ABSTRACT

Low-molecular-weight heparin (LMWH) has been used in cancer patients with venous thromboembolic complications, resulting in a higher survival rate and an inhibitory action on experimental metastasis. In the present study, human umbilical vein endothelial cells (HUVECs) were treated with LMWH for 24 h. We found that the resulting HUVECs could significantly inhibit the highly metastatic human prostate cancer cell line (PC-3M) in terms of its adhesion to the endothelium and migration across the endothelium, according to scanning electron microscopy. We also determined the elevated levels of endothelial intercellular Ca\(^{2+}\) concentration after the adhesion of PC-3M cells to HUVECs was greatly reduced by incubation with LMWH. Using proteomics, we surveyed the global protein changes in HUVECs after LMWH treatment and identified four down-regulated proteins that were possible isoforms of cytoskeletal vimentin intermediate filaments, cartilage-derived C-type lectin, and serine/threonine protein phosphatase 1β (PP-1B). LMWH affected the morphology of vimentin and the expression levels of α\(_5\) integrin and PP-1B in HUVECs bound to PC-3M cells. Vimentin assists in the adhesion of PC-3M cells, which was confirmed by short interfering RNA experiments. Furthermore, the direct binding of purified vimentin protein with LMWH was detected with surface plasmon resonance methods. However, when we used fluorescence-labeled heparin for 24 h to identify whether this binding occurred within cells, heparin was distributed principally around endothelial cells. Taken together, these findings suggest that the mono-incubation of LMWH with HUVECs could inhibit PC-3M cell adhesion to, and migration through, endothelium. LMWH’s regulation of vimentin plays a role in the antimetastatic action.

 Role of Vimentin in the Inhibitory Effects of Low-Molecular-Weight Heparin on PC-3M Cell Adhesion to, and Migration through, Endothelium

Yan Pan, Tianluo Lei, Bao Teng, Jihong Liu, Jianzhao Zhang, Yu An, Yuan Xiao, Jing Han, Xueyang Pan, Junhua Wang, Heming Yu, Hong Ren, and Xuejun Li

State Key Laboratory of Natural and Biomimetic Drugs, Department of Pharmacology, School of Basic Medical Sciences, and Institute of System Biomedicine, Peking University, Beijing, China

Received March 28, 2011; accepted June 30, 2011

ABbreviations: LMWH, low-molecular-weight heparin; CFDA, carboxyfluorescein diacetate; DTT, dithiothreitol; EC, endothelial cell; HUVEC, human umbilical vein endothelial cell; IEF, isoelectric focusing; IPG, immobilized pH gradient; MALDI-TOF-MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; PBS, phosphate-buffered saline; RU, response units; PAGE, polyacrylamide gel electrophoresis; SEM, scanning electron microscopy; siRNA, short interfering RNA; SPR, surface plasmon resonance; TGF-β, transforming growth factor-β; PP-1B, protein phosphatase 1β; 2D, two-dimensional; FITC, fluorescein isothiocyanate; RT-PCR, reverse transcription-polymerase chain reaction; [Ca\(^{2+}\)]\(_{i}\), intracellular calcium concentration; ns-siRNA, nonsilencing siRNA; siVimentin, vimentin-targeted siRNA.
and plasminogen. Tumor cell adhesion to, and migration through, the EC layer both are essential components of tumor metastasis (Kramer and Nicolson, 1979). The vascular endothelium constitutes an anatomical barrier between circulating tumor cells and extravascular tissues, and its integrity is important in maintaining this function (Kusama et al., 1995). Some reports have shown that LMWH can protect ECs from activation and that it can inhibit the expression of cell adhesion molecules when used as an anticoagulant in patients with coronary artery disease (Lindmark and Siegbahn, 2002; Manduteanu et al., 2002). We proposed that the protective effects of LMWH on ECs could possibly also aid its antimetastatic pharmacological actions.

In this study, we investigated 1) the effects of incubating endothelium with LMWH and 2) the adhesion to and migration through the endothelium of a type of tumor cell, high metastasis human prostate cancer cells (PC-3M), to analyze and confirm the effects of LMWH on tumor metastasis and explore its molecular mechanisms.

Materials and Methods

Cell Culture. Human umbilical vein endothelial cells (HUVECs) were isolated using collagenase I (1 mg/ml; Invitrogen, Carlsbad, CA) digestion of umbilical veins from undamaged sections of fresh cords. HUVECs were grown in M199 (Invitrogen) supplemented with 20% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 200 g/ml streptomycin, and 2 mM L-glutamine. The identification of HUVECs was confirmed by their polygonal morphology and detection of their immunoreactivity for factor VIII-related antigens (Andrews et al., 2001). PC-3M cells were cultured in RPMI medium 1640 containing 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 g/ml streptomycin in a humidified incubator with 5% CO2 in air at 37°C.

