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VEGF (Vascular Endothelial Growth Factor) Functionalized Magnetic Beads in a Microfluidic Device to Improve the Angiogenic Balance in Preeclampsia

Laura Trapiella-Alfonso,* Lucile Alexandre,* Camille Fraichard, Kelly Pons, Simon Dumas, Lucie Huart, Jean-François Gaucher, Marylise Hebert-Schuster, Jean Guibourdenche, Thierry Fournier, Michel Vidal, Isabelle Broutin, Laurence Lecomte-Raclet, Laurent Malaquin, Stéphanie Descroix, Vassilis Tsatsaris, Nathalie Gagey-Eilstein,† Edouard Lecarpentier†

Abstract—Preeclampsia is a hypertensive pregnancy disease associated with a massive increase in sFlt-1 (soluble form of the vascular endothelial growth factor 1) in the maternal circulation, responsible for angiogenic imbalance and endothelial dysfunction. Pilot studies suggest that extracorporeal apheresis may reduce circulating sFlt-1 and prolong pregnancy. Nonspecific apheresis systems have potential adverse effects because of the capture of many other molecules. Our concept is based on a specific and competitive apheresis approach using VEGF (vascular endothelial growth factor) functionalized magnetic beads to capture sFlt-1 while releasing endogenous PlGF (placental growth factor) to restore a physiological angiogenic balance. Magnetic beads were functionalized with VEGF to capture sFlt-1. Experiments were performed using PBS, conditioned media from human trophoblastic cells, and human plasma. The proof of concept was validated in dynamic conditions in a microfluidic device as an approach mimicking real apheresis. Magnetic beads were functionalized with VEGF and characterized to evaluate their surface ligand density and recognition capabilities. VEGF-coated magnetic beads proved to be an efficient support in capturing sFlt-1 and releasing PlGF. In static conditions, sFlt-1 concentration decreased by 33±13%, whereas PlGF concentration increased by 27±10%. In dynamic conditions, the performances were improved, with 40% reduction of sFlt-1 and up to 2-fold increase of free PlGF. The sFlt-1/PlGF ratio was reduced by 63% in the plasma of preeclamptic patients. Apheresis was also associated with VEGF release. A ligand-based approach using VEGF-coated beads is an effective approach to the capture of sFlt-1 and the release of endogenous PlGF. It offers new perspectives for the treatment of preeclampsia.

Key Words: endothelium ■ morbidity ■ preeclampsia ■ placenta ■ pregnancy

Preeclampsia is a hypertensive disorder of pregnancy associated with substantive maternal and perinatal mortality and morbidity.[1] There is currently no curative treatment for preeclampsia, and only childbirth and delivery of the placenta alleviate maternal symptoms. The development of therapeutic strategies for preeclampsia is one of the highest priorities in perinatal medicine.

During preeclampsia, a massive amount of a sVEGFR1 (soluble form of the vascular endothelial growth factor receptor 1), also named sFlt-1, is released by the placenta into the maternal circulation, resulting in inhibition of the proangiogenic effects of its ligands, VEGF (vascular endothelial growth factor)-A, and PlGF (placental growth factor) on the maternal endothelium.[2] These angiogenic factors are essential for the survival of endothelium, thus explaining the endothelial dysfunction during preeclampsia.[3,4]

On the basis of compelling evidence that circulating sFlt-1 is a critical and potentially rate-limiting step in the pathobiology of preeclampsia, it is supposed that by reducing its circulating concentrations with an extracorporeal device the disease

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progression could be limited. Based on the positive charge of sFlt-1, dextran sulfate columns have been used to remove excess circulating maternal sFlt-1 during preeclampsia.[5] Thadhani et al[6] have shown that this nonspecific method of apheresis for women with preterm preeclampsia can reduce sFlt-1 concentrations by 18%. A proof-of-concept trial on selective removal of sFlt-1 in healthy volunteers and preeclamptic women using an sFlt-1 antibody-specific adsorption column is ongoing to test the safety and efficacy of this procedure.[7]

The objective of this study was to develop and provide the proof of concept of a selective and competitive apheresis approach to reduce circulating sFlt-1 while increasing free PlGF to restore the physiological angiogenic balance in the maternal circulation. To shift the equilibrium of sFlt-1/PlGF binding, we grafted magnetic beads with ligands of sFlt-1 which competes with PlGF. To increase sFlt-1 capture and optimize equilibrium displacement, we chose to use VEGF as a competitive ligand, which has a >10× greater affinity for sFlt-1 than does PlGF.[8] This competitive biomimetic binding approach captures circulating sFlt-1 while releasing endogenous PlGF, thereby increasing the bioavailability of PlGF and potentiating its proangiogenic effects on maternal endothelial function. To go one step further in validating the potential of our approach, it was integrated in a microfluidic device mimicking a miniaturized extracorporeal circulation system (Figure S1 in the online-only Data Supplement).

Material and Methods

Data that support the findings of this study are available from the corresponding author on reasonable request. Magnetic beads Dynabeads M-280 streptavidin, magnetic support Dynal Dynabeads MPC-DynaMag-96 Side Skirted Magnet, and DynaMag-5 Magnet were from Invitrogen. BSA fraction IV (BSA) and PBS without Ca^{2+} and Mg^{2+} were from Eurobio. Tween 20 was from Sigma-Aldrich. Human recombinant sFlt-1 with an antibody constant fraction (rhVEGFR1/Fc Chimera, Ser27-His687) and human recombinant PlGF (rh PlGF) were from R&D Systems. Kits from R&D systems (Quantikine) or from Roche Diagnostics (Elecsys) were used for the assay of free VEGF, free PlGF, sFlt-1, and sVEGFR-2 (soluble form of the vascular endothelial growth factor receptor 2).

