Local Retention Versus Systemic Release of Soluble VEGF Receptor-1 Are Mediated by Heparin-Binding and Regulated by Heparanase

Shay Sela, Shira Natanson-Yaron, Eyal Zcharia, Israel Vlodavsky, Simcha Yagel, Eli Keshet

Rationale: The vascular endothelial growth factor (VEGF) decoy receptor soluble VEGF-R1 (sVEGF-R1) is thought to protect the cells that produce it from adverse VEGF signaling. To accomplish this role, a mechanism for pericellular retention of sVEGF-R1 is required. Local retention may also prevent the accumulation of high circulating levels of sVEGF-R1 and resulting interference with homeostatic VEGF functions in remote organs.

Objective: To reveal natural storage depots of sVEGF-R1 and determine mechanisms underlying its pericellular retention. To uncover natural mechanisms regulating its systemic release.

Methods and Results: We show that both the canonical and human-specific isoforms of sVEGF-R1 are strongly bound to heparin. sVEGF-R1 produced by vascular smooth muscle cells is stored in the vessel wall and can be displaced from isolated mouse aorta by heparin. Another major reservoir of sVEGF-R1 is the placenta. Heparin increases the level of sVEGF-R1 released by cultured human placental villi, and pregnant women treated with low molecular weight heparin showed markedly elevated levels of sVEGF-R1 in the circulation. Heparanase is expressed in human placenta at the same locales as sVEGF-R1, and its transgenic overexpression in mice resulted in a marked increase in the levels of circulating sVEGF-R1. Conversely, heparanase inhibition, by either a neutralizing antibody or by inhibition of its maturation, reduced the amounts of sVEGF-R1 released from human placental villi, indicating a natural role of heparanase in sVEGF-R1 release.

Conclusions: Together, the findings uncover a new level of regulation governing sVEGF-R1 retention versus release and suggest that manipulations of the heparin/heparanase system could be harnessed for reducing unwarranted release of sVEGF-R1 in pathologies such as preeclampsia. (Circ Res. 2011;108:1063-1070.)

Key Words: soluble VEGF-R1 ■ heparan sulfate ■ vascular smooth muscle cell ■ heparanase ■ preeclampsia
sVEGF-R1 by showing that heparanase indeed plays a natural role in the above processes.9 Here, we extended this biological principle to the HS-bound decoy receptor and actively subjected to regulation. An attractive candidate for such regulation is heparanase, an endo-

We further reasoned that to exert dynamic regulation, binding of sVEGF-R1 to HS is not rigid but rather flexible and actively subjected to regulation. An attractive candidate for such regulation is heparanase, an endo-β-d-glucuronidase known to be implicated in diverse processes such as angiogenesis, metastasis and inflammation. It has been shown that heparanase-mediated release of HS-bound growth factors plays a major role in the above processes.9 Here, we extended this biological principle to the HS-bound decoy receptor sVEGF-R1 by showing that heparanase indeed plays a natural role in liberating sVEGF-R1 from its placental reservoir.

Methods

Recombinant Expression of sVEGF-R1 Isoforms
cDNAs encompassing the entire coding region of both soluble receptor isoforms (GenBank sequences EU368830.1 and U01134.1) were subcloned into Bluescript expression vectors and transfected onto T7 polymerase-expressing Hela cells. Twenty-four hours later, media containing the secreted proteins were collected. Protein concentration was determined with sVEGF-R1 ELISA.

Displacement From Heparin Sepharose Columns
Heparin sepharose (GE Healthcare, CL-6B) was packed on chromatography columns according to manufacturer instructions. A 1-μg sample of each sVEGF-R1 isoform was loaded and bound to the columns. Proteins were displaced from the columns using increasing NaCl concentrations. Fractions were collected and evaluated for sVEGF-R1 amount by Western blotting as described below.

