpler and faster way to modify their behavior and functions as compared with clathrin-mediated or caveolae-dependent endocytosis or to proteasome-mediated degradation.45 SHP2 has been generally considered a positive downstream signal activated by membrane receptors.46 However, emerging evidences suggest that it may positively or negatively regulate signaling pathways depending on the specific type of signaling network. For example, SHP2 may act as a negative regulator of platelet-derived growth factor receptor by inducing dephosphorylation of the receptor itself or of its cognate substrates.47–49 However, SHP2 is also implicated in positive regulation of this receptor through regulation of Ras or focal adhesion kinase.50,51 Similarly, SHP2 exerts both negative and positive influences on T-cell receptor52,53 and JAKs/STATs pathway.54,55 Finally, SHP2 exclusively acts as negative regulator in angiotensin II receptor A1-mediated signals.56

Here, we do not investigate the precise mechanism by which collagen I favors SHP2-mediated VEGFR-2 dephosphorylation. Recently, we have demonstrated that SHP2 is involved in the negative control of VEGFR-2 triggered by GAS6-dependent Axl stimulation.28 Furthermore, our observation parallels the results that VEGFR-2 may be negatively regulated by TIMP-2 through SHP1.13 In ECs stimulated by TIMP-2, SHP1 shifts from αβ3 integrin to VEGFR-2, which is dephosphorylated.

It has been reported that in vascular smooth muscle cells β3 engagement by vitronectin results in tyrosine phosphory-lation of its cytosolic domain and recruitment of SHP2, which modulate insulin growth factor I receptor.57 Thus, we hypothesize a protective role on VEGFR-2 signaling by vitronectin-engaged αβ3, which recruits SHP2 and preserves the receptor from phosphatase activity. In contrast, EC adhesion on collagen I, which does not depend on αβ3 integrin, could allow SHP2 interacting with VEGFR-2. Preliminary evidences demonstrate that a phosphatase activity is associated to β3 in ECs plated on vitronectin and stimulated by VEGF-A165. However, β1 immunocomplexes do not contain any phosphatase activity both in cells plated on collagen I and vitronectin (S. Mitola, unpublished).

Then we showed that SHP2 plays a relevant role in VEGFR-2 internalisation. VEGFR-2 undergoes endocytosis by a Cbl-dependent ubiquitination process58 or through a mechanism caveolin-1-dependent.58 Here, we extend these observations by showing overexpression of SHP2C459D dramatically decreases VEGFR-2 internalization, suggesting that dephosphorylation is involved in regulating its trafficking.

VEGF-A165 induced tyrosyl phosphorylation of dynamin II, a GTPase regulator of vesicle fission required for the control of both clathrin- and caveolae-mediated endocytosis.38 Previous studies showed that Src-dependent dynamin II phos-phorylation is required for endocytosis.39,59,60 Furthermore, SHP2 activates Src kinase by inhibiting the recruitment of C-terminal Src kinase, which exerts its inhibitory role by phosphorylating Tyr-529 of Src.41 Here, we show that overexpression of SHP2C459D significantly reduced the effect of VEGF-A165 on dynamin II phosphorylation and Src activation. These data suggest that SHP2 activation dephosphorylates VEGFR-2 and concomitantly activates Src kinase, which triggers a dynamin-dependent receptor internalization. SHP2 binds Tyr-1173 of stimulated VEGFR-2. This residue seems important in internalisation process as demonstrated by studying truncated receptors lacking Tyr-1173 and Tyr-1212.61 This process occurs in ECs growing on native ECM, as demonstrated by the data showing that this phosphatase is also activated by VEGF-A165 in ECs adherent to this ECM (Figures 2A, 2B, and 3) as well as in cells on collagen I. Here, we did not address the behavior of the internalized VEGFR-2 in EC adhering on collagen I. VEGFR-2 could either be accumulated in the endosomal compartment and degraded or recycled to the membrane, thus allowing a different degree of EC activation or a modified spatial regulation of the signal. It is known that tyrosine kinase receptors may be translocated to the nucleus.52 Notably, it has been recently reported that VEGF-A through VEGFR-2 may be transferred to the nucleus of migrating ECs.33 We may speculate that collagen I mediated internalization of VEGFR-2 results in attenuation of VEGFR-2–mediated signals or in activation of undefined EC responses and that a modulation of SHP2-dependent mechanism of internalization by ECM characterizes specific steps of angiogenesis.