Adhesion Assay. PC-3M cells were labeled with 100 g/ml carboxyfluorescein diacetate (CFDA; Sigma-Aldrich) for 30 min at 37°C (Woodward et al., 2002). Cells were washed with phosphate-buffered saline (PBS) twice to remove residual fluorescent dye. Cell viability was not compromised by this labeling protocol, as indicated by trypan blue exclusion.

HUVECs were cultured in 96-well dishes coated with 1% gelatin. When HUVECs reached an approximate confluence of 80%, they were treated with 0.05 to 500 g/ml heparin (LMWH; Sigma-Aldrich) for 24 h. Then, tumor cells in suspension were pretreated with 0.05 to 500 g/ml heparin (LMWH; Sigma-Aldrich; deaminated sodium salt from porcine intestinal mucosa, average molecular mass 5 kDa) for 24 h; HUVECs were then cultured in 96-well dishes coated with 1% gelatin (treated with or without LMWH) containing 55 mM fluoride (Sigma-Aldrich). The protein concentration in lysates was measured using the Bradford method (Bradford, 1976). Two samples were plated onto a confluent Petri dish (105 cells) and incubated with 50 g/ml LMWH for 24 h. Confluent HUVEC monolayers were washed three times with M199 solution and loaded with 5 mM Fluo-3/AM (Invitrogen) in M199 for 1 h at 37°C and used for intracellular Ca2+ measurement (Minta et al., 1998; Lewalle et al., 1998). Tumor cells to be plated on Fluo-3-loaded HUVECs were detached using 0.1% EDTA in PBS and added to HUVEC monolayers at a final concentration of 5 105 cells in 50 l per chamber. Ca2+-dependent changes in fluorescence intensity were recorded continually in individual cells using a TCS-NT laser scanning confocal microscope (Leica, Wetzlar, Germany) with an excitation wavelength of 490 nm.

Two-Dimensional Electrophoresis. After HUVECs reached subconfluence, they were treated with 50 g/ml LMWH for 24 h. Immediately after treatment, cells were placed on ice, washed with ice-cold PBS, suspended at a concentration of approximately 105 cells/ml, and lysed with a buffer consisting of 40 mM Tris, 8 M urea, 4% (w/v) 3-[[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate, 60 mM dithiothreitol (DTT), and 1 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich). The protein concentration in lysates was measured using the Bradford method (Bradford, 1976). Two samples (treated with or without LMWH) containing 55 g of protein, respectively, were added to the rehydration solution (8 M urea, 2% 3-[[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate, 0.5% immobilized pH gradient (IPG) buffer, 0.28% DTT, and trace bromophenol blue). The final volume was 125 l.

Isoelectric focusing (IEF) was performed in the initial dimension, using commercially available preformed IPGs (linear pH gradient 3–10, 7 cm). Gels were rehydrated overnight by placing strips gel side down in a solution that contained rehydration solution in an IPGphore strip holder (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) and covered with mineral oil. IEF was conducted using an IPGphore Isoelectric Focusing System (GE Healthcare). IEF was run at 20°C using the step-and-hold and gradient methods as follows: IPG strips were rehydrated with sample at 0 V for 6 h, then IEF was performed at 30 V for 6 h, linear increase to 200 V for 0.5 h, held at 500 V for 0.5 h, followed by 1000 V for 0.5 h, then at 8000 V for 4 to 5 h. Before second-dimension electrophoresis [SDS-polyacrylamide gel electrophoresis (PAGE)], the IEF strips were equilibrated for 15 min in solution (6 M urea, 30% glycerol, 50 mM Tris-HCl, pH 8.8, 2% SDS) containing 10 mg/ml DTT for 15 min, and cells on the upper face of the membrane were scraped using a cotton swab, and tumor cells that had migrated to the lower face of the filter were trypsinized and counted using a flow cytometer (BD-LSR; BD Biosciences, San Jose, CA).

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Assay. HUVECs were plated into 96-well dishes (1 105 cells/well) and incubated in M199 medium for 24 h. The culture medium was changed to a medium containing a range of concentrations of LMWH and incubated for 24 h. At the end of these treatments, 20 l of sterile 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium dye (Sigma-Aldrich) was added. Cells were incubated at 37°C for 2 h. After removing medium, 100 l of dimethyl sulfoxide was added and thoroughly mixed. Spectrometric absorbance at 540 nm (for formazan dye) and 690 nm (as background level) was measured using a microplate reader (Bio-Rad Laboratories, Hercules, CA) (Tozawa et al., 2003).