Bead Functionalization and Characterization

Dynabeads M-280 streptavidin were functionalized with homemade bt-VEGF (biotinylated VEGF, see online-only Data Supplement for $VEGF_{95}$-AVI-biotin expression and purification, AVI being a C-terminus tag sequence of 15 amino acids) or with human anti–Flt-1 biotinylated antibody (R&D Systems). The functionalization was performed following the supplier's information with slight modifications (see SI).

After bead functionalization, 2 tests were performed for their characterization: one to assess the ligand surface density and the other to ensure the ability of the grafted ligand to bind sFlt-1 (see SI).

Proof of Concept With Recombinant Protein

Formation and Assay of sFlt-1/PlGF Complexes

Increasing concentrations of rhVEGFR1/Fc (from 0 to 1000 ng/mL) in PBS/0.1% BSA were added to the same volume of a solution at 4 ng/mL of rhPlGF in PBS/0.1% BSA and the mixture was incubated for 1 hour at 37°C. Complex formation was quantified after assay of free rhPlGF with Quantikine kits. The maximum absorbance signal (S_{max}) was given by the solution of rhPlGF without rhVEGFR1/Fc. The amounts of free and complexed rhPlGF were obtained using equations 1 and 2:

$$\%rhPlGF_{free} = \frac{S}{S_{max}} \times 100 \quad (1)$$

$$\%rhPlGF_{complexed} = \frac{S_{max} - S}{S_{max}} \times 100 \quad (2)$$

Capture of rhVEGFR1/Fc and Release of rhPlGF With VEGF-Beads

Two hundred microliters of the solution of rhVEGFR1/Fc/rhPlGF complexes (see Formation and Assay of sFlt-1/PlGF Complexes) was incubated in a tube with 5 µL (50 µg) of VEGF-coated beads (V-beads) at 105 pmol of bt-VEGF/mg beads. For the negative control, nonfunctionalized beads (C-beads) were used. After 1-hour incubation at 37°C with stirring, the concentrations of free rhPlGF and rhVEGFR1/Fc were measured with Quantikine kits.

Ethical Statements and Biological Samples

The local ethics committee (Comité de Protection des Personnes Ile de France 3) approved the studies of cultures of human primary cells from placenta tissue (Perinat Collection ANR-10-EQPX-0010) and the human plasma sample from the Angiogenic switch to Physiological state by Extracorporeal Removal of sFlt1 and release of free PlGF case/control prospective study (http://www.clinicaltrials.gov. Unique identifier: NCT03188900). All patients gave written consent for participation in these studies. For the primary cell culture, cytotrophoblasts were isolated as previously described, [9]and the conditioned media (CM) were collected after 72 hours of culture and then stored at −80°C.

Microfluidic Experiments

The characteristics and procedures for the chip fabrication and the microfluidic set-up were previously described and are summarized in the SI.[10] PBS containing 1% BSA (Sigma-Aldrich) was used for the conditioning of the chip.

Sample Treatment

The chip and tubing system was initially filled with washing buffer, and the temperature was adjusted to 37°C using an indium tin oxide glass slide with feedback regulation from a voltage controller (Eurotherm 3508) via a thermocouple gauge installed inside a slot in the polydimethylsiloxane chip. When using plasma samples, preliminary 3-minute spin centrifugation was used to remove any solid aggregates, and only supernatant was then collected.

The sample (150 µL) flow in the chip chamber was 1 µL/min. A Tygon tube (Saint Gobain AAD04103 Tygon Non-DEHP Medical Microbore Tubing, 0.020" ID, 0.060" OD) connected at the outlet was used to collect the emerging solution.

Results

Functionalization and Characterization of Streptavidin Magnetic Beads

Functionalization

To study the impact of VEGF density on the capacity of the beads to bind sFlt-1, it was necessary to estimate precisely the surface saturation of the magnetic beads because steric hindrance, repulsion, or interactions may play a role in protein recognition.[11,12] Based on previous work,[13] 4 different grafted V-beads with ligand densities between $11.7 \cdot 10^3$ and $158 \cdot 10^3$ VEGF/µm^2 were prepared by changing the initial quantity of VEGF, and then characterized (Table S1).

Binding of sFlt-1

To evaluate how the VEGF grafted on the beads was still able to recognize human recombinant sFlt-1 with good affinity, a binding assay was developed that yielded the dissociation constant of the immobilized VEGF/rhVEGFR1/Fc complex (Figure S2). The binding assay was performed with the 35% saturated beads (105 pmol VEGF/mg of beads) and revealed

a K_d value of 1.0±0.4 nmol/L (n=5). This value is in good agreement with the value obtained when VEGF is coated in a plate for a classical ELISA test[14] and confirms the high ligand-receptor affinity.

Proof of Concept

Capture and Release of Recombinant Proteins

The ability of V-beads simultaneously to capture sFlt-1 and release PlGF was first demonstrated in static conditions with recombinant proteins.