Acknowledgments
This study was supported by Associazione Italiana per la Ricerca sul Cancro, Istituto Superiore di Sanità. (AIDS and Stem Cells National Projects), Ministero dell’ Università e della Ricerca (MIUR) (60% and PRIN 2004 projects), Fondi incentivazione della Ricerca di Base (RBN01814P, RBN01MAWA, RBN01ITC8), Ministero della Salute (Ricerca Finalizzata 2002 and 2003 to M.A. and F.B.), Regione Piemonte, and European Community (LSHM-CT-2003-503251) (http://www.engl.org). We thank Letizia Lanzetti, Luca Primo, and Guido Serini for their suggestions, and Vincenzo Bagnardi for statistical analysis.

References
inducing apoptosis of angiogenic blood cells. Cell. 1994;79:
1157–1164.


Type I Collagen Limits VEGFR-2 Signaling by a SHP2 Protein-Tyrosine Phosphatase–Dependent Mechanism 1
Stefania Mitola, Barbara Brenchio, Marco Piccinini, Leon Tertoolen, Luca Zammataro, Georg Breier, Maria Teresa Rinaudo, Jeroen den Hertog, Marco Arese and Federico Bussolino

Circ Res. 2006;98:45-54; originally published online December 8, 2005;
doi: 10.1161/01.RES.0000199355.32422.7b
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2005 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/98/1/45

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2005/12/08/01.RES.0000199355.32422.7b.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/
Supplemental Methods

Reagents and Plasmids

Rabbit polyclonal antibodies (Ab) VEGFR-2 (N-terminal: N931; C-terminal: C-1158), anti-SHP1, anti-SPH2, anti-dynamin II were purchased from Santa Cruz (Santa Cruz Biotecnology, Inc., Santa Cruz, CA), anti-src and anti-phospho(Tyr-416) src from Cell Signaling Technology, (Beverly, MA), mAb anti-phosphotyrosine (pY) from Upstate Biotechnology (Lake Placid, NY).

SHP2C459S cDNA mutant, in which Cys 459 is substituted by Ser1, was subcloned into the BamHI/EcoRI site of a modified Pinco retroviral vector and expressed under the control of the two long terminal repeats2. ECs were infected by Pinco retroviral vector encoding SHP2 C459S. Expression of transgene was evaluated by western blot analysis with Ab anti-SHP2. Infection conditions did not modify EC morphology and cell cycle, which was analysed by propidium iodide fluorescence with FACS flow cytometer (FACS Advantage SE, Mountain View, CA).

SHP2 cDNA was inserted between the BamHI and EcoRI sites of pGEX2TK (Pharmacia Biotech, Piscataway, NJ) to yield GSH -S- transferase (GST) fusion protein and expressed in E. coli. Protein was purified by GST-sepharose affinity chromatography (Pharmacia-Biotech).

VEGFR-2 and SHP2 cDNAs were respectively cloned in ClaI and StuI sites of pCS2MT-CFP and in XhoI/ and NotI sites of pCS2MT-YFP to create constructs encoding the labeled fusion proteins VEGFR-2-CFP and YFP-SHP2, respectively.