sVEGF-R1, Heparanase, and Cathepsin L Western Blotting
Human recombinant proteins that were displaced from heparin sepharose columns were run on a separating gel and blotted with the ab9540 antibody (Abcam). Mouse endogenous sVEGF-R1 was immuno-precipitated using the V4262 antibody (Sigma), and immuno blotted using the sc-9029 antibody (Santa-Cruz biotechnology). C2970 antibody (Sigma) was used for human cathepsin L blotting. Human heparanase was detected using the monoclonal antibody 01385-126 (kindly provided by ImClone Systems Inc., New York, NY), recognizing both the 50-kDa subunit and the 65-kDa proheparanase.10

sVEGF-R1 In Situ Hybridization
For in situ detection of mouse sVEGF-R1 RNA, paraffin-embedded sections of mouse heart were hybridized with 35S-labeled riboprobe complementing the first 600 nucleotides of intron 13 of VEGF-R1, as previously described.11

sVEGF-R1 and Active Heparanase Immunohistochemistry
Paraffin-embedded sections of mouse heart, or human term placenta were used. Antigen retrieval was performed by microwave heating in pH 6 citrate buffer. The 36-1100 antibody (Zymed) was used at a 1:100 dilution to detect sFlt1. The 733 antibody was used at a 1:100 dilution to detect the active form of heparanase.12

Heparin Displacement of sVEGF-R1 From Aortic Arches
Mouse aortic arches were isolated, cleaned from adventitia, cut into cylinder segments, and incubated for 24 hours with or without 10 U/mL heparin (Sigma, H0777). Medium was collected and evaluated for sVEGF-R1 levels with sVEGF-R1 ELISA.

Term Human Placental Explants Culture
Term human placentas were excised to small sections, and incubated according to guidelines detailed elsewhere.13 In short, 75 mg of placental villi were placed in each well, and incubated in DMEM/F12 medium supplemented with 10% FCS, in 8% oxygen. Incubation time varied according to the different protocols used in each experimental setting.

Heparanase-Overexpressing Transgenic Mice
The generation and characterization of these mice are described elsewhere.14

Real-Time PCR
RNA and cDNA were generated from embryonic day (E)16.5 placentas of Wt and heparanase Tg mice. sVEGF-R1 levels were evaluated using SYBR green real time PCR, and normalized according to β-actin levels. The primers used were as follows: sVEGF-R1 forward primer: GGCCCAAGAGGAAAAGCCTTCT; sVEGF-R1 reverse primer: AATCCCAAACACACACCGGC; β-actin forward primer: AC-CGGCAGCAGCAGCAGTCT; β-actin reverse primer: GAACCTGGTGCCGGGTTGGA.

Heparanase Inhibition in Human Placental Villi Explants
Heparanase processing and thereby enzymatic activity was indirectly inhibited via a 72-hour incubation with 8 μmol/L of the specific non-standard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>E</td>
<td>embryonic day</td>
</tr>
<tr>
<td>GBM</td>
<td>glomerular basement membrane</td>
</tr>
<tr>
<td>HS</td>
<td>heparan Sulfate</td>
</tr>
<tr>
<td>HSPG</td>
<td>heparan sulfate proteoglycan</td>
</tr>
<tr>
<td>LMWH</td>
<td>low-molecular-weight heparin</td>
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<tr>
<td>PE</td>
<td>preeclampsia</td>
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<tr>
<td>sFlt1</td>
<td>generic soluble VEGF receptor 1 isoform</td>
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<tr>
<td>sFlt1-14</td>
<td>human-specific soluble VEGF receptor 1 isoform</td>
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<tr>
<td>sVEGF-R1</td>
<td>soluble VEGF receptor 1</td>
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<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
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<td>VEGFR</td>
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cathespin L inhibitor (Calbiochem, catalog no. 219427). Heparanase was directly inhibited via a 48-hour incubation with the neutralizing antibody 733 added to the medium at concentration of 10 μg/mL. Heparanse inhibition required a longer incubation time than the 24 hours used for the heparin displacement experiment to elicit an inhibition effect: Heparanse neutralization was evaluated after 48 hours, whereas cathespin L inhibitor required 72 hours of incubation, as active heparanse can be found in the villi, and longer incubation time allows diminishing its effect.

Enzyme-Linked Immunosorbent Assay
Human sVEGF-R1 levels were determined using the DVR100b sVEGF-R1 ELISA kit (R&D Systems). Mouse sVEGF-R1 levels were determined using the MVR100 sVEGF-R1 ELISA kit (R&D Systems).

Serum and Placentas From Pregnant Women
Serum samples and term placentas were taken with Institutional Review Board approval with the informed consent of pregnant women.

Statistics
Data were analyzed by 2-sample t test. Standard error of the mean (SEM) was calculated as sample standard deviation divided by the square root of the sample size.

