

Low Molecular Weight Fucoidan Increases VEGF₁₆₅-induced Endothelial Cell Migration by Enhancing VEGF₁₆₅ Binding to VEGFR-2 and NRP1*

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Therapeutic induction of angiogenesis is a potential treatment for chronic ischemia. Heparan sulfate proteoglycans are known to play an important role by their interactions with pro-angiogenic growth factors such as vascular endothelial growth factor (VEGF). Low molecular weight fucoidan (LMWF), a sulfated polysaccharide from brown seaweeds that mimic some biological activities of heparin, has been shown recently to promote revascularization in rat critical hindlimb ischemia. In this report, we first used cultured human endothelial cells (ECs) to investigate the possible ability of LMWF to enhance the actions of VEGF₁₆₅. Data showed that LMWF greatly enhances EC tube formation in growth factor reduced matrigel. LMWF is a strong enhancer of VEGF₁₆₅-induced EC chemotaxis, but not proliferation. In addition, LMWF has no effect on VEGF₁₂₁-induced EC migration, a VEGF isoform that does not bind to heparan sulfate proteoglycans. Then, with binding studies using ¹²⁵I-VEGF₁₆₅, we observed that LMWF enhances the binding of VEGF₁₆₅ to recombinant VEGFR-2 and Neuropilin-1 (NRP1), but not to VEGFR-1. Surface plasmon resonance analysis showed that LMWF binds with high affinity to VEGF₁₆₅ (1.2 nM) and its receptors (5–20 nM), but not to VEGF₁₂₁. Pre-injection of LMWF on immobilized receptors shows that VEGF₁₆₅ has the highest affinity for VEGFR-2 and NRP1, as compared with VEGFR-1. Overall, the effects of LMWF were much more pronounced than those of LMW heparin. These findings suggested an efficient mechanism of action of LMWF by promoting VEGF₁₆₅ binding to VEGFR-2 and NRP1 on ECs that could help in stimulating therapeutic revascularization.

In recent years, a great deal of vascular research has been focused on understanding angiogenesis, which is the process of stimulating endothelial cells (ECs)⁴ to form new capillaries

from preexisting blood vessels. Angiogenesis plays a beneficial role in diverse physiological processes including embryonic development, tissue growth, menstrual cycle, and wound repair (1–4). It also plays a major role in pathological processes such as tumor growth, cancer cell metastases, and diabetic retinopathy (1, 5–8).

Therapeutic induction of angiogenesis is a potential treatment for chronic ischemia (9, 10). Recently, a low molecular weight fraction of fucoidan (LMWF) has been shown *in vivo* to promote revascularization in rat critical hindlimb ischemia (11). Fucoidan is a sulfated polysaccharide extracted from brown seaweeds that possesses some biological activities similar to those of heparin. Previous studies have shown that it can act as a potent inhibitor of vascular smooth muscle cell proliferation *in vitro* (12), and prevent neointimal hyperplasia of these cells during artery restenosis *in vivo* (13, 14). Fucoidan is of particular pharmacological interest because, in addition to its non-animal origin, it exhibits anti-inflammatory activities, is a potent modulator of connective tissue proteolysis (15), but has low anticoagulant activity compared with heparin (16–18). LMWF is also devoid of direct antithrombin effect (19).

Similar to heparin, fucoidan is also known to bind some pro-angiogenic growth factors, such as fibroblast growth factor-1 and 2 (FGF-1 and 2), protecting them from proteolysis and enhancing their activity on ECs (20–23). FGFs are known to induce angiogenesis *in vivo* by promoting proliferation and migration of ECs to form new capillaries (10, 24). Fucoidan has been shown to enhance FGF-2 induced EC tube formation in matrigel (23), a process that resembles *in vivo* vessel formation, and is a good index of angiogenic activity (25). While these studies present one explanation for the LMWF ability to aid in angiogenesis induction after ischemic damage, it is possible that this polysaccharide influences EC activity and angiogenesis in multiple ways. For instance, among the molecules implicated in the induction of and the control of angiogenesis, is the potent angiogenic vascular endothelial growth factor-A (VEGF-A) (26).

VEGF-A is a well known angiogenic growth factor whose expression can be induced in response to hypoxia and ischemic damage (26, 27). VEGF-A can act both by inducing EC proliferation and by stimulating EC migration (8, 26, 28). It has also been shown to be a pro-survival factor for ECs (29). It is a secreted glycoprotein with multiple splice variants (30, 31). The most abundant splice variant, VEGF₁₆₅, is known to bind hep-

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⁴ The abbreviations used are: EC, endothelial cells; LMWF, low molecular weight fucoidan; VEGF, vascular endothelial growth factor; PBS, phosphate-buffered saline; FCS, fetal calf serum; BSA, bovine serum albumin; NRP1, Neuropilin-1.

arin and heparan sulfates while another widely expressed form, VEGF₁₂₁ lacks a heparin binding motif (26). VEGF₁₂₁ also has reduced mitogenic and migration inducing effects compared with VEGF₁₆₅ (26). While the consequences of VEGF₁₆₅/heparin interaction are not fully understood, it is thought to help anchor the protein to the extracellular matrix and possibly influence presentation of VEGF₁₆₅ to its receptors on the EC surface (26). VEGF₁₆₅ binds to two tyrosine kinase high affinity receptors, Flt-1 or VEGFR-1, and KDR/Flk-1 or VEGFR-2. The binding of VEGF₁₆₅ to VEGFR-2 is enhanced by Neuropilin-1 (NRP1) expressed on endothelial cells, which acts a co-receptor, forming a complex with VEGFR-2 (32).

In the current *in vitro* study, we have tested the ability of LMWF to influence the cellular processes important to angiogenesis in ECs (25). We show that LMWF greatly enhances EC tube formation and migration, but not proliferation. Through the use of binding studies in purified systems, we demonstrate that LMWF binds to VEGF₁₆₅ and its receptors and that LMWF promotes binding of VEGF₁₆₅ to VEGFR-2 and NRP1.

EXPERIMENTAL PROCEDURES

Materials—Recombinant human VEGF₁₆₅, VEGF₁₂₁, FGF-2, recombinant human VEGFR-1 and VEGFR-2/Fc chimera, recombinant rat Neuropilin-1/Fc chimera used for these studies were obtained from R&D Systems (Minneapolis, MN). Unless specified, all chemicals and reagents were purchased from Sigma.

Polysaccharides—The LMW fucoidan (LMWF) used for these studies was obtained by radical processing of HMW extracts from brown seaweed according to a protocol previously described (11) and patented (33). Number-average molecular mass (Mn) of LMWF was 5.1 ± 0.3 kDa, compared with 5.6 ± 0.3 kDa for LMW heparin (Sigma). All other polysaccharides (unfractionated heparin, unfractionated fucoidan, sulfated dextran, chondroitin sulfate) were from Sigma.