VEGFR-2Y1173F and VEGFR-2Y1212F were generated by PCR site-directed mutagenesis. The antisense oligonucleotide for VEGFR-2Y949F was CCAG GGC AAG GAC TTC GTT GGG GAA CTC, for VEGFR-2Y994F GCT TCT GAA GAA CTG TTT AAA GAC TTC CTG, for VEGFR-2Y1052-1057F GAC ATT TTT AAG GAT CCG GAT TTT GTC AGA AAA, for VEGFR-2Y1173F GCAG GAT GGC AAA GAC TTT ATT GTC CTT CCA. These mutants were cloned in KpnI and XhoI sites of pCDNA3.1. Porcine aorta ECs cells were grown in Hams-F12 medium
with 10% FCS (BioWhittaker Walkersville, MD) and transfected with VEGFR-2 mutants, VEGFR-2-CFP and YFP- SHP2 by Lipofectamine Plus reagent technique (Invitrogen Ltd, Paisley, UK).

**Cells**

Experiments were performed with human ECs from umbilical cord veins in two different conditions: a) cells grown on gelatin for 3-5 days to allow them to produce a native ECM and then starved for 20 h in M199 containing 1% foetal calf serum (FCS) and 1% human serum albumin; b) cells treated as in a) detached by trypsin-EDTA solution (Sigma) (2 min at 4°C), except when differently indicated, and then plated for 90-120 min on human collagen I or vitronectin (Sigma-Aldrich). Trypsin treatment does not modify expression of VEGFR-2, αv, α2, β1, β2 and β5 integrin subunits, as monitored by FACS analysis. Cells were stimulated with VEGF-A_{165} (0,23 nmol/L) (R&D System Minneapolis, MN). Plastic surface was coated with ECM proteins at 10 µg/ml phosphate-buffered saline (PBS).

**Cell motility**

ECs (150 cells/ mm²) motility plated on collagen I or vitronectin and stimulated in M199 with VEGF-A_{165} (0,23 nmol/L) or vehicle was analyzed by time-lapse videomicroscopy. Cell paths were generated from centroid positions and migration parameters were computed with DIAS software (Solltech Inc., Oakdale, IA, USA). The x and y coordinates of the cell centroids were recorded every 10 min. The reported cell speed for each condition is an average of 100 cells. Directional persistence was calculated by determining the ration between the net path length and the total path length.

**Immunoprecipitation and immunoblotting**

ECs were lysed as previously described with the sole exception of the absence of phosphatase inhibitors. After pre-clearing, samples (1-3 mg of proteins) were immunoprecipitated with the
indicated Abs. Denatured proteins were separated by sodium dodecylsulphate polyacrilamide gel electrophoresis (SDS-PAGE) (8 or 10%) and probed with the indicated Abs or with the SHP2-GST recombinant protein. The enhanced chemiluminescence technique (PerkinElemer/Cetus, Norwalk, CT) was used for detection. Routinely, VEGFR-2 was immunoprecipitated by using an Ab raised against a N-terminal peptide and immunodetected by the same Ab.

Bands were analyzed by densitometric scanning (Bio-Rad, model GS-700) and were normalized for differences in protein loading per lane. Statistical analysis was performed by one-way analysis of variance (ANOVA) and p value was calculated by Bonferroni’s multiple comparison test (GraphPad Prism; GraphPad Prism Software).