Formation of rhVEGFR1/Fc /rhPlGF Complexes

The first step was to produce artificial samples of varying compositions of complexed and free proteins. To do so, it was necessary to control the formation of recombinant rhVEGFR1/Fc/rhPlGF complexes (Figure 1A). To estimate the initial amount of each protein required to form the complex, a theoretical study was performed (see SI). Taking into account these calculations, the initial concentration of *rh*PlGF was kept constant at 6.9 pmol/L, and the selected initial *rh*VEGFR1/Fc concentrations ranged from 0 to 500 pmol/L. The estimated K_d value for the *rh*PlGF/*rh*VEGFR1/Fc complex was 22±3 pmol/L (n=3; Figure S3).

sFlt-1 Capture and PlGF Release

The second step of the proof of concept was to evaluate the ability of V-beads to capture rhsFlt-1 and to disrupt the rhVEGFR1/Fc/*rh*PlGF complex (Figure 1A). Thus, 3 different complex solutions (C1, C2, and C3) obtained from 3 different initial concentrations of rhVEGFR1/Fc (ranging from 52.3 to 29.2 pmol/L), were incubated with C-beads as the negative control and V-beads. The final concentrations of rhVEGFR1/Fc and rhPlGF were thus determined in the supernatants. Figure 1B and 1C show the molar quantity of total rhVEGFR1/Fc and free rhPlGF in each solution before (sample bar) and after incubation (C- and V-beads bars). No significant effects were observed when C-beads were used, showing that the nonspecific interactions were minimized. In contrast, a significant decrease in rhVEGFR1/Fc concentration accompanied by an increase of PlGF concentration in the presence of V-beads was observed in all the samples tested. In addition, because the total amount of rhPlGF used to form the complexes is known (6.9 pmol/L), the % of PlGF initially and finally complexed could be calculated. Although around 90% of initial rhVEGFR1/Fc was captured, only 20% of initial rhPlGF complexed to rhVEGFR1/Fc was released. This could be explained by the great difference in the amounts of proteins used to form the complexes (Table S2). Note that, in this study, we chose conditions where almost all the rhPlGF was complexed (>90%) and rhVEGFR1/Fc was in high excess, which is the case during severe preeclampsia.

These results with recombinant proteins demonstrate our competitive approach for the capture of sFlt-1 and the release of PlGF and allow us to move on to more complex media to validate the strategy.

Impact of the Saturation Rate on the Capture of sFlt-1 and Release of PlGF

The efficiency of sFlt-1 capture and PlGF release was assessed according to the surface ligand density of the V-beads. The amount of bt-VEGF coated on streptavidin Dynabeads was evaluated from 30 to 259 pmol/mg of beads corresponding to a surface ligand density ranging from $11.7 \cdot 10^3$ to $102 \cdot 10^3$ VEGF/μm^2 (Table S1). To investigate how our system was able to accommodate complex samples, these experiments were performed with CM obtained from human trophoblastic primary cells in which the initial concentrations of sFlt-1_{total} and $PlGF_{free}$ were 0.27 pmol/mL (20868 pg/mL) and 0.015 pmol/mL (292 pg/mL), respectively. Two hundred microliters of this CM was incubated with the necessary amount of beads to ensure the required VEGF excess compared with initial sFlt-1 in the sample. The amount of sFlt-1 captured increased when the ligand surface density decreased, from 30% capture at $100 \cdot 10^3$ VEGF/μm^2 to 60% capture at $12 \cdot 10^3$ VEGF/μm^2 for the case of a 500 molar excess of VEGF (Figure 2A). This could be explained by a high steric hindrance at higher saturation rate limiting the capture.[12] Then, at constant surface ligand density, the effect of VEGF in excess compared with the initial concentration of sFlt-1 was evaluated. We increased the mass of beads to obtain a 10 to 500 molar excess of VEGF. As expected, the capture increased with the excess of VEGF. However, over 100 molar excess only a slight improvement of capture was observed. For PlGF release, the excess VEGF plays a more important role than for sFlt-1 because this molecule is initially complexed to sFlt-1 and has to be displaced. In addition, the levels of PlGF are considerably lower than those of sFlt-1 (sometimes ≥1 order of magnitude). For example, in the CM used for this study, the initial quantity of free PlGF in solution was 3 fmol, whereas the total quantity of sFlt-1 was 54 fmol, corroborating the great difference in concentration between the 2 molecules and the fact that most of the PlGF will be complexed. Thus, we can observe in Figure 2B how a molar excess of at least 100 is required to have a significant release of PlGF.

Based on these series of experiments, the selected surface ligand density for further experiments was fixed at $41 \cdot 10^3$ VEGF/μm^2 (105 pmol VEGF/mg of beads; 35% saturation rate) as the better compromise in terms of beads and VEGF consumption and performance of capture/release. In patient samples, the quantities of these factors vary considerably and consequently the VEGF excess from beads may affect the efficiency of apheresis differently. Nevertheless, previous experiments show that this efficiency is not negatively affected if we ensure at least a 100 molar excess (VEGF/sFlt-1).

Valorization of the Competitive Strategy

To emphasize the features of this new specific and competitive strategy for restoring the angiogenic balance in preeclampsia, we compared the performances of the proposed approach with the specific strategy based on the use of antibodies for the capture of sFlt-1. Magnetic beads were functionalized either with VEGF or with anti–sFlt-1 antibody in a similar range of ligand surface density. Their performances in restoring the angiogenic balance in complex media (cell culture supernatants and mixed plasma of pregnant women) were compared using an equivalent amount of beads during the incubation. In Figure 3, we see that both systems capture sFlt-1 in the same range. However, the competitive approach, by releasing PlGF, is more efficient for the final objective of restoring the angiogenic balance.