Isolation of HUVEC—Human umbilical vein endothelial cells (HUVEC) were isolated from umbilical cords with 0.1% collagenase as described (21). Briefly, the umbilical vein was cannulated and perfused with PBS to remove blood. It was then filled with 0.1% collagenase in PBS, and incubated for 15 min at 37 °C. Cells were flushed from the vein with PBS, centrifuged for 5 min at $180 \times g$ and resuspended in 6 ml of endothelial cell basal medium II with all supplements (ECBM; Promocell, Germany) except heparin, and cultured in T25 flasks previously coated with collagen I (Iwaki, Japan). Throughout this study, cells of either passage 2 or 3 were used.

Tube-forming Assay—Growth factor-reduced (GFR) Matrigel (BD Biosciences, New Bedford, MA) was added to wells of a cold 96-well plate (45 μ l/well), and then incubated at 37 °C for 1 h to allow gelling. HUVEC previously starved overnight were seeded onto the Matrigel with various concentrations of LMWF in either 1% FCS medium, 1% FCS + 20 ng/ml VEGF₁₆₅, or full endothelial cell medium. Cell culture was carried out at 37 °C for 18 h in a humidified 5% CO₂ atmosphere. Cells were observed directly and scored on a scale of 0–5 for tube formation using dark field illumination on an inverted light microscope at low power ($\times 40$). Based on quality and number of the tubes, they were assigned numeric values: 0, no real tubes; 1,

some poorly formed tubes; 2, some formed tubes; 3, network of tubes both formed and poorly formed; 4, network of formed tubes; and 5, network of well formed tubes. Score results from four random fields in duplicate wells were averaged. Photographs were also taken with a digital camera under the same conditions.

Proliferation Assay—HUVEC were plated at 1×10^4 cells/well overnight into collagen coated 24-well dishes (Iwaki, Japan). After 24 h, the culture medium was replaced with medium containing 5% FCS with either 20 ng/ml VEGF₁₂₁, 10 ng/ml VEGF₁₆₅, or 5 ng/ml FGF-2, with or without 10 μ g/ml LMWF. EC medium and 5% FCS medium, with and without LMWF, were also tested. Cells in some wells were detached and counted immediately to obtain time = 0 counts. After 48 h of treatment, cells were detached and counted. Net cell number was determined by subtracting the time = 0 counts from the later counts. Experimental conditions were performed in triplicate on two separate occasions.

Transwell Migration Assay—HUVEC motility was measured using BioCoat 24-Multiwell insert system (BD Biosciences) with 3- μ m pore size polycarbonate filter insert that divides the chamber into upper and lower portions. The filters were coated with human recombinant fibronectin. Briefly, HUVEC that were previously starved overnight in 5% FCS, were trypsinized and resuspended in 0.1% FCS medium at a density of 4×10^5 cells/ml. 250 μ l of this suspension were then added to the upper chamber of the insert while 750 μ l of 0.1% FCS were added to the lower chamber. After 20 h in the absence or presence of polysaccharides (LMWF, unfractionated fucoidan, LMW heparin, unfractionated heparin, sulfated dextran, chondroitin sulfate), cells on the top of the filter were removed by gentle swabbing and the remaining cells on the bottom side of the filter were stained using 0.25% cresyl violet, and counted under a light microscope with an eyepiece grid to visualize set fields. At least four fields in duplicate wells were counted for each condition.

VEGF Binding to Recombinant VEGF Receptors—Immulon-1B removal well strips (Dynatech Laboratories) were coated overnight at 4 °C with 100 μ l of 5 mg/ml anti-human IgG (Fc-specific) in PBS buffer. Nonspecific interactions were blocked with PBS containing 0.1% BSA in PBS by a further overnight incubation at 4 °C. Then, 5 ng/well of recombinant Neuropilin-1/Fc, VEGFR-1/Fc or VEGFR-2/Fc chimera were added followed by 32 pM ¹²⁵I-VEGF₁₆₅ as previously described (34). Incubations were performed overnight at 4 °C in the absence or presence of LMWF or LMW heparin (0.001 to 1000 μ g/ml). The wells were then washed and radioactivity was measured with a γ -counter. B and B₀ are the bound radioactivity in the presence or in the absence of polysaccharide, respectively. Data are the average of 2–4 independent experiments carried out in triplicates. The EC₅₀, concentration that produces 50% of the maximum response, was calculated from log-dose response curves.

For competition experiments, biotinylated albumin-heparin (0.3 μ g/ml) was incubated overnight at 4 °C with the immobilized VEGF receptors (8.3 ng/immulon well), in the absence or presence of LMWF or LMW heparin. The wells were then washed and binding was revealed by streptavidin-peroxidase

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reaction at 405 nm on a multiskan UV reader. The IC₅₀, concentration that produces 50% inhibition, was calculated from log-dose response curves.

Seldi TOF Analysis—Cationic chip arrays CM10 and anionic chip arrays Q10 (Ciphergen Biosystems, CA) were employed. Spots were pre-wetted twice for 5 min with 10 μ l of PBS. Samples were prepared by mixing 1 μ g of recombinant VEGF receptors and/or 10–100 ng of VEGF₁₆₅ in the presence or absence of 1 μ g of LMWF, and applied to the spots. The samples were incubated for 30 min at 37 °C. The spots were washed, air-dried, and a saturated solution of sinapinic acid was applied twice to each spot. The chip arrays were analyzed using a protein chip reader (PBS II, Ciphergen Biosystems), calibrated using purified peptide and protein standards (Ciphergen Biosystems). Spectra were analyzed with proteinchip software (3.1.1 Ciphergen Biosystems). Normalization was performed by total ion current normalization function.

Surface Plasmon Resonance Experiments—VEGF receptors (recombinant Neuropilin-1, VEGFR-1 and VEGFR-2/Fc chimera), VEGF₁₂₁ or VEGF₁₆₅ were coupled to the carboxymethylated dextran surface of a CM5 sensor chip using standard amine coupling chemistry for analysis of ligand binding using a BIAcore 3000 optical biosensor (BIAcore, Uppsala, Sweden). Bovine serum albumin and goat anti-human Fc were used as controls. Following immobilization, residual activated ester groups were blocked by treatment with 1 M ethanolamine. NaCl, 3 M, was used to regenerate the sensor surface between analysis for LMWF and LMW heparin binding. Samples were diluted in running buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 3.4 mM EDTA, and 0.005% Tween-20). Flow cell, temperature, flow rate, sample volume, and mixing were selected with the BIAcore control software (BIAcore). The apparent binding affinities of LMWF and LMW heparin for VEGF₁₆₅ and VEGF receptors were determined by analysis of the kinetic of the association assuming a 1:1 Langmuir model using BIAevaluation software. Results are means of triplicate determinations.

Statistical Analysis—For migration, proliferation, and tube formation assays, two tailed paired Student's *t* tests were performed. For binding experiments, curves were analyzed by fitting a logistic curve (Graph Pad software). Multiple statistical comparisons were performing using analysis of variance multivariable linear model or Mann-Whitney *t* test. *p* < 0.05 was considered statistically significant.