**Fluorescence resonance energy transfer analysis**

For fluorescence resonance energy transfer (FRET), PAE cells carrying VEGFR-2-CFP and YFP-SHP2 were observed by a Leitz orthoplan upright microscope (Leitz GMBH, Wetzlar, Germany) equipped with an epi-illumination fluorescence detection system and a temperature-controlled specimen holder at 33°C. Measurements were made in a DMEM containing 2% FCS and 10 mmoles/L HEPES (pH 7.4) exactly as previously described. As an excitation source, a SPEX Fluorolog fluorimeter (SPEX Industries, Edison, NJ) (slit width: 8 nm) with two excitation monochromators was used. Two filter sets (Ploemopak) were used, the "CFP" filter set (filter #1) with an RKP510 dichroic mirror and a 490 nm long-pass emission filter, and the "FRET" filter (filter #2), equipped with a dichroic mirror RKP510 (reflection short-pass filter) and a BP530-560 (band-pass) emission filter (Leitz GMBH, Wetzlar, Germany). Excitation of CFP and YFP was respectively performed at 430±2 and 490±2 nm. The YFP spectra were measured with a dichroic mirror >510 nm. Spectral data were recorded with an integration time of 0.5 s/nm, slit 8 nm. The fluorescence intensity was quantified with a Photon Counting Tube (type 9862, EMI Limited, Middlesex, England). The fluorescence intensities were corrected for differences in excitation light.
intensities, using the reference photomultiplier. Real-time dual excitation measurements were made by alternating excitation at 430 and 490 nm and analysis of YFP emission at 535±5 nm. Fluorescence intensities were recorded from single living cells and corrected for background, using adjacent non-transfected cells (Scion Imaging Software (Scion Corporation)). $E$ is the FRET efficiency in a CFP and YFP labelled proteins, which is dependent on the distance and orientation of the fluorophores. $\alpha$ is the degree of interaction, ranging from 0 (no interaction) to 1 (100% interaction)\(^6\). Cells were analyzed for 30 minutes before the addition of VEGF-A, and then FRET were recovered. All measurements were made with a Leitz 50x NA 1.0 water immersion objective.

**Binding Assay and Analysis**

For specific binding studies ECs were detached by ethylenediamine tetracetate sodium salt (1 mmol/L) in cold PBS and then plated at confluence on collagen I or vitronectin for 2 hours. ECs were incubated in an orbital shaker at 4° C for 2 h in 200µl/well of binding medium [medium 199 containing 20 mmoles/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) buffer, pH 7.4, 14 µmoles/L bovine serum albumin, 4,9 µmoles/L soybean trypsin inhibitor] with increasing concentrations of $^{125}$I-VEGF-A (44TBq/mmol, Amersham-Biotech-Pharmacia, Milano, I) in the presence of a 100-fold excess of unlabeled ligand. Cells were washed three times with ice-cold binding buffer and lysed in 200µl/well of sodium dodecyl sulphate 2% in phosphate buffered saline\(^7\). Triplicate samples under each condition were obtained for each experiment. Specific binding, calculated subtracting from the total cpm bound after incubation with a 100-fold excess of unlabeled ligand, was approximately 80%. The $K_d$ was estimated by Scatchard plot using the Ligand program (Elsevier-Biosoft, Cambridge, UK).

**VEGFR-2 internalization**

Cells were washed twice with PBS and then incubated on ice for 7 min in ice-cold 20 mmol/L sodium acetate, pH 3.7. After washes, cells were incubated with trypsin (47 µmoles/L in PBS) in ice
for 30 min. Soybean trypsin inhibitor (49 µmoles/L) was added to stop the reaction. VEGFR-2 was immunoprecipitated as described below by using a Ab raised against the C-terminal domain and the trypsin resistant 205 kDa protein is the internalized receptor. Alternatively, confluent cells (12-well plate) were equilibrated for 1 hour in binding medium [medium 199 containing 20 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) buffer, pH 7.4, 14 µmoles/L bovine serum albumin, 4.9 µmoles/L soybean trypsin inhibitor], twice washed with the same medium and then incubated at 37°C with 0.1 nmole/L ¹²⁵I-VEGF-A (44TBq/mmol, Amersham-Biotech-Pharmacia, Milano, I) for the indicated times. Cells were put on ice to block further internalization and washed twice with PBS. Subsequently, cells were incubated for 5 min in 0.2 moles/L sodium acetate, pH 4.0, 0.5 moles/L NaCl. Samples were collected and cells were lysed in 1 mole/L NaOH for 10 min. Acid wash samples representing the membrane bound ligand fraction and cell lysates representing the internalized ligand fractions were counted. The ratio of internalized : surface radioactivity was plotted against time.