Results
sVEGF-R1 Is a Heparin-Binding Protein
To test the hypothesis that sVEGF-R1 might be retained in the vicinity of the producing cell through binding to HS, we first examined its ability to bind heparin. Because 2 distinct splice variants of sVEGF-R1 are known to exist, namely the “generic” soluble receptor (sFlt1) and the recently identified human-specific variant (sFlt1-14), we wished to determine whether the previously reported heparin-binding capability of the generic isoform also applies to sFlt1-14. To this end, the respective recombinant proteins were produced in a cell culture, loaded on heparin sepharose columns and subsequently displaced by increasing NaCl concentrations. Relative amounts of displaced sFlt1-14 and sFlt1 were determined by Western blotting using an antibody (ab9540) recognizing a shared epitope.

To determine whether this sVEGF-R1 store is displaceable by heparin, the aortic segment was excised and incubated ex vivo for 24 hours in the presence or absence of heparin. sVEGF-R1 ELISA was used to determine the amount of sVEGF-R1 released to the conditioned medium. As shown in Figure 2C, heparin treatment resulted in a 6-fold increase in the amount of sVEGF-R1 secreted to the culture medium.

Natural Stores of sVEGF-R1 in the Human Placenta Are Displaceable by Heparin
Reasoning that there are additional physiological arenas where sVEGF-R1 is retained in the tissue, we turned to the human placenta. It is known that the human placenta is a rich source of sVEGF-R1 secreted to the circulation during normal pregnancy, which is even greatly increased in PE. Yet, the notion that the placenta is also a HS-bound depot of sVEGF-R1 has not been considered.

To test this, human placental villi explants were incubated for 24 hours with increasing concentrations of heparin and the amounts of sVEGF-R1 released to the medium (Figure 3A) or remaining in the tissue (Figure 3B) were determined. As shown, heparin treatment led to release of sVEGF-R1 to the medium in a dose-dependent manner and was mirror-imaged by a parallel decrease in the protein remaining in the villi. The apparent increase in the amount of sVEGF-R1 released to the medium was not merely a reflection of increased sVEGF-R1 production, as the total amount of sVEGF-R1 was not increased in heparin-treated explants and the only difference was in its relative distribution between the tissue and medium.
To extend this finding to an in vivo setting, we resorted to analysis of serum samples obtained from pregnant women treated with the low molecular weight heparin (LMWH) clexane, which is routinely used for prophylaxis of coagulation abnormalities during pregnancy. As shown in Figure 3C, clexane increased the level of sVEGF-R1 released to the circulation by 4-fold. Together, these results established that the level of sVEGF-R1 detectable in the circulation represent only a small fraction of the total amount of bodily sVEGF-R1, the majority of which is stored in tissue reservoirs.

Heparanase Is a Potential Regulator of sVEGF-R1 Release

The presence of sVEGF-R1 tissue stores, on one hand, and its presence in the circulation, on the other hand, argues for an active mechanism governing the retention versus release of this natural VEGF inhibitor. Reasoning that heparanase might play such a regulatory role by virtue of releasing HS-bound sVEGF-R1, we first determined whether heparanase is indeed produced in the relevant tissue environment. To this end, we analyzed term human placentas for heparanase production, as the placenta is known to be the major source of circulating sVEGF-R1 in pregnancy.

Figure 2. sVEGF-R1 stored in the vessel wall and its displacement by heparin. A, Left, In situ mRNA hybridization of an aortic section (outflow tract region) with a sVEGF-R1–specific riboprobe (see Methods). Upper and lower images, Dark field and bright field images, respectively, of serial sections. The section shown in the lower image was immunostained with αSMA to highlight VSMCs, the apparent source of sVEGF-R1 in the vessel wall. Right, Visualizing sVEGF-R1 in coronary vessels by immunostaining with an antibody directed against the 31 unique carboxy-terminal 31 aa of sFlt1. Note (in the inset), a positive signal in VSMCs and a negative signal in endothelial cells. B, A Western blot of aortic arch–extracted proteins with an antibody detecting both VEGF-R1 and sVEGF-R1. Note exclusive expression of sVEGF-R1 but not of the signaling transmembrane receptor. Both VEGF-R1 and sVEGF-R1 are detected in kidney tissue, applied as control. C, Mouse aortic arches were isolated and incubated for 24 hours with or without 10 U/mL of heparin. Data are presented as means±SEM n=3 for each group; *P<0.05.