RESULTS

LMWF Enhances EC Tube Formation—An important aspect of angiogenesis is the formation of ECs into functional capillaries. To study the effect of LMWF on this process *in vitro*, wells containing growth factor-reduced Matrigel were used in a tube-forming assay. HUVEC were observed 18 h after seeding and analyzed for the quality and number of the tubes formed in the gel with either full EC medium, 1% FCS, or 1% FCS+20 ng/ml VEGF₁₆₅ (Fig. 1). Overall, conditions in EC medium had a high level of tube formation. The highest quality networks of tubes formed in EC medium with 10 μ g/ml LMWF (score = 4.8 \pm 0.5; Fig. 1C; *p* < 0.01). Low serum content (1% FCS) by itself induced a very low level of tube formation (score = 0.8 \pm 0.5; Fig. 1E). The presence of 1 μ g/ml LMWF in 1% FCS significantly

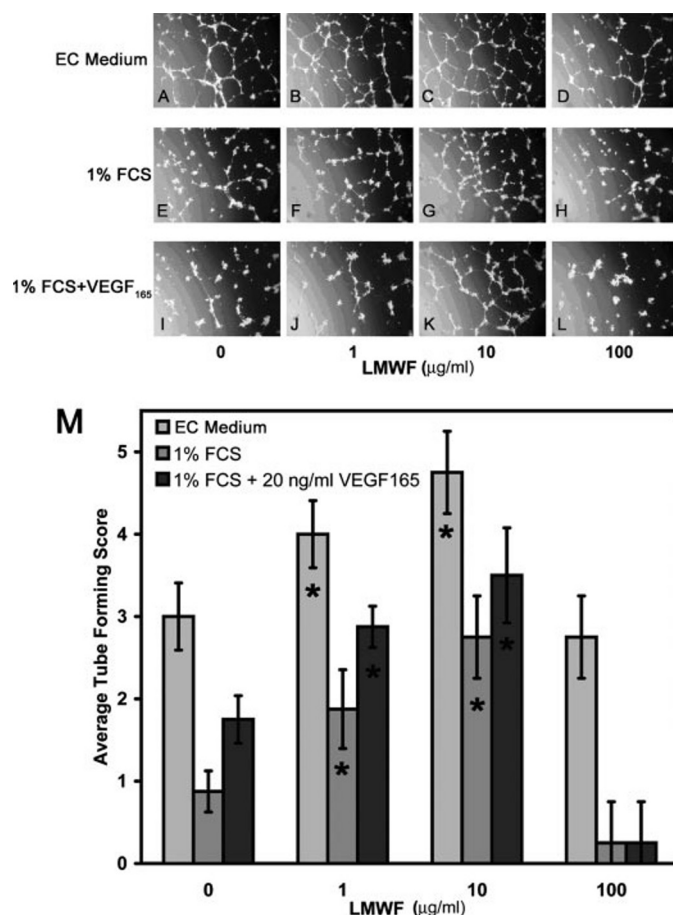


FIGURE 1. LMWF enhances HUVEC tube forming in Matrigel. HUVEC which were previously starved overnight were seeded with the indicated concentrations of LMWF onto growth factor-reduced Matrigel in either EC medium (A–D), 1% FCS (E–H), or 1% FCS+20 ng/ml VEGF₁₆₅ (I–L). Cell culture was carried out at 37 °C for 18 h. Cells were observed directly and scored as indicated under “Experimental Procedures” for tube formation using dark field illumination on an inverted light microscope ($\times 40$). A–L, representative photographs from tube forming assay. M, graph representing mean scores \pm S.D. for tube forming in each condition and averaged from four random fields in duplicate wells in two independent experiments. *, *p* value < 0.05 as compared with the absence of LMWF in each condition.

cantly (*p* < 0.05) increased the amount of tube formation. Addition of 10 μ g/ml LMWF in 1% FCS significantly (*p* < 0.01) increased the amount of tube formation (score = 2.8 \pm 0.5; Fig. 1G) reaching values obtained with fully supplemented commercial EC medium. The highest concentration (100 μ g/ml) of LMWF in 1% FCS had a negative effect on tube formation (score = 0.3 \pm 0.5; Fig. 1H). Addition of 20 ng/ml VEGF₁₆₅ in 1% FCS increased tube formation. The presence of 1 μ g/ml or 10 μ g/ml LMWF in this medium significantly (*p* < 0.05) increased the amount of tube formation (Fig. 1M). Again, the highest LMWF concentration (100 μ g/ml) had an inhibitory effect on tube formation (Fig. 1M).

LMWF Has No Effect on HUVEC Proliferation Stimulated by VEGF₁₆₅—Given the ability of LMWF to influence tube forming of HUVEC, it is also possible that this polysaccharide could influence other processes important to the progression of angiogenesis such as cellular proliferation. To test the ability of LMWF to influence HUVEC proliferation, we performed cell growth experiments for 48 h in commercial full EC medium, or