References


**LEGEND TO SUPPLEMENTAL FIGURES**

**Figure 1S. Specific binding of radiolabeled VEGF-A165 to EC plated on collagen I or vitronectin.**

ECs were detached by ethylenediamine tetracetate sodium salt (1 mmol/L) in cold PBS and then plated at confluence on collagen I (○-----○) or vitronectin (●——●) for 2 hours A, specific ligand binding curve. The binding studies were performed at equilibrium by incubating endothelial cells with 125I-VEGF-A concentrated to 50 pM or higher. This experimental condition excluded the analysis of the binding of VEGF-A165 to VEGFR-1, which has a $K_d$ ranging from 9 to 16 pM$^9,10$. Cells were incubated with indicated concentrations of 125I-VEGF-A for 2 h at 4°C in the presence of a 100-fold excess of cold ligand. B, Scatchard plot of the data reported in A indicates that a $K_d$ of 104 pmoles/L in ECs on collagen I and of 102 pmoles/L on vitronectin. The number of binding sites expressed on cell surface was also similar in both conditions: on collagen I, $B_{max}=63$ fmoles; on vitronectin, $B_{max}=61$. The data shown are representative of three experiments.

**Figure 2S. Densitometric analyses of experiments shown in Figure 2**

Panel A. ECs (2x10$^7$) grown on native ECM were stimulated with VEGF-A165 for the indicated times and cell lysates were immunoprecipitated with anti-SHP2. SHP2 immunocomplexes were incubated for 1 hour a 37°C with phosphorylated VEGFR-2 immunopurified from ECs stimulated...
with VEGF-A_{165} (0.23 nmol/L for 10 min). Denatured proteins were separated by SDS-PAGE and blotted with mAb anti-pY. Mean ± S.D. of 5 experiments. ANOVA gave F=67.01.

Panel B. ECs (2x10^7) grown on native ECM were stimulated with VEGF-A_{165} (0.23 nmol) for the indicated times and cell lysates were immunoprecipitated by Ab anti-SHP2. Denatured proteins were separated by SDS-PAGE and blotted with mAb anti-pY. Mean ± S.D. of 3 experiments. ANOVA gave F=61.08.

Panel C. ECs (10^7) plated for 90 minutes on collagen I or on vitronectin were stimulated with VEGF-A_{165} (0.23 nmol/L for 10 min.) and cell lysates were immunoprecipitated by Ab anti-SHP2. Denatured proteins were separated by SDS-PAGE and blotted with mAb anti-pY. Mean ± S.D. of 3 experiments. ANOVA gave F=105.00.

Figure 3 S. Densitometric analyses of experiments shown in Figure 3

Panel A. ECs (2×10^7) grown on native ECM were stimulated with VEGF-A_{165} and cell lysates were immunoprecipitated by Ab anti-VEGFR-2. Denatured proteins were separated by SDS-PAGE and probed with mAb anti-pY or with GST-SHP2 protein. Mean ± S.D. of 4 experiments. ANOVA performed on data about pY-VEGFR-2 gave F=26.97. §: p<0.05 versus unstimulated cells; *: p<0.05 versus 3 min. VEGF-A_{165} stimulated cells; ^: p<0.05 versus 10 min. VEGF-A_{165} stimulated cells.

ANOVA performed on data about SHP-GST bound to VEGFR-2 gave F=10.42. §: p<0.05 versus unstimulated cells; *: p<0.05 versus 10 min. VEGF-A_{165} stimulated cells.

Panel B. ECs (10^7) plated for 90 minutes on collagen I or on vitronectin, were stimulated with VEGF-A_{165} (0.23 nmol/L). Cells were lysed, immunoprecipitated with anti-VEGFR-2. Denatured proteins were separated by SDS-PAGE and blotted with mAb anti-pY or with anti-SHP2. Mean ± S.D. of 3 experiments.

ANOVA performed on data about pY-VEGFR-2 gave F=173.60. §: p<0.05 versus unstimulated cells plated on collagen I or vitronectin; *: p<0.05 versus cells plated on collagen I and stimulated
for 5 min with VEGF-A_{165}; ^ p<0.05 versus cells plated on collagen I and stimulated for 15 min with VEGF-A_{165}.