Figure 3. sVEGF-R1 stored in the placenta and its displacement by heparin. A and B, Human term placental villi were explanted and grown in culture for 24 hours in the absence or presence of different concentrations of heparin. Levels of sVEGF-R1 released to the medium (A) and remaining in the tissue (B) were determined by ELISA. Data are presented as means±SEM (n=3 for each group); *P<0.01, **P<0.05. C, Serum levels of sVEGF-R1 from untreated and clexane-treated pregnant women were determined as above. Serum samples were obtained at a mean gestational age of 35.8 weeks for the clexane-treated group and 37.9 weeks for the normal pregnancy group. Data are presented as means±SEM n=5 for each group; *P<0.004.
Western blot analysis revealed that both the precursor inactive protein, as well as the proteolytically processed active enzyme, are readily detected in the placenta (Figure 4A). Correspondingly, cathepsin L, the protease responsible for processing of the 65 kDa precursor protein,10 was also detectable in the placenta (Figure 4A).

To determine localization of active heparanase within the placenta, in situ immuno-histochemical analysis with an antibody that recognizes primarily active heparanase was performed. As shown in Figure 4B, heparanase was predominantly detected in syncytiotrophoblasts and in placental syncytial knots. Remarkably, these locales completely coincide with the sites of sVEGF-R1 production,15 consistent with the proposition that heparanase may function in sVEGF-R1 release.

Transgenic Heparanase Overexpression Increases the Level of Circulating sVEGF-R1
To examine whether heparanase is capable of releasing sVEGF-R1 into the systemic circulation, transgenic mice in which heparanase expression is controlled by a ubiquitous promoter driving its overexpression in all tissues14 was used. Higher levels of sVEGF-R1 were detected in the serum of heparanase-overexpressing mice in comparison to littermate controls (230 pg/mL and 140 pg/mL, respectively) (Figure 5A). This significant 65% increase likely reflects sVEGF-R1 release from different tissue depots.

A similar analysis was also performed in pregnant mice where circulating sVEGF-R1 can reach levels >180-fold higher than in nonpregnant mice. Control mice showed a gestational stage-dependent increase in circulating sVEGF-R1 reaching 25 ng/mL on average at E16.5. Remarkably, heparanase overexpression resulted in a significant increase in the levels of circulating sVEGF-R1 at all stages and was 3.5-fold higher by E16.5 (Figure 5B). This result could not be explained by a difference in embryo number, which was comparable to controls (data not shown). Note-worthy, at this time point, the level of placental sVEGF-R1 expression was indistinguishable between control and heparanase-overexpressing transgenic mice, indicating that heparanase indeed act to release sVEGF-R1 from its placental stores (Online Figure II).

These results corroborate the notion that only a minor fraction of placentally produced sVEGF-R1 is reaching the circulation and further suggest that heparanase might play a role in sVEGF-R1 release.

Heparanase Inhibition Decreases sVEGF-R1 Release From the Placenta
To determine whether heparanase plays a natural role in regulated release of placental sVEGF-R1, we used 2 independent approaches for inhibiting endogenous heparanase: pre-
venting processing and activation of the proenzyme via cathepsin L inhibition and direct inhibition of heparanase through the use of a neutralizing antibody. Inhibition of cathepsin L activity in human placental villi explants resulted in a 35% reduction in sVEGF-R1 secreted to the medium (Figure 6A). Likewise, heparanase neutralizing antibody reduced the level of sVEGF-R1 secreted to the culture medium by 32% (Figure 6B). This was associated with a parallel similar increase in sVEGF-R1 retained in the villi (data not shown). These results indicate that heparanase is a natural regulator of sVEGF-R1 release from the placenta.

Discussion

Here, we showed that the majority of sVEGF-R1 is retained in the vicinity of the producing cell in spite of it being a secreted protein. Further, we showed that pericellular retention of this natural VEGF decoy receptor is mediated through its high affinity binding to HS and that its release is controlled at least in part by heparanase.

Existence of tissue depots of sVEGF-R1 provide a basis for the notion that the primary physiological role of sVEGF-R1 is to sequester VEGF locally thereby granting the producing cell protection against unwarranted VEGF signaling. Thus, it can be argued that the abundant presence of sVEGF-R1 in the vessel wall shown here has a functional role in protecting VSMCs from adverse VEGF signaling. Consistent with this proposition is our previous observation that the mRNA transcribed from the VEGF-R1 locus in VSMCs is spliced in a mode yielding exclusively sVEGF-R1 (but not the signaling transmembrane receptor) and results shown here that only sVEGF-R1 protein is produced and deposited in the VSMC layer (Figure 2). Because VEGF is known to cause vasodilatation, it could be speculated that its local titration keeps VEGF-induced vasodilatation in check.