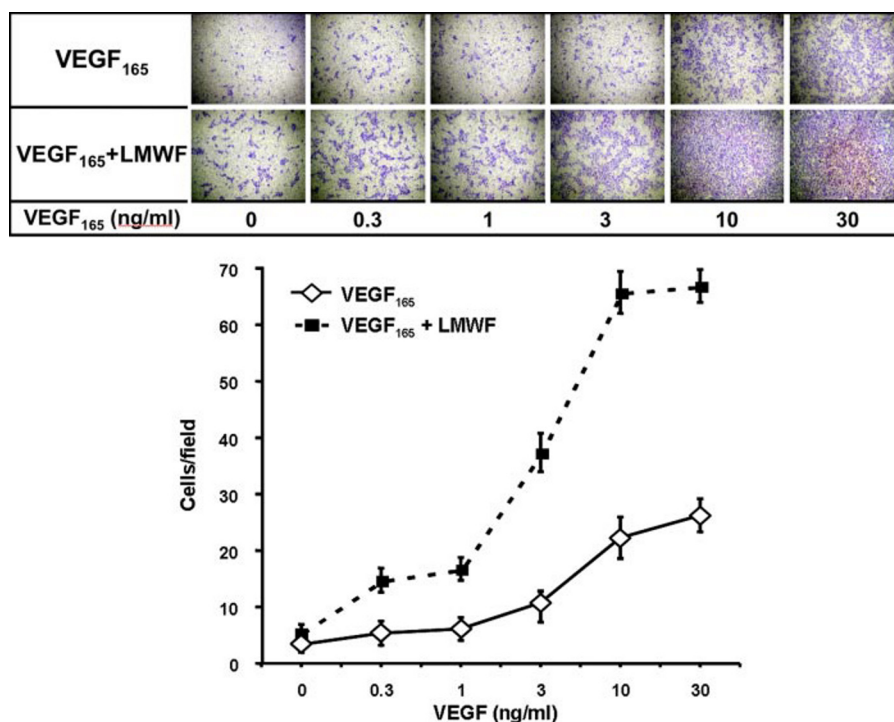


FIGURE 2. LMWF enhances VEGF₁₆₅-induced HUVEC migration. Representative photographs of stained filters from the transwell migration assay (top panel). Previously starved HUVECs were seeded into the upper chambers of a 3- μ m filter, fibronectin-coated, transwell plate, and allowed to migrate for 20 h in response to the indicated concentrations of VEGF₁₆₅ in 0.1% FCS with or without 10 μ g/ml LMWF. Cells remaining in the upper chamber were removed and the migrated cells were visualized by staining with cresyl violet. The first row contained no LMWF, while the second row contained 10 μ g/ml LMWF. Bottom panel, HUVEC on the filters were counted under a light microscope with an eyepiece grid to visualize set fields. Data are represented as average cells/field. Values are means \pm S.D. for each condition in duplicate wells counted in quadruplicate fields.

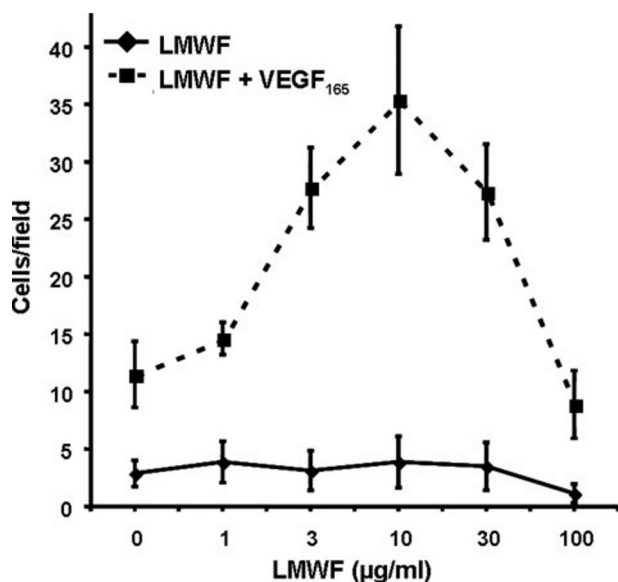


FIGURE 3. Optimization of LMWF concentration on HUVEC migration. Starved HUVECs were seeded into the upper chambers of a 3- μ m filter, fibronectin coated, transwell plate and allowed to migrate for 20 h in response to various concentrations of LMWF in 0.1% FCS with or without 3 ng/ml VEGF₁₆₅. LMWF concentrations tested ranged from 0–100 μ g/ml. Cells remaining in the upper chamber were removed and the migrated cells on the bottom side of the filters were visualized by staining with cresyl violet and counted under a light microscope. Data are represented as average cells/field, and values are means \pm S.D. for each condition in duplicate wells counted in quadruplicate fields.

in 5% FCS supplemented with FGF-2 or VEGF₁₆₅. The presence of 10 μ g/ml LMWF increased proliferation of HUVEC when added to EC medium, 5% FCS or FGF-2. EC medium gave the highest cell number when combined with LMWF ($7.7 \pm 0.43 \times 10^4$ cells/well versus $6.3 \pm 0.18 \times 10^4$ cells/well; $p < 0.001$). The lowest amount of proliferation occurred in 5% FCS alone (net cell number of $1.0 \pm 0.45 \times 10^4$ cells/well). Addition of 10 μ g/ml LMWF in 5% FCS increased the net cell number to $2.9 \pm 0.25 \times 10^4$ cells/well. FGF-2 produced a net cell number of $2.1 \pm 0.21 \times 10^4$ cells/well, that was increased ($4.9 \pm 0.64 \times 10^4$ cells/well) when FGF-2 was combined with 10 μ g/ml LMWF ($p < 0.001$). In contrast, LMWF did not influence the VEGF₁₆₅ effect on HUVEC proliferation: VEGF₁₆₅ produced a net cell number of $2.0 \pm 0.4 \times 10^4$ cells/well and addition of 10 μ g/ml LMWF had no significant effect on cell number ($2.1 \pm 0.33 \times 10^4$ cells/well).

LMWF Increases VEGF₁₆₅-induced EC Migration—High molec-

ular weight fucoidan has been shown previously to enhance motility of HUVEC induced by FGF-1 and FGF-2 (21). To examine the ability of LMWF to influence EC migration in response to VEGF₁₆₅, transwell assays were employed with HUVEC allowed to migrate through fibronectin-coated 3- μ m filters for 20 h. VEGF₁₆₅ induced chemotaxis of HUVEC in a dose-dependent manner (Fig. 2, top panel, first row). As compared with the baseline value of migration (3.4 cells/field), the amount of cell migration induced by VEGF₁₆₅ concentrations of 0.3, 1, 3, 10, and 30 ng/ml were 5.4, 6.1, 10.8, 22.3, and 26.3 cells/field, respectively (Fig. 2). Over the range of VEGF₁₆₅ from 0–30 ng/ml, significant increases in HUVEC migration were observed upon addition of 10 μ g/ml LMWF when compared with VEGF₁₆₅ alone (Fig. 2). The enhancing effect of LMWF was pronounced for 3 ng/ml and maximal for 10 ng/ml VEGF₁₆₅ (Fig. 2).

To find the optimum concentration of LMWF increasing VEGF₁₆₅-induced migration, a range of LMWF concentrations in the presence or absence of 3 ng/ml VEGF₁₆₅ was tested for the ability to induce HUVEC migration in the transwell assay (Fig. 3). The largest increase in migration induced by VEGF₁₆₅ was found for 10 μ g/ml LMWF. At this concentration, migration was increased 3.1-fold (increase from 11.5 to 35.4 cells/field). Slightly lesser increases were observed for 3 and 30 μ g/ml LMWF with values of 27.8 and 27.4 cells/field counted respectively (2.4-fold increase for both). When the highest concentration of LMWF (100 μ g/ml) was added to VEGF₁₆₅, a slight