ANOVA performed on data about SHP2-bound to VEGFR-2 gave F=105.1.

§: p<0.05 versus unstimulated cells plated on collagen I or vitronectin; *: p<0.05 versus cells plated on collagen I and stimulated for 5 min with VEGF-A_{165}; ^ p<0.05 versus cells plated on collagen I and stimulated for 15 min with VEGF-A_{165}; + p<0.05 versus cells plated on collagen I and stimulated for 30 min with VEGF-A_{165}.

**Figure 4S. Effect of retroviral infection on VEGFR-2 expression.**

Basal expression of VEGFR-2 in ECs overexpressing vector alone or SHP2_{C459S} was detected by immunoprecipitation and immunoblotting with Ab - anti VEGFR-2. The data shown are representative of 5 experiments.

**Figure 5S. Densitometric analyses of experiments shown in Figure 6**

Panel A. ECs (2x10^7) overexpressing SHP2_{C459S} were plated on collagen I and stimulated for the indicated times with VEGF-A_{165} (0.23 nmol/L). Cells were lysed, immunoprecipitated with anti-dynamin II. Denatured proteins were separated by SDS-PAGE and blotted with mAb anti-PY. Mean ± S.D. of 7 experiments. ANOVA gave F=87.89. §: p<0.05 versus unstimulated cells; *: p<0.05 versus EC-PINCO stimulated for 10 min with VEGF-A_{165}; ^ p<0.05 versus EC-PINCO stimulated for 15 min with VEGF-A_{165}.

Panel B. ECs (2x10^7) overexpressing SHP2_{C459S} were plated on collagen I and stimulated for the indicated times with VEGF-A_{165} (0.23 nmol/L). Cells were lysed and denatured proteins separated by SDS-PAGE were blotted with mAb anti-pY416-Src. Mean ± S.D. of 4 experiments. ANOVA gave F=57.60. §: p<0.05 versus unstimulated cells; *: p<0.05 versus EC-PINCO stimulated for 10 min with VEGF-A_{165}; ^ p<0.05 versus EC-PINCO stimulated for 15 min with VEGF-A_{165}.
Figure 1S; Mitola et al
A. Densitometric analysis of phosphorylated VEGFR-2 incubated with immunocomplexes anti-SHP2 (see Figure 2B)

% decrease of VEGFR-2 phosphorylation

Minutes

0 5 10 20

B. Densitometric analysis of phosphorylated SHP2 immunoprecipitated from ECs plated on native ECM (see Figure 2C)

% increase of SHP2 phosphorylation

0 5 10 20

C. Densitometric analysis of phosphorylated SHP2 immunoprecipitated from ECs plated on collagen I or vitronectin (see Figure 2E)

% increase of SHP2 phosphorylation

Vitronectin Collagen I

- - + - +

VEGF-A_{165}
A
Densitometric analysis of phosphorylated VEGFR-2 and of the amount of SHP2-GST bound to VEGFR-2 in ECs plated on native ECM (see Figure 3B)

Minutes

% Increase

0 3 10 20 45

pY-VEGFR-2
SHP2-GST bound to VEGFR-2

B
Densitometric analysis of phosphorylated VEGFR-2 and of the amount of SHP2-GST bound to VEGFR-2 in ECs plated on collagen I or vitronectin (see Figure 3E)

Collagen I
Vitronectin

% Increase

0 5 15 30

pY-VEGFR-2
SHP2-GST bound to VEGFR-2

Figure 3S
Densitomeric analysis of phosphorylated dynamin II in EC-PINCO and EC-SHP2
C459S plated on collagen I (see Figure 6C).

[Bar graph showing % increased of dynamin II phosphorylation over time for EC-PINCO and EC-SHP2 C459S.]

Densitomeric analysis of pY416-Src in EC-PINCO and EC-SHP2 C459S plated on collagen I (see Figure 6D).

[Bar graph showing % increase of pY416-Src over time for EC-PINCO and EC-SHP2 C459S.]

Figure 5S