It should also be mentioned that both systems suffer a loss of efficiency in the capture of sFlt-1 when the sample matrix

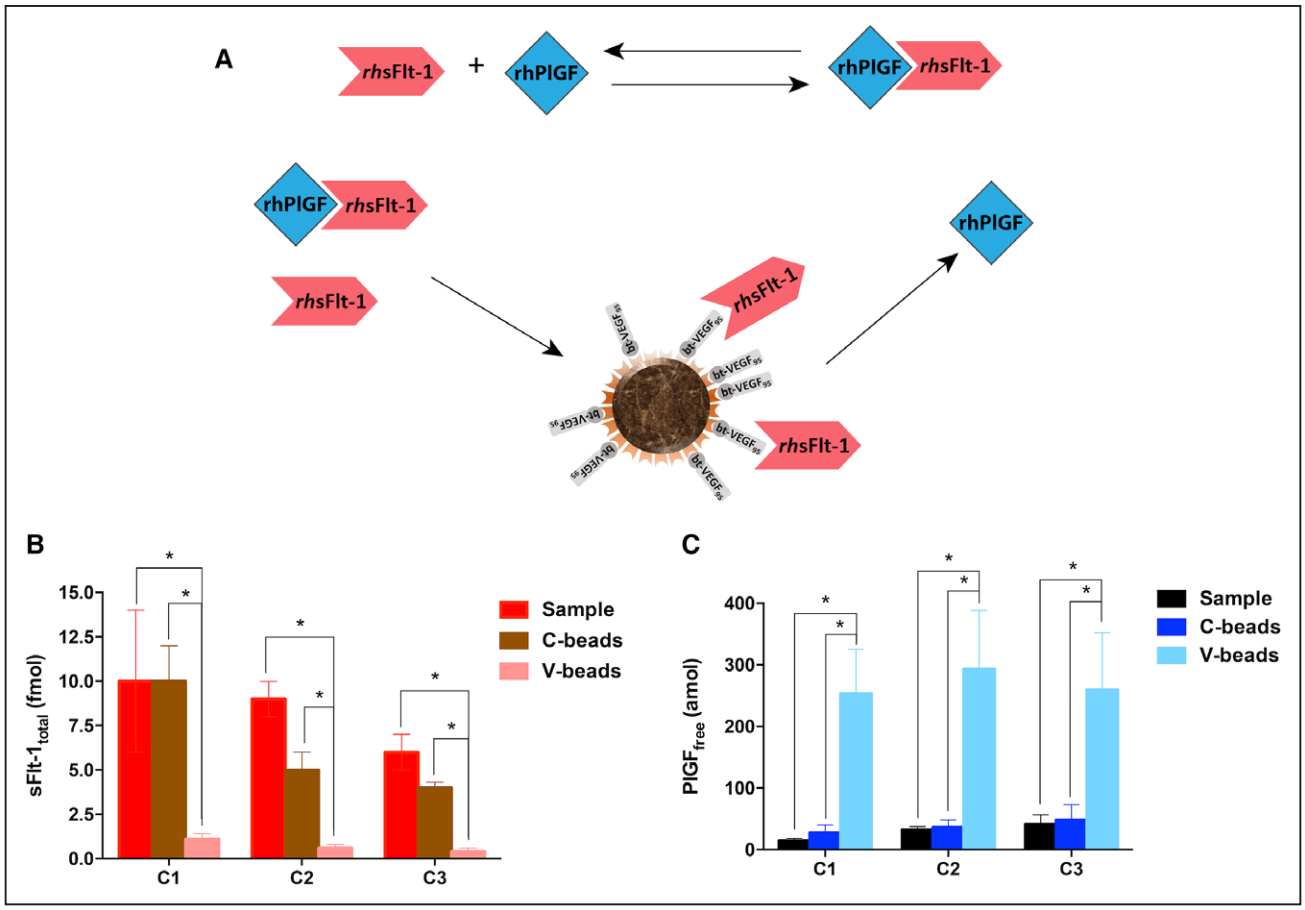

Figure 1. Proof of concept of the specific and competitive apheresis approach using recombinant proteins in physiological buffer medium (PBS at pH 7.4). **A**, Schematic representation of the rhVEGFR1/Fc/rhPlGF (recombinant human sFlt-1 with an antibody constant fraction) complex formation and its interaction with VEGF (vascular endothelial growth factor)-coated beads (V-beads), showing the hypothesis of our work (the ability to displace the PlGF complexed to sFlt-1); **B**, Evolution of the concentration of *rh*sFlt-1 after being incubated with V-beads; **C**, Evolution of the concentration of *rh*PlGF after incubation with V-beads. Please note that in **B** and **C**, i, C1, C2, and C3 are 3 solutions of complexes with different initial concentrations of *rh*VEGFR1/Fc (ie, 52.3; 43.7; and 29.2 pmol/L, respectively); obtained after 1 h incubation at 37°C of rhVEGFR1/Fc in PBS/0.1% BSA to the same volume of 4 ng/mL rhPlGF solution in PBS/0.1% BSA. Complex formation was quantified after assay of free rhPlGF with Quantikine kits. **B** and **C**, ii, Two hundred microliters of the solution of rhVEGFR1/Fc/rhPlGF complexes was incubated with 5 µL (50 µg) of V-beads at 105 pmol of bt-VEGF (biotinylated VEGF)/mg beads. Nonfunctionalized beads (C-beads) were used for negative control; concentrations of free rhPlGF and rhVEGFR1/Fc were measured with Quantikine kits. Sample denotes initial rhVEGFR1/Fc/rhPlGF formed complexes. rhsFlt-1 indicates recombinant human sFlt-1. *$P<0.001$.

is more complex. Thus, antibody anti-sFlt-1 grafted beads capture 37% of sFlt-1 in CM and 23% in plasma, whereas V-beads capture 51% in CM and 15% in plasma capture. This fact could be explained by the complexification of the sample matrix.