Local retention of sVEGF-R1, thereby preventing its systemic release, might also be required to avoid indiscriminate neutralization of VEGF in remote organs. This may include interference with homeostatic function of VEGF in the kidney and in additional organs where constitutive VEGF signaling is needed to maintain endothelial fenestrations. Indeed, it was shown that excessive systemic release of placenta-produced sVEGF-R1 may cause PE through interfering with normal VEGF kidney function. It may be generalized that high levels of circulating sVEGF-R1 detectable in pathological settings likely reflect a failure to retain it locally (see below). It is important to keep in mind that in the human, 2 isoforms of the decoy receptor coexist, namely the generic sFlt1 and the human-specific sFlt1-14, with the latter predominating in normal and PE pregnancies.

Local retention of sVEGF-R1 is mediated by cell-bound or ECM deposited HS. This was shown in 2 independent ways: First, by showing sVEGF-R1 displacement and release by heparin and LMWH and second, by demonstrating increased levels of sVEGF-R1 in the circulation of transgenic mice overexpressing heparanase. These experiments allow for the evaluation of the relative amounts of retained versus released sVEGF-R1 in respective tissues. For example, the finding that the amount of sVEGF-R1 displaceable from the vessel wall of the aortic arch by heparin largely exceeds the normally secreted amount by 6-fold (Figure 2C) indicates that the majority of the decoy receptor is stored rather than released. Interestingly, levels of sVEGF-R1 detectable in the vessel wall were found to vary depending on the anatomic location of the respective arterial segment (data not shown). Likewise, the finding that heparanase overexpression in pregnant mice results in 3 to 4-fold increase of circulating sVEGF-R1 indicates that only a minor fraction of sVEGF-R1 produced by the placenta, apparently the richest storage depot of sVEGF-R1, finds its way to the circulation. Because circulating sVEGF-R1 has been causally implicated in the development of PE, existence of a large placental reservoir of this potentially harmful agent may greatly impact the pathogenic process. In this regard, mechanisms accounting for balancing retained versus secreted sVEGF-R1 are of great importance.

It is likely that increased levels of circulating sVEGF-R1 in PE reflects a limited retention capacity by placental HS which is overwhelmed by excessive production of sVEGF-R1. Several parameters may determine the retention capacity of secreted sVEGF-R1. First is the repertoire of placental HSPGs and their levels, which may vary. For example, placental expression of the key HSPG syndecan 1 was shown
to diminish in preeclampsia and thereby possibly reduce overall retention capacity. Second, heparanase activity is responsible for release of HSPG-bound proteins in general, but might also participate in sVEGF-R1 release. Indeed, heparanase is expressed in the same locales shown previously to produce sVEGF-R1, and has a natural role in regulating sVEGF-R1 release, as demonstrated in loss-of-function experiments described above (Fig 6). The observed 32% to 35% decrease in sVEGF-R1 secretion is likely an underestimation considering that the 2 reagents used, ie, heparanase neutralizing antibody and a cathepsin-L inhibitor, do not fully block heparanase activity (not shown). The fraction of sVEGF-R1 released by heparanase in the normal and PE placenta is yet to be determined. Heparanase is constitutively expressed in the placenta in small fetal vessels and in a variety of trophoblast subpopulations with different potentials for invasion. Interestingly, heparanase expression was shown to be progressively up regulated during pregnancy, which grossly correlates with the progressive accumulation of sVEGF-R1 in the circulation. Yet, a comparison of heparanase levels between normal and PE placenta did not reveal additional increase in PE (data not shown). Regardless of the mechanisms accounting for sVEGF-R1 retention, the fact that only circulating sVEGF-R1 is pathogenic, suggests that inhibition of its systemic release (eg, via inhibition of heparanase activity) could be harnessed therapeutically.