LMW Fucoidan Enhances VEGF₁₆₅ Activity on EC Migration

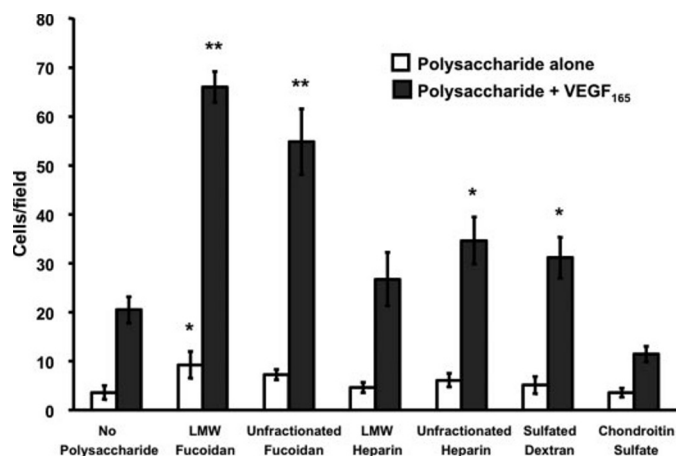


FIGURE 4. Migration enhancing effect is maximal with LMWF. Starved HUVECs were seeded into the upper chambers of a 3 μ m filter, fibronectin coated, transwell plate and allowed to migrate for 20 h in response to various polysaccharides (10 μ g/ml) in 0.1% FCS with or without addition of 10 ng/ml of VEGF₁₆₅. Cells remaining in the upper chamber were removed and the migrated cells visualized by staining with cresyl violet on the bottom side of the filters were counted. Data are represented as average cells/field and values are means \pm S.D. for each condition in duplicate wells counted in quadruplicate fields. *, $p < 0.01$ as compared with no addition; **, $p < 0.001$ as compared with no addition.

inhibitory effect was observed (decrease to 8.9 from 11.5 cells/field).

Migration Enhancing Effect Was Optimal with LMWF—To determine if the VEGF₁₆₅-induced migration enhancing effect shown above is specific to LMWF or shared with other sulfated polysaccharides, unfractionated fucoidan, LMW heparin, unfractionated heparin, LMW-sulfated dextran and chondroitin sulfate were tested for the ability to influence chemotaxis induced by 10 ng/ml VEGF₁₆₅ (Fig. 4). Cell migration induced by VEGF₁₆₅ alone was 4-fold increased as compared with the absence of VEGF₁₆₅. Among the sulfated polysaccharides tested with VEGF₁₆₅, LMWF had the most potent migration enhancing effect (3.2-fold increase over VEGF₁₆₅; $p < 0.001$) followed by unfractionated fucoidan (2.7-fold increase over VEGF₁₆₅; $p < 0.001$). Unfractionated heparin (1.7-fold increase over VEGF₁₆₅), LMW-sulfated dextran (1.5-fold increase over VEGF₁₆₅) and LMW heparin (1.3-fold increase over VEGF₁₆₅) had lower migration enhancing effects. Chondroitin sulfate had no significant effect on HUVEC migration through the transwell (Fig. 4).

LMWF Migration Enhancing Effect Was Specific to VEGF₁₆₅—VEGF₁₆₅ is known to bind heparin (26). Because LMWF was previously assumed to be a heparin mimetic, we have examined the ability of LMWF to enhance migration induced by the VEGF₁₂₁ splice variant, which lacks a heparin binding domain. VEGF₁₂₁ on its own, while less potent than VEGF₁₆₅, still induces the chemotaxis of HUVEC in a dose-dependent manner (Fig. 5). In contrast to VEGF₁₆₅, no significant differences in HUVEC migration were observed over the range of VEGF₁₂₁ from 0.3–30 ng/ml upon addition of 10 μ g/ml LMWF as compared with VEGF₁₂₁ by itself (Fig. 5).

LMWF Enhanced VEGF₁₆₅ Binding to VEGFR-2 and NRP1—LMWF could exert the effect on VEGF₁₆₅ activity by modulating its binding to VEGF receptors. We tested the effect of LMWF on ¹²⁵I-VEGF₁₆₅ binding to the three VEGF receptors

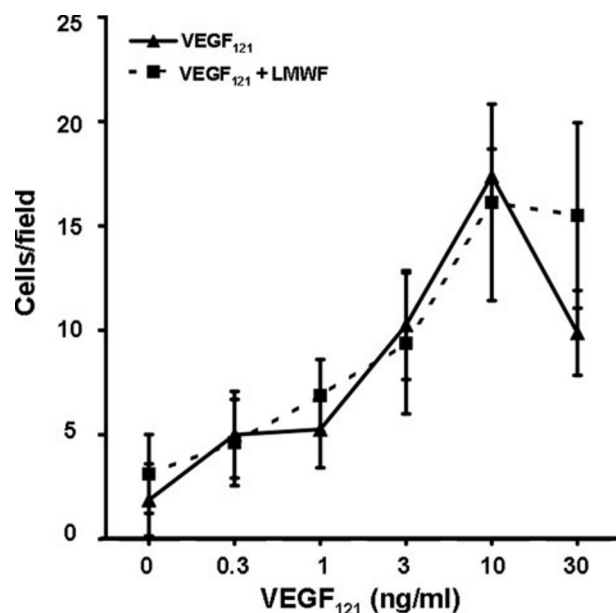


FIGURE 5. VEGF₁₂₁-induced migration is unaffected by LMWF. Previously starved HUVECs were seeded into the upper chambers of a 3- μ m filter, fibronectin-coated, transwell plate, and allowed to migrate for 20 h in response to various concentrations of VEGF₁₂₁ in 0.1% FCS with or without 10 μ g/ml LMWF. VEGF₁₂₁ concentrations tested ranged from 0–30 ng/ml. Cells remaining in the upper chamber were removed, and the migrated cells were counted. Data are represented as average cells/field and values are means \pm S.D. for each condition in duplicate wells counted in quadruplicate fields.

present on ECs, VEGFR-1, VEGFR-2, and NRP1. Binding studies performed on recombinant receptor/Fc chimera demonstrated that LMWF has a dose-dependent enhancing effect on both VEGFR-2 and NRP1 (Fig. 6). No enhancing effect was observed on VEGFR-1 (Fig. 6A). Interestingly, the maximum enhancing effect of LMWF on the binding of VEGF₁₆₅ to VEGFR-2 (180%) was much higher than with LMW heparin (120%). The enhancing effect of LMWF started at concentrations as low as 0.001 μ g/ml (0.2 nM). Calculated EC₅₀ of LMWF for VEGFR-2 and NRP1 were \sim 0.01–0.02 μ g/ml (2–4 nM). The highest concentrations (\geq 100 μ g/ml) of LMWF or LMW heparin inhibited VEGF₁₆₅ binding.

LMWF Binding to VEGF Receptors—Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) was first used to investigate a putative complex formation between LMWF, VEGF₁₆₅, and VEGF receptors on cationic and anionic surfaces as mimics of an extracellular matrix. It was not possible to conclusively demonstrate the presence of complexes due to the diverse molecular properties, some species being anionic (sulfated polysaccharides, VEGFR-2, NRP1), while others are globally neutral but with a cationic domain (VEGF₁₆₅), or mostly cationic (VEGFR-1). They also range from very low molecular weights (LMW polysaccharides), medium sizes (unfractionated polysaccharides, VEGF₁₆₅) and very high molecular weights (VEGFR-1, VEGFR-2, NRP1).