Optimization of the Microfluidic Conditions

To test this approach in a dynamic setting, a microfluidic polydimethylsiloxane chip of the magnetic fluidized bed previously described[10] was used to increase the surface-to-volume

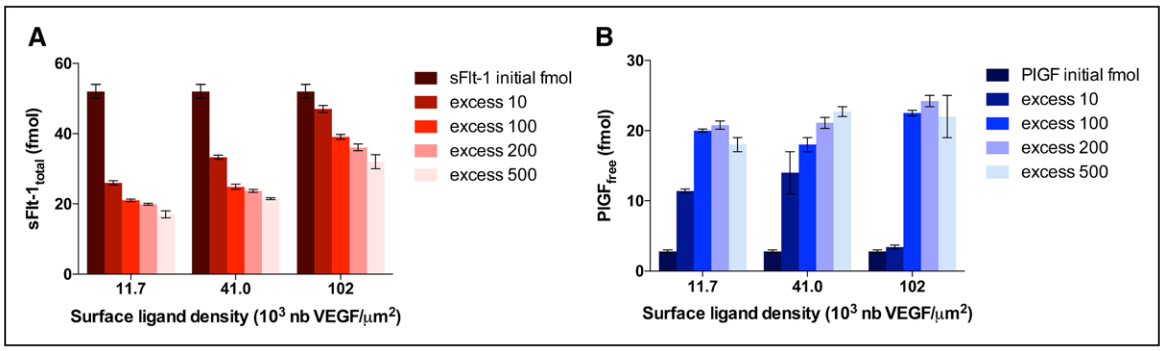

Figure 2. Impact of the grafting rates of VEGF (vascular endothelial growth factor)-coated beads (V-beads) on their capture/release performances in conditioned media (CM) of human trophoblastic cells. The amount of bt-VEGF (biotinylated VEGF) coated on streptavidin Dynabeads was evaluated from 30 to 259 pmol/mg of beads corresponding to a surface ligand density ranging from $11.7 \cdot 10^3$ to $102 \cdot 10^3$ VEGF/µm². Grafting of V-beads is done following the protocol in Bouzas-Ramos et al.[13] **A**, sFlt-1 capture and (**B**) PlGF (placental growth factor) release after 2.5-h incubation at 37ºC with stirring. Note that the marked excess is the molar excess of VEGF compared with the initial concentration of sFlt-1.

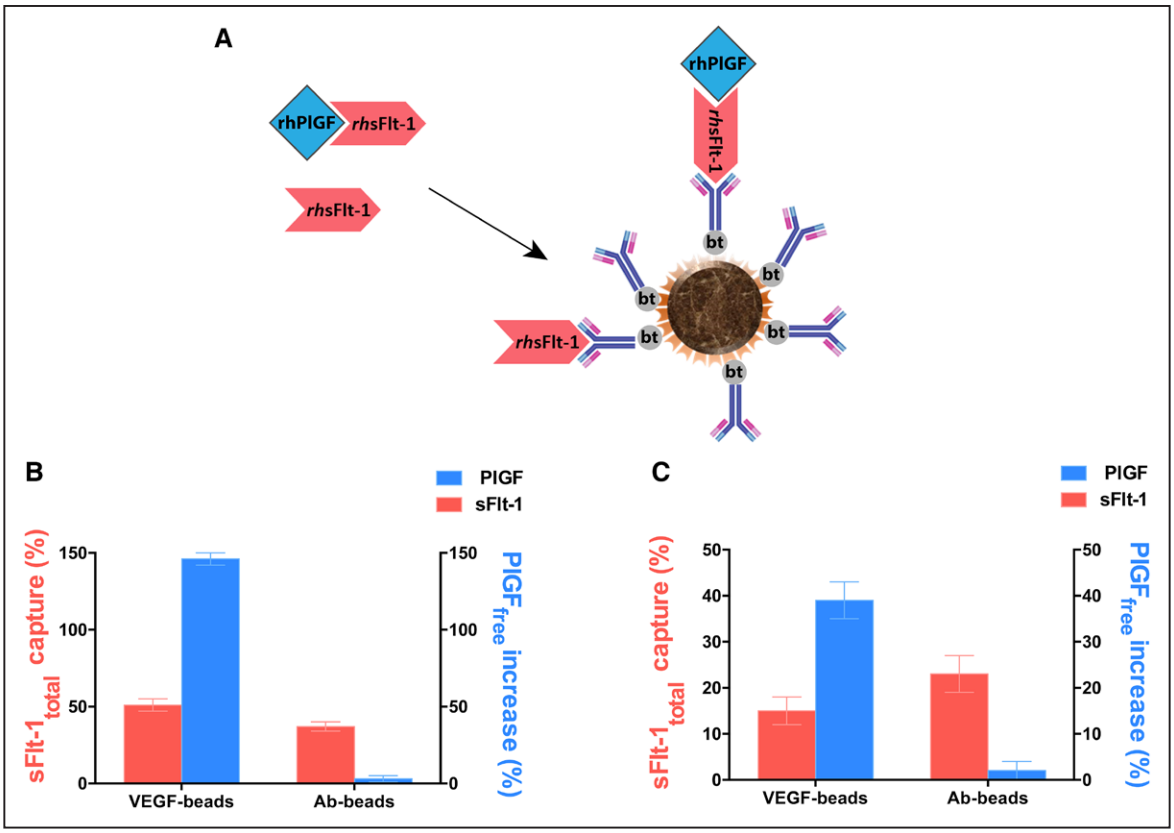

Figure 3. Comparison of specific systems to restore the angiogenic balance, one based on anti–sFlt-1 antibodies grafted beads (Ab-beads) and the other on competitive ligand (VEGF [vascular endothelial growth factor]) grafted beads (V-beads) in complex media. **A**, Schematic representation of the antibody-grafted beads during the capture of sFlt-1. **B**, Capture/release performances of both specific systems in conditioned media (CM); **C**, Capture/release performances of both specific systems in plasma. Note: The beads were grafted at 105 pmol VEGF and 25 pmol anti–sFlt-1 Ab per mg of beads ensuring an excess of biomolecule respect to the initial amount of sFlt-1 in the sample of 98× and 23×, respectively, in the case of CM; and 890- and 212-fold, respectively, in the case of the pooled plasma. (CM: 265 pg/mL PlGF (placental growth factor); 20 692 pg/mL sFlt-1. Pooled plasma: 502 pg/mL PlGF; 2250 pg/mL sFlt-1). bt indicates biotinylated; rhPlGF, recombinant human PlGF; and rhsFlt-1, recombinant human sFlt-1.

interactions during the treatment of the CM and the plasma samples with the functionalized beads. Here, the solid phase of V-beads is continuously recirculated (fluidization) during the percolation of a liquid phase (the biological samples). In the following sections, we present the different steps of the optimization of the microfluidic device-based approach.