Neutralization of VEGF function in the kidney glomerular basement membrane (GBM) is known to be the primary cause of PE-associated renal damage. Specifically, it has been shown that podocyte-derived VEGF acts on VEGF receptors displayed on nearby endothelial cells. Thus, sVEGF-R1 entrapped in the HS-rich GBM is bound to interfere with this homeostatic VEGF function. Accordingly, inhibition of sVEGF-R1 binding to HS (eg, with LMWH) in the target organ may also be beneficial. Consistent with this proposition, a recent report showing that the low molecular weight heparin dalteparin is effective in decreasing the recurrence of placental-mediated complications in women without thrombophilia. Noteworthy, HSPG-bound proteins released by heparanase are still bound to a sugar moiety of an average size of 10 to 20 sugar units, which may alter its biological activity. Thus, sVEGF-R1 released by heparanase may also have different affinity for GBM binding than sVEGF-R1 released by another mode.

In conclusion, this study uncovered a previously unrecognized level of VEGF regulation, namely, bioavailability of its decoy receptor determined by its local storage versus its systemic release. The first situation, we argue, is intended for keeping in check local VEGF action, whereas the second situation is largely associated with pathological VEGF neutralization. Further mechanistic insights on factors governing retention versus release of sVEGF-R1 might be harnessed for ameliorating the pathological consequences of excessive circulating sVEGF-R1 acting on remote organs.

Sources of Funding

This work was supported by grants from the Israel Science Foundation (grant 593/10) and the National Cancer Institute, NIH (grant CA106456) to I.V.

Disclosures

None.

References

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Novelty and Significance

What Is Known?

- The soluble vascular endothelial growth factor (VEGF) receptor 1 (sVEGF-R1) is a heparin-binding, naturally secreted decoy receptor functioning as a VEGF trap.
- sVEGF-R1 could accumulate in the circulation and interfere with normal VEGF functioning in remote organs, notably with kidney function in the context of preeclampsia (PE).

What New Information Does This Article Contribute?

- There are large tissue stores of extracellular sVEGF-R1, primarily in the vessel wall and in placenta that are displaceable by heparin and heparin mimetics.
- Heparanase, a heparan-sulfate endoglycosidase, plays a natural regulatory role governing the retention vs. the systemic release of sVEGF-R1.

The sVEGF-R1 is thought to play a protective role against adverse VEGF signaling, which requires a mechanism for its pericellular retention. Extending previous studies showing that sVEGF-R1 binds heparin, we show that sVEGF-R1 is efficiently retained in the vicinity of its producer cells in multiple tissues via binding to heparan sulfates. sVEGF-R1 could, however, also accumulate in the circulation under pathophysiological conditions like PE, raising the possibility of an active mechanism regulating its systemic release. Here, we show that heparanase releases sVEGF-R1 from its cellular stores. Using a combination of transgenic heparanase manipulations with different modes of heparanase inhibition, we show that heparanase is coexpressed with sVEGF-R1 and that it plays a natural role in determining the biodistribution of sVEGF-R1. These findings suggest that heparanase inhibitors might be therapeutically useful for reducing pathogenic levels of circulating sVEGF-R1 and for normalizing essential homeostatic VEGF functions in multiple organs.
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*Circ Res.* 2011;108:1063-1070; originally published online March 17, 2011; doi: 10.1161/CIRCRESAHA.110.239665
*Circulation Research* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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Expanded Methods:

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**sVEGF-R1 in situ hybridization** - For in situ detection of mouse sVEGF-R1 RNA, paraffin-embedded sections of mouse heart were hybridized with 35S-labeled riboprobe complementing the first 600 nucleotides of VEGF-R1's intron 13, as previously described. In brief, 10-gm-thick frozen sections were collected on poly(L-lysine)-coated glass slides, refixed in 4% paraformaldehyde, and dehydrated in graded ethanol solutions. Before hybridization, sections were pretreated successively with 0.2 M HCl, 2x SSC (Ix SSC is 0.15 M NaCl/0.015 M sodium citrate), Pronase (0.125 mg/ml), 4% paraformaldehyde, and acetic anhydride in triethanolamine buffer. Hybridization was carried out at 50°C overnight in 50% (vol/vol) formamide/0.3 M NaCl containing 10% (wt/vol) dextran sulfate, Ix Denhardt's solution (0.02% Ficoll/0.02% polyvinylpirrrolidone/0.02% bovine serum albumin), carrier tRNA (1 mg/ml), 10 mM dithiothreitol, 5 mM EDTA, and 35S-labeled RNA probe (2 x 108 cpm/ml). Washing was performed under stringent conditions that included an incubation at 50°C for >14 hr in 50% formamide/0.3 M NaCl and a 30-min incubation at 37°C with RNase A (20 ug/ml). Autoradiography was performed using Kodak NTB-2 nuclear track emulsion and autoradiographic exposure was for 4-5 days. Control hybridizations with "sense" RNA probes were carried out in all experiments.