We further investigated the affinity of LMWF for VEGF receptors by competition of LMWF with the binding at 4 $^{\circ}$ C of biotinylated heparin to immobilized VEGFR-1, VEGFR-2, and NRP1. As previously reported (34), VEGF binding is saturable and addition of 50 nM cold VEGF completely abolish this bind-

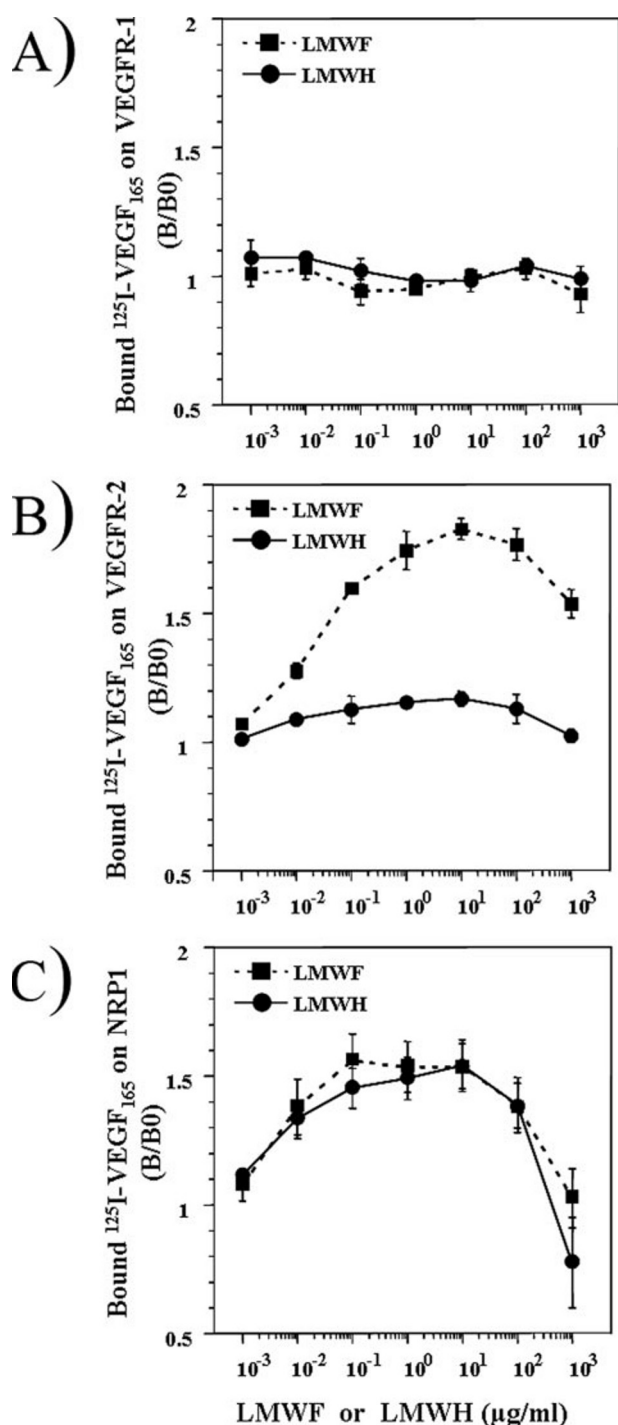


FIGURE 6. LMWF enhances VEGF₁₆₅ binding to VEGFR-2 and NRP1. Immulon-1B removal well strips were coated with 5 ng/ml human recombinant VEGFR-1 (A), VEGFR-2 (B), or NRP1 (C) and treated or not overnight with LMWF or LMW heparin (0.001–1000 μg/ml) and 32 pM ¹²⁵I-VEGF₁₆₅. After washing, the bound radioactivity, *B*, was measured. *B*₀ is the bound radioactivity in the absence of polysaccharide. Data are the average ± S.D. of 2–4 independent experiments made in triplicate.

ing. Surprisingly, LMWF was a very potent ($IC_{50} < 1 \mu\text{g/ml}$) competitor for all three VEGF receptors. LMWF at 1 μg/ml was able to displace $85 \pm 2\%$ of biotinylated heparin on VEGFR-1, $69 \pm 3\%$ on VEGFR-2, and $67 \pm 1\%$ on NRP1. In contrast, LMW heparin was a poor inhibitor with $IC_{50} > 10 \mu\text{g/ml}$ for all three receptors.

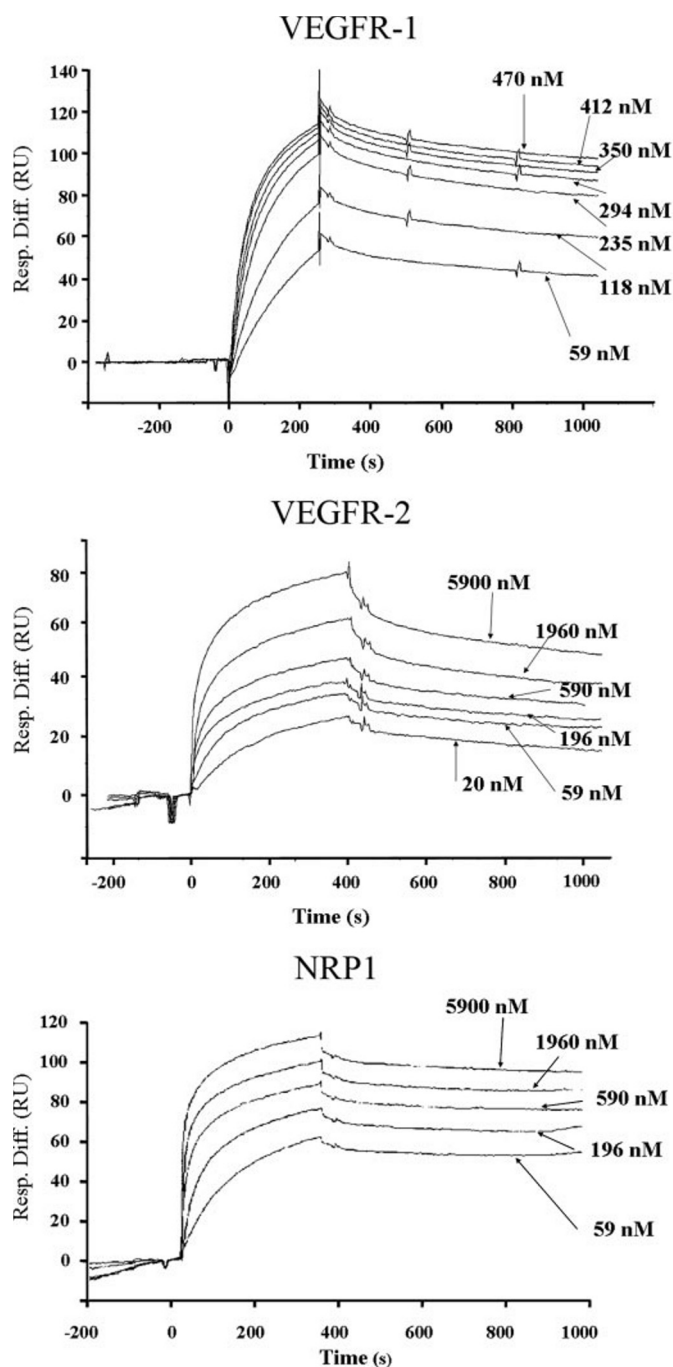


FIGURE 7. Representative sensorgrams showing the association and dissociation profiles of LMWF on immobilized VEGF receptors. LMWF was injected over surface plasmon resonance sensor surfaces immobilized with VEGFR-1 (first row), VEGFR-2 (second row), or NRP1 (third row). Binding parameters for LMWF on VEGF receptors are reported in Table 1, and compared with those of LMW heparin. Kinetic studies with the indicated polysaccharide concentrations were determined at a flow rate of 30 μl/min. Representative sensorgrams in response units (RU) are overlaid for the indicated concentrations of LMWF.