Time of Residence of the Sample Inside the Bed

We assessed the impact of the sample residence time inside the bed of magnetic beads. This residence time is directly correlated with the flow rate and the bed porosity. We used a CM of primary human trophoblastic cells where concentrations of sFlt-1 and PlGF were, respectively, 0.26 and 0.013 pmol/mL. Experiments with 150 µL of biological samples were performed at a flow rate ranging from 0.5 µL/min (residence time of 7.7 s) to 3 µL/min (residence time of 2.9 s). As shown in Figure 4A, increasing the residence time of each molecule inside the chip improved the capture of sFlt-1 and the release of PlGF in a nonlinear way because the optimum capture/release mechanism was reached at 6.5 s of residence time, representing a flow rate of 1 µL/min and a total experiment time of 2.5 hours. This residence time is in agreement with the results obtained in static conditions (Figure S4). As shown in Figure 4B, 6.5 s of residence time is enough to reach a final sFlt-1/PlGF ratio at a physiological level below 38 for a sample with an initial ratio around 80. Indeed, Zeisler et al[15] identified an sFlt-1/PlGF ratio cutoff of 38 as having important predictive value in clinical practice.

For the following experiments, a flow rate of 1 µL/min was chosen to percolate the sample through the bed, which means a residence time of 6.5 s of the biological sample inside the chip. When the experiment was performed with C-beads (the abscissa 0 in Figure 4), no capture and no release could be measured and the sFlt-1/PlGF ratio was not affected by the sample percolation through the bed of beads.

Surface Ligand Density of the Beads

The performance of capture/release of sFlt-1/PlGF in the microfluidic device was evaluated considering the surface ligand density of the magnetic beads. The amount of bt-VEGF coated on streptavidin Dynabeads was evaluated as $11.7 \cdot 10^3$ to $158 \cdot 10^3$ VEGF/µm^2. We showed that increasing the VEGF surface density on the beads from 11.7 to $41.0 \cdot 10^3$ VEGF/µm^2 beads increased the capture of sFlt-1. However, large ligand surface densities induced a decrease of capture of sFlt-1 (Figure S5). Thus, $41.0 \cdot 10^3$ VEGF/µm^2 was the final surface ligand density selected for the experiments. These findings are in agreement with the static experiments (Figure 2).

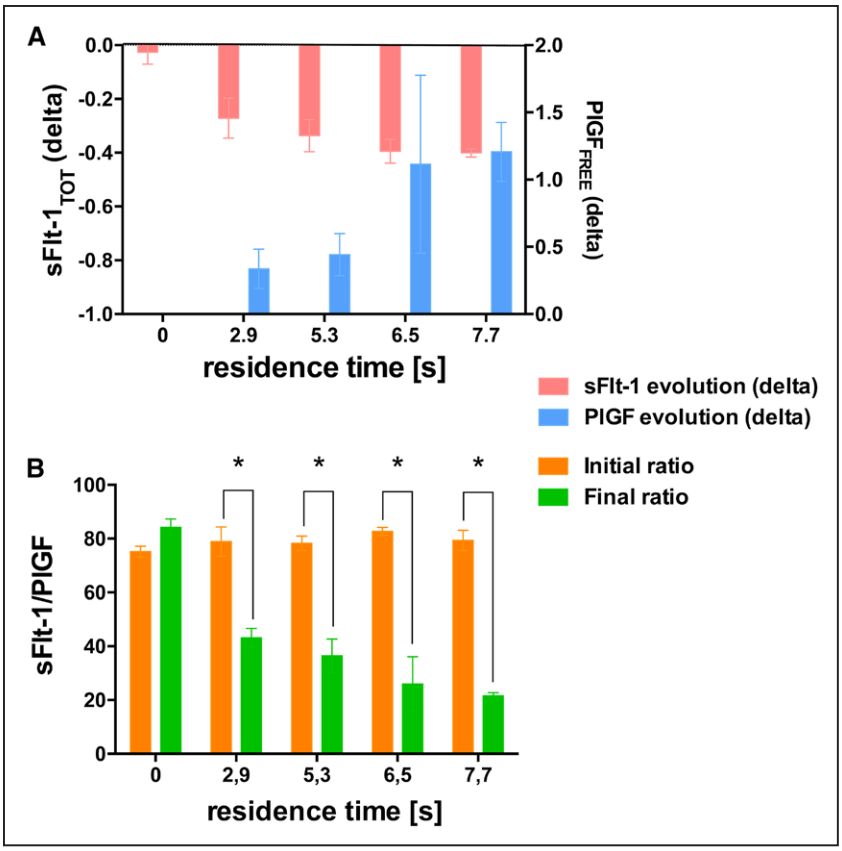

Figure 4. Evolution of concentrations of sFlt-1 and PlGF (placental growth factor) as a function of the residence time of each molecule inside the matrix (50 µg) of magnetic beads (beads ligand density: $41 \cdot 10^3$ VEGF (vascular endothelial growth factor)/µm^2; 105 pmol VEGF/mg beads) in the microfluidic device. **A**, the capture of sFlt-1 and the release of free PlGF and (**B**) the comparison between the initial and final sFlt-1/PlGF ratio. The abscissa 0 represents a control experiment done with Streptavidin Dynabeads M-280 without coating. Evolution of the concentration of molecules was evaluated as delta=$\frac{[C]_{fin}-[C]_{init}}{[C]_{init}}$ where concentrations are measured in pmol/mL. *P<0.001.