**sVEGF-R1 and active heparanase immunohistochemistry** - Paraffin-embedded sections of mouse heart, or human term placenta were used. Antigen retrieval was performed by microwave heating in citrate buffer (10mM Citric Acid, 0.05% Tween 20, pH 6.0). The 36-1100 antibody (Zymed) was used at a 1:100 dilution to detect sFlt1. The 733 antibody was used at a 1:100 dilution to detect the active form of heparanase.

**Heparin displacement of sVEGF-R1 from aortic arches** – Mouse aortic arches were isolated, cleaned from adventitia, cut into cylinder segments and incubated for 24 hours with or without 10 units/ml heparin (Sigma, H0777). Medium was collected and evaluated for sVEGF-R1 levels with sVEGF-R1 ELISA.

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each well, and incubated in DMEM/F12 medium supplemented with 10% FCS, in 8% oxygen. Incubation time varied according to the different protocols used in each experimental setting.

**Heparin displacement of sVEGF-R1 from human placental villi explants** – Human term placental villi were explanted for 24 hours with different heparin concentrations. sVEGF-R1 levels were determined in media and explanted placental villi using ELISA. Protein concentration of each villi sample was determined using the Bradford assay. Similar protein amount of each villi sample was used to determine sVEGF-R1 concentration.

**Heparanase over-expressing transgenic mice** – The generation and characterization of these mice are described elsewhere.

**Real Time PCR** - RNA and cDNA were generated from E16.5 placentas of Wt and heparanase Tg mice. sVEGF-R1 levels were evaluated using SYBR green real time PCR, and normalized according to beta actin levels.

sVEGF-R1 F primer: GGCCCAGAGGAACGCTCCTC; sVEGF-R1 R primer: AATCCAAACACACACACGGCCG. Beta actin F primer: ACCCGCGAGCACAGCTTCTT; Beta actin R primer: GAACTGGTGCGGTGTGGA.

**Heparanase inhibition in human placental villi explants** - Heparanase processing and thereby enzymatic activity was indirectly inhibited via a 72-hour incubation with 8 µmol/L of the specific cathepsin L inhibitor (Calbiochem, cat. 219427). Heparanase was directly inhibited via a 48-hour incubation with the neutralizing antibody 733 added to the medium at concentration of 10µg/ml. Heparanase inhibition required a longer incubation time than the 24 hours used for the heparin displacement experiment in order to elicit an inhibition effect. Heparanase neutralization was evaluated after 48 hours, whereas cathepsin L inhibitor required 72 hours of incubation, as active heparanase can be found in the villi, and longer incubation time allows diminishing its effect.

**ELISA** - Human sVEGF-R1 levels were determined using the DVR100b sVEGF-R1 ELISA kit (R&D systems). Mouse sVEGF-R1 levels were determined using the MVR100 sVEGF-R1 ELISA kit (R&D systems).

**Serum and placentas from pregnant women** - Serum samples and term placentas were taken with Institutional Review Board approval with the informed consent of pregnant women.

**Statistics** - Data was analyzed by two sample t-test. Standard error of the mean (SEM) was calculated as sample standard deviation divided by the square root of the sample size.

**References:**


Online Figure I: Heparin changes the ratio of secreted vs. retained sVEGF-R1 in placental villi explants.

The total amounts of sVEGF-R1 detected in the medium (Secreted sVEGF-R1) and villi (Bound sVEGF-R1) in the heparin displacement experiments (see Fig.3 for details) are shown. Note that a dose-dependent increase in released sVEGF-R1 is not accompanied by a statistically significant increase in overall sVEGF-R1 production. Data are presented as mean±SEM. n=3 for each group.
Online Figure II: sVEGF-R1 mRNA expression in placentas of wild type (Wt) and heparanase over-expressing transgenic mice (Tg). Real time PCR was used to evaluate the relative expression levels of sVEGF-R1 mRNA in E16.5 placentas of Wt and Tg mice (normalized to β-actin levels). Note no significant difference in sVEGF-R1 levels. Data are presented as mean±SEM. n=5 and n=6 for Wt and Tg group, respectively.