We also performed surface plasmon resonance analysis on immobilized VEGF receptors. Sensorgrams are presented for the binding of LMWF on VEGFR-1, VEGFR-2 and NRP1 (Fig. 7). Binding capacities, calculated association/dissociation rate kinetic constants and dissociation constants are reported in Table 1 for LMWF and LMW heparin. Affinity constants of

TABLE 1

Binding analysis of LMWF and LMW heparin on receptors using surface plasmon resonance

VEGFR-1, VEGFR-2, and NRP1 were immobilized on BIAcore sensor chips. The association rate constants k_{on} , dissociation rate constants k_{off} , dissociation constants K_d (k_{off}/k_{on}), and binding capacities (B_{max}) are means of three experiments (error ~20%). RU, response units.

		K_d	$k_{on}/10^4$	$k_{off}/10^{-4}$	B_{max}
		nM	$M^{-1} s^{-1}$	s^{-1}	RU
LMWF	VEGFR-1	5.3	3.19	1.7	137
	VEGFR-2	17.6	3.21	5.7	53
	NRP1	19.8	6.13	12.1	86
LMW heparin	VEGFR-1	660	0.08	5.6	42
	VEGFR-2	148	0.47	6.9	23
	NRP1	6.3	35.8	22.7	11

LMWF for all three receptors are very high for a polysaccharide species (3 orders of magnitude higher than on immobilized anti-human Fc or BSA used as negative controls). The quantitative results also indicated that LMWF has a higher affinity than LMW heparin for VEGFR-1 and VEGFR-2. For NRP1, a closer inspection of the BIAcore data revealed that the lower K_d of LMW heparin than LMWF, was largely the result of the reduction in the k_{on} value, but binding capacity of LMWF for NRP1 was 5 times higher than for LMW heparin (Table 1).

We then carried out surface plasmon resonance experiments by analyzing VEGF₁₆₅ binding on immobilized receptors with and without a primary injection of LMWF. Pre-injection of LMWF decreases by a factor of 2.4 and 2.2 the affinity constants of VEGF₁₆₅ to VEGFR-1 and VEGFR-2, respectively. This decrease of affinity arises both from a decrease of k_{on} , and from an increase of k_{off} (data not shown). This indicated that LMWF does not help presenting VEGF₁₆₅ in a better configuration to VEGFR-1 and VEGFR-2. In contrast, preinjection of LMWF increases by a factor of 2.4 the affinity constant of VEGF₁₆₅ to NRP-1. All together, the presence of LMWF gives VEGF₁₆₅ a higher affinity for VEGFR-2 and NRP1 as compared with VEGFR-1.

LMWF Binding to VEGF₁₆₅ but Not to VEGF₁₂₁—On immobilized VEGF₁₆₅, exposure of various concentrations of LMWF to the sensor chip gave a calculated association rate constant of $k_{on} = 47.7 \times 10^3 M^{-1} s^{-1}$, a dissociation rate constant of $k_{off} = 0.57 \times 10^{-4} s^{-1}$, and a consequent dissociation constant of K_d (k_{off}/k_{on}) = 1.26 nM. On immobilized VEGF₁₆₅, the affinity of LMW heparin was 10 times lower (data not shown). Surface plasmon resonance experiments evidenced that LMWF binds to VEGF₁₆₅, but not to VEGF₁₂₁ or BSA (Fig. 8). This observation is in agreement with the effect observed on LMWF migration enhancement induced with VEGF₁₆₅ but not with VEGF₁₂₁.

DISCUSSION

In this report we provide functional evidence that LMWF, a low molecular weight algal polysaccharide, enhances cellular processes that could be relevant to angiogenesis. Understanding how this polysaccharide influences EC response to angiogenic stimuli, could contribute to the development of new agents for therapeutic revascularization.

Our experiments indicate that LMWF enhances the ability of EC to form tubes in growth factor-reduced Matrigel. LMWF improves tube forming either in full growth factor supple-

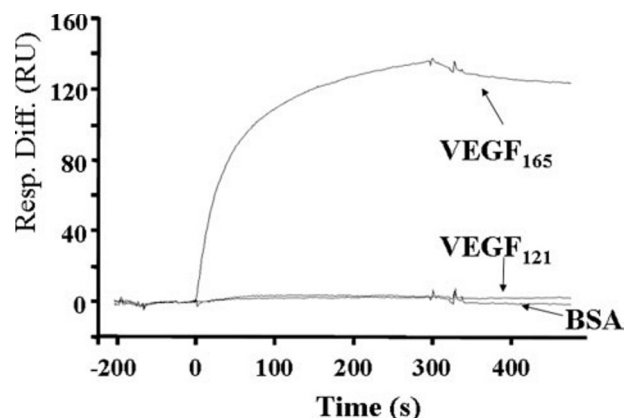


FIGURE 8. Specificity of LMWF binding for VEGF₁₆₅. LMWF was injected over sensor surfaces immobilized with VEGF₁₆₅, VEGF₁₂₁, or BSA. The figure overlaid a representative binding curve in response units (RU) obtained by surface plasmon resonance analysis (BIAcore) with injection of 150 ng of LMWF at a flow rate of 30 μ l/min on each immobilized species.

mented EC medium, in 1% FCS medium, or 1% FCS + VEGF₁₆₅ (Fig. 1). To our knowledge, this is the first report that showed the efficacy of LMWF for enhancing EC tube formation with only a small amount of serum. Soeda *et al.* (42) show that a high molecular weight fraction of fucoidan has no effect on FGF-2 tube formation at a concentration of 10 μ g/ml whereas it inhibits FGF-2 tube formation at 100 μ g/ml (23). Matou *et al.* (22) showed that 10 μ g/ml of 20-kDa fucoidan enhances FGF-2 induced tube forming of HUVEC (22). Another study by Chabut *et al.* (23) has shown a positive effect for fucoidan on tube formation but in the presence of both FGF-2 and 5% FCS (23). The above studies seem to indicate that the molecular size of fucoidan with relation to concentration influence its effect on tube formation. The current study supports the hypothesis that a low molecular weight species of fucoidan has a generally positive effect on this process, whereas HMW fucoidan and/or high concentrations are usually inhibitory.