Validation of the Approach in Complex Matrices: From Cell Culture to Human Plasma

To validate our system, we used 2 types of biological samples, CM of human trophoblastic cells (n=3) and plasma from pregnant women with preeclampsia (n=3). The details on these samples are summarized in Table S3.

First, we tested our device with CM from human primary trophoblastic cells. For each of the samples tested, we were able to capture sFlt-1, release PlGF and consequently decrease the sFlt-1/PlGF ratio. As shown in Figure 5A, we captured >70% of the sFlt-1 of the sample and released half to more than double the initial quantity of PlGF. The final sFlt-1/PlGF ratio in CM was significantly reduced and systematically below the physiological level of 38 (Figure 5C).

Finally, with plasma samples from pregnant women with preeclampsia (Figure 5B), we observed a ≈40% reduction of sFlt-1 and an increase in free PlGF concentration from one-sixth to more than double in all the tested samples. Thus, our microfluidic system with V-beads decreased the sFlt-1/PlGF ratio in plasma samples by 63% on average (Figure 5D).

We also assessed the potential release of VEGF after sFlt-1 apheresis. Free VEGF was almost undetectable in our samples (CM and maternal plasma) and systematically increased after incubation with V-beads (Figure S6). In addition, because sVEGFR-2 is detectable in plasma samples from pregnant women, but not in CM, we evaluated the ability of our V-beads to trap some sVEGFR-2. After incubation of plasma samples with V-beads, depletion in sFlt-1 and sVEGFR-2 was observed, showing that beads are also able to capture sVEGFR-2 significantly (Figure S7). Note that the initial concentrations of both receptors are in the same range. However, in all cases sFlt-1 were preferentially captured (23±5% for sFlt-1 versus 12±5% for sVEGFR-2, mean values).

Discussion

Extracorporeal strategies to treat preeclampsia are evaluated for 3 main reasons:[1] the suspected primary role of circulating sFlt-1 in the pathogenesis of the disease,[2] the development of drugs that act specifically on the soluble form and not on the membrane form is particularly complex, and[3] apheresis methods avoid the adverse effects of molecules that cross the placenta. We decided to target sFlt-1 because of mounting evidence of its involvement in the pathogenesis of preeclampsia.[16–19] Soluble Flt-1 has been postulated to exert inhibitory effects on angiogenic signaling via 2 mechanisms: direct sequestration of angiogenic ligands, such as VEGF and PlGF and dominant-negative heterodimerization with surface VEGFRs.[20]

In vivo, the angiogenic effects are mainly related to the binding of VEGF to VEGFR-2. PlGF does not bind to VEGFR-2, but regulates VEGF bioavailability for VEGFR-2 by competing with VEGF-A in binding to VEGFR-1.[21] PlGF may also have direct angiogenic activity via intracellular signaling triggered by its binding to the VEGFR-1 membrane receptor.[22] During normal pregnancy, massive amounts of PlGF are produced by the placenta, reaching concentrations of free PlGF around 400 pg/mL, whereas during preeclampsia, free PlGF is extremely low due to the release of sFlt-1 into the maternal circulation.[23] Our sFlt-1 capture strategy consists of a specific and competitive approach

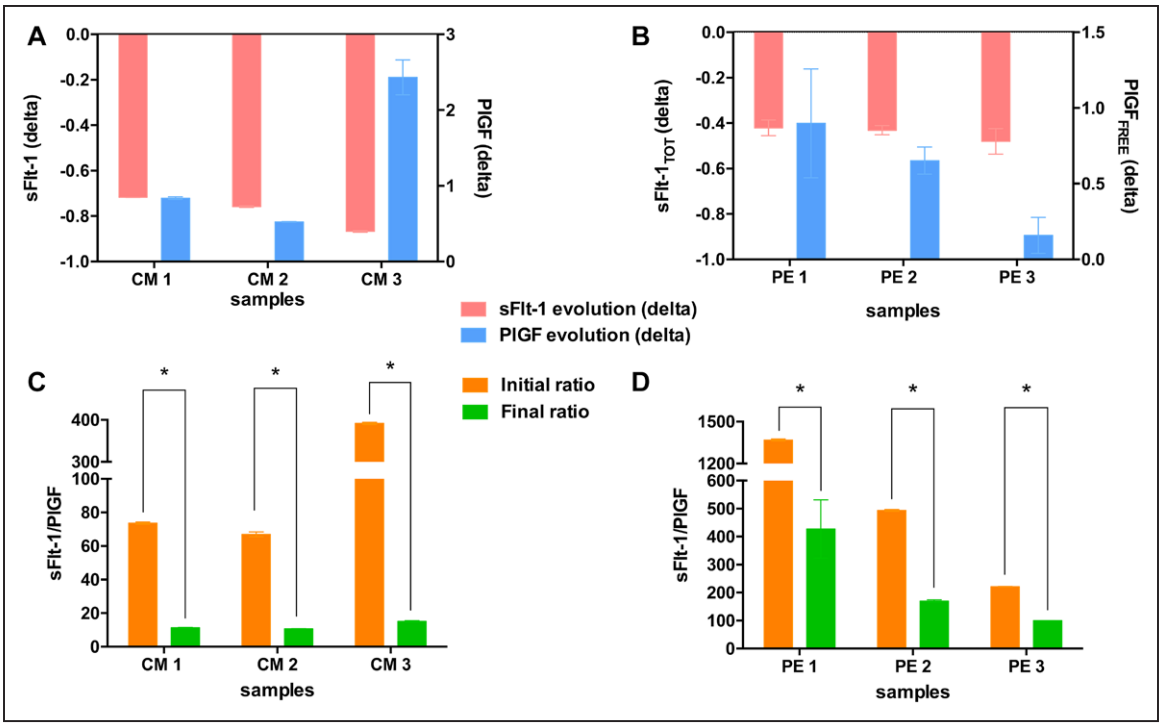

Figure 5 . Performance of sFlt-1 capture and PlGF (placental growth factor) release in conditioned media (CM) from human trophoblastic primary cell culture (**A**) and human plasma from preeclamptic (PE) women (**B**) after passage through the microfluidic device at the optimized conditions (1 µL/min, 37°C). Evolution of the sFlt-1/PlGF ratio CM (**C**) and plasma samples (**D**) after passage through the microfluidic device at the same conditions. Beads ligand density: $41 \cdot 10^3$ VEGF (vascular endothelial growth factor)/um^2 (105 pmol VEGF/mg beads). Performances are evaluated as delta=$\frac{[C]_{fin} - [C]_{init}}{[C]_{init}}$ with concentrations measured in pmol/mL. *P<0.001.

between the VEGF coated on the beads and the PlGF bound to the sFlt-1. The decline in total sFlt-1 is, therefore, as important as the increase in free PlGF for our study end point, which is why we used the sFlt-1/PlGF ratio for the expression of our results. To capture sFlt-1 in vitro in static and dynamic conditions, we used streptavidin magnetic beads, which are easy to functionalize with specific ligands (eg, biotinylated VEGF, Figure S2). Before application, we performed several tests to evaluate surface ligand density (Table S1) and the ability to recognize sFlt-1 by the grafted ligand (Figure S2), as well as the storage stability of the V-beads (up to 2 weeks at 4°C, Figure S8). Then, the sFlt-1 capture combined with PlGF release was proven mainly by 2 series of experiments. First, with the rhVEGFR1/Fc/rhPlGF complexes in static conditions, we checked the total amount of PlGF used in the experiments, which is not possible with more complex media (CM, plasma) because human PlGF ELISA kits only measure free PlGF. These experiments demonstrated the ability of V-beads to capture rhVEGFR1/Fc and also to release rhPlGF significantly (20% of the initial complexed PlGF). Second, our approach was compared with another specific capture strategy based on beads coated with an sFlt-1 antibody. We demonstrated an equivalent capture rate of sFlt-1, but with the addition of increased free PlGF in solution, which proves the potential interest of our competitive approach. The increase in free PlGF in solution after incubation with the V-beads may be caused by the combination of 2 phenomena. The initially free sFlt-1 is trapped by the V-beads, resulting in a shift of the biochemical equilibrium towards free PlGF (see equilibrium equation in Figure 1A). In addition, the V-beads compete with the PlGF bound to the sFlt-1, and due to the excess VEGF used for the incubation, we can displace the bound PlGF (Figure 2 and Figure S5). Finally, maybe because of this large excess of VEGF grafted on the beads, our approach is also able to displace the bound VEGF. This release of VEGF is sample-dependent (Figure S6), which shows the complexity of the VEGFs/VEGFRs system and the importance of the biochemical equilibria. During normal pregnancy, circulating free VEGF levels are very low, almost undetectable with noncompetitive ELISA. Whether these extremely low levels of VEGF have a physiological role during pregnancy is not known. Eremina et al[24] have shown that tight regulation of VEGF-A signaling is critical for establishment and maintenance of the glomerular filtration barrier. A slight elevation of circulating endogenous free VEGF could be beneficial, especially for the glomerular dysfunction characterized by proteinuria and endotheliosis, the renal lesion seen in preeclampsia.

The second part of our work was performed under dynamic conditions. To mimic the dynamic binding and release on the solid phase that take place in extracorporeal circulation and to evaluate the potential of our approach, we used the microfluidic fluidized bed model. The microfluidized bed allows the use of the same magnetic beads as for the static conditions while limiting the consumption of samples and reagents. Moreover, the magnetic fluidized bed has many advantages in applications involving fluid-solid exchanges, such as high surface-to-volume ratio, constant mixing, and low flow resistance.[10] Our results demonstrate the proof of concept of significant sFlt-1 capture combined with the release of PlGF in these

dynamic conditions, with a 68% reduction of sFlt-1/PlGF ratio in plasma from preeclamptic women. The flow rates and the fluid volumes are low in such conditions and not compatible with application in pregnant women (50–60 mL/min),[6] but this dynamic microscale technique is of value in testing different molecules in the capture of sFlt-1 in preeclamptic plasma because of the limited amount of human material. This microfluidic device could also be of interest for a proof of concept of this approach in animal models.

Using VEGF as a competitive ligand for capture of sFlt-1 and release of PlGF, not only sFlt-1 is captured in the plasma of preeclamptic women but also sVEGFR-2 (Figure S7). The physiological impact of sVEGFR-2 in normal and pathological pregnancies is unclear. Mothers destined to develop preeclampsia have lower plasma sVEGFR-2 concentrations than those who have a normal pregnancy.[25] Work is currently underway to develop ligands with an affinity that is both greater than that of PlGF for sFlt-1 and significantly lower for sVEGFR-2 than for sFlt-1.

Perspectives

The nest steps of our strategy are: (1) to optimize the grafting with a VEGF-like molecular ligand, specific for sFlt-1, allowing no binding of sKDR; (2) to evaluate the biological effects of the release in the maternal circulation of the endogenous VEGF; and (3) to develop apheresis columns based on our ligand-based approach and usable in vivo with an animal model.

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Disclosure

None.

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