Given the ability of LMWF to enhance tube formation, we explored other cellular processes important to angiogenesis such as proliferation and motility. We observe that LMWF increases proliferation of HUVEC at low serum concentration or in presence of FGF-2, but does not increase the ability of VEGF₁₆₅ to induce HUVEC proliferation. This observation is consistent with other reports using 20 kDa or HMW fucoidan on HUVEC proliferation induced by FGF-2 and VEGF₁₆₅ (21, 35). The difference in the effect of fucoidan on proliferation induced by these growth factors suggests distinct signaling pathways (36). As a result, they could also influence other cellular processes important to angiogenesis, such as cellular motility, differently. In a well established assay using HUVEC (25, 37), we demonstrate that VEGF₁₆₅-induced chemotaxis is greatly enhanced by LMWF (Figs. 2 and 3). We show that the combined effect of LMWF and VEGF₁₆₅ is synergistic since the chemotaxis effect induced by LMWF and VEGF₁₆₅ in combination is greater than the addition of their individual activities. Furthermore, among the sulfated polysaccharides tested in this report, LMWF is the most potent, followed by unfractionated fucoidan, which has a slightly lesser, but still synergistic effect on migration of HUVEC when combined with VEGF₁₆₅. Unfractionated heparin, LMW heparin, and LMW-sulfated

dextran slightly increase VEGF₁₆₅-induced migration, but the effect is not synergistic. Chondroitin sulfate has no effect. Based on these experiments, it appears that LMW fucoidan possesses structural properties beyond sulfation that allows it to specifically influence the effect of VEGF₁₆₅ on HUVEC.

To our knowledge this is the first report of LMW fucoidan enhancing VEGF₁₆₅-induced migration. It has been previously reported that fucoidan can increase HUVEC motility induced by FGF-1 and FGF-2 (21) and that fucoidan has no effect on VEGF₁₆₅-induced migration of HUVEC in two studies (21, 35). In the first study, Giroux *et al.* (21) used scratch wound assays to show that 20-kDa fucoidan had no effect on VEGF₁₆₅-induced motility (21). While the scratch wound assay is a great tool for observing increase or decrease in overall cell motility when surrounded by stimuli, the transwell assay measures cell movement toward an isolated stimulus. In the second study by Koyanagi *et al.* (35), transwell assays were used with HUVEC and they used a high molecular weight fraction of fucoidan. In addition to differences in polysaccharide size that can greatly influence biological activity (38–40), it is also likely that differences in the assays could account for variable results (coating on the filters was collagen IV *versus* fibronectin; time allowed for migration of 8 h *versus* 20 h; pore size in transwell filters of 8 μ m *versus* 3 μ m). For instance, a 3 μ m pore size used in our study represents a more selective barrier for ECs, thus accenting differences in chemotaxis *versus* an increase in overall motility. Our experiments also show that migration induced by a VEGF-A splice variant that lacks the heparin binding domain, VEGF₁₂₁, is unaffected by LMWF. Taken together with the fact that neither unfractionated heparin nor LMW heparin enhance VEGF₁₆₅-induced migration synergistically, these data suggest that LMWF interacts with VEGF₁₆₅ in a more complex way than by simply binding to the protein. It is also quite interesting that LMWF can greatly enhance HUVEC chemotaxis in response to VEGF₁₆₅, while not increasing cellular proliferation. This finding led us to hypothesize that this polysaccharide must have a unique mode of interaction with VEGF₁₆₅ and its receptors on endothelial cells.

VEGF-A is known to have three types of receptors on the EC surface, VEGFR-1 (Flt-1), VEGFR-2 (Flk-1), and neuropilin-1 (NRP1). While the detailed mechanisms for these receptors are still under intense investigation, they all are known to play important roles in vasculogenesis and angiogenesis (26, 28, 41). The absence of effect of LMWF for VEGF₁₆₅ binding on VEGFR-1 correlated with the absence of effect of this receptor on endothelial cell migration. While VEGFR-1 is thought to act as a “decoy” receptor that serves as a negative regulator for VEGF-A signaling, VEGFR-2 serves as the primary mediator of the mitogenic, pro-migratory, and survival effects (26, 29). NRP1 is thought to enhance the binding of VEGF₁₆₅ to VEGFR-2 and also to potentiate the chemotaxis of EC (26, 28). Interestingly, the VEGF₁₂₁ splice variant does not bind NRP1, suggesting at least a partial explanation for the reduced potency of this form (26). We demonstrate that LMWF does not enhance migration induced by VEGF₁₂₁, and that LMWF does not bind to the VEGF₁₂₁ isoform.

We show in this report that LMWF increases the binding of VEGF₁₆₅ to VEGFR-2 and NRP1. Consistently, the concentra-

tion range for the binding effect matches the range for which we observe effects on EC migration. The enhancing effect started at LMWF concentrations of 0.2 nM. Surprisingly, the enhancing effect on the binding of VEGF₁₆₅ to VEGFR-2 was much higher with LMWF than with LMW heparin. Surface plasmon resonance (Figs. 7 and 8) evidenced the high affinity of LMWF for VEGF₁₆₅ and VEGF receptors. Our results are also consistent with the data that NRP1 enhances VEGFR-2 activity by forming a multimeric complex with VEGF₁₆₅ (32). By binding to both VEGF₁₆₅ and VEGF receptors, LMWF may form various types of bridges VEGF-receptor/LMWF/VEGF₁₆₅ and thus modulate differently the number and affinity of available VEGF molecules in the vicinity of each type of receptor. The high efficiency of LMWF on cell migration could thus be linked to the dual effect on VEGFR-2 and NRP1.

Heparan sulfate proteoglycans have been shown to regulate the binding of VEGF to its receptors in a complex way which may depend on VEGF isoforms, the cell types involved, and the specific VEGF receptors present on the cell surface (26). A possible role of LMWF could be to increase the local concentration of VEGF₁₆₅ at the cell surface, thus greatly enhancing the probability of receptor binding. Moreover, the affinity of LMWF for VEGF₁₆₅ is quantitatively comparable with the physiological affinities of growth factors for heparan sulfate proteoglycans present on cells, further supporting the use of LMWF as a functional analog of heparan sulfates. In conclusion, our *in vitro* results indicate that the presence of LMWF increases VEGF₁₆₅-induced EC migration and enhances the binding of VEGF₁₆₅ to VEGFR-2 and NRP1. LMWF effect on VEGFR-2 and NRP1 act in a highly efficient synergistic manner to enhance endothelial cell migration but not cell proliferation. This unique polysaccharide may help to elucidate precise roles played by polysaccharides in the complex process of angiogenesis. Additionally, the specific and potent activity of this polysaccharide from a vegetal origin could also be a good candidate for aiding therapeutic revascularization as already suggested by our previous *in vivo* experiments (11).

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