Electron Scanning Microscopy Assay. SEM analysis was used to detect the effect of LMWH on morphological changes in HUVECs when bound by PC-3M cells. Cell culture and drug incubation methods were similar to those used in the adhesion assay. After 30 min, the coculture was rinsed and fixed in 37°C with 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.3, for 1.5 h. After fixation, cells were stored at 4°C in buffer until final processing, which was accomplished by dehydrating cells in a graded series of acetones and then drying out in liquid CO2. Cells were coated with a thin layer of gold-palladium before viewing under a JEOL JSM-5600LV scanning electron microscope (JEOL, Peabody, MA) (Douglas et al., 1999).

The Measurement of Intracellular Ca2+ in HUVECs. HUVECs were plated on a confluent Petri dish (105 cells) and incubated with 50 g/ml LMWH for 24 h. Confluent HUVEC monolayers were washed three times with M199 solution and loaded with 5 mM Fluo-3/AM (Invitrogen) in M199 for 1 h at 37°C and used for intracellular Ca2+ measurement (Minta et al., 1998; Lewalle et al., 1998). Tumor cells to be plated on Fluo-3-loaded HUVECs were detached using 0.1% EDTA in PBS and added to HUVEC monolayers at a final concentration of 5 105 cells in 50 l per chamber. Ca2+-dependent changes in fluorescence intensity were recorded continually in individual cells using a TCS-NT laser scanning confocal microscope (Leica, Wetzlar, Germany) with an excitation wavelength of 490 nm.
then for another 15 min in equilibrium solution containing 25 mg/ml iodoacetamide. SDS-PAGE was run in a 10% gel, without stacking gels, using the Pharmacia IsoDalt system. The IPG gel strips were embedded on top of the gels with 0.5% agaroose. Electrophoresis was conducted at a constant current of 30 mA/gel. The protein isoelectric point and molecular mass, respectively, were assigned by calibration of 2D PAGE gels with carboxymethyl isoelectric point calibration markers and molecular mass markers (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). Gels were stained with Coomassie Blue. The experimental procedures were repeated three times. The differentially expressed protein spots were excised manually from the SDS-PAGE gels and subject to in-gel tryptic digestion.

**Mass Spectrometry Identification of Proteins.** The protein spot of interest was analyzed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS). The proteins from the selected spots were reduced with DTT and alkylated with iodoacetamide before overnight digestion with a sequence-grade modified trypsin (Promega, Madison, WI). The peptide mixture was concentrated and desalted using Millipore ZipTip m-C18 pipette tips (Millipore Corporation, Billerica, MA). The peptide mass fingerprints were measured with a Biflex III MALDI-TOF mass spectrometer (Bruker, Newark, DE) in a positive ion reflector mode using α-cyano-4-hydroxycinnamic acid as a matrix. The database searches were performed using Mascot (www.matrixscience.com).

**Immunofluorescence Assay.** HUVECs were grown on glass coverslips in 5% CO₂ in air at 37°C and then incubated in 50 g/ml LMWH for another 24 h. Next, 3000 CFDA-labeled PC-3M cells were added to the coverslips and incubated with HUVECs for 30 min. After they were gently washed in PBS, the coverslips were fixed in 3.7% formaldehyde for 10 min. Then, the fixed cells were incubated with anti-human vimentin monoclonal antibodies (Clone V9; NeoMarkers, Fremont, CA) at 37°C in a humid chamber for at least 1 h, washed three times in PBS, and incubated with goat anti-mouse IgG secondary antibody for an additional hour at 37°C. For samples stained with FITC, HUVECs were incubated with 0.625 µg/ml of propidium iodide for 15 min at room temperature. After several washes, stained specimens were viewed using a TCS-SP2 laser scanning confocal microscope (Leica) (Gonzales et al., 2001).

**RNA Isolation and Reverse Transcription-Polymerase Chain Reaction.** HUVECs were grown to at least 80% confluence and then incubated in different concentrations of LMWH for another 24 h. Then total RNA was isolated using TRIzol reagent (Invitrogen, Groningen, The Netherlands). A further set of samples was investigated as follows: HUVECs were grown to at least 80% confluence and then incubated with different concentrations of LMWH for another 24 h. Next, PC-3M cells were added to the culture wells and incubated with HUVECs for 2 min. Total RNA was immediately isolated with the use of TRIzol reagent. First-strand cDNAs were generated from RNA samples by reverse transcription using oligo(dT)s. The following primers were used: β-actin forward, 5'-ATCATGGTTCGACCTCACAACA-3' and reverse, 5'-CATCCTTGTCGAGGACTCC-3'; PP-1B forward, 5'-CGTCTGCTACTCCACCTC-3' and reverse, 5'-TCTTTTACCCGCTCCT-3'; vimentin forward, 5'-GACAATGCTGTCGTTCCGCTT-3' and reverse, 5'-TCCCTCGGTCCGGACTTCT-3'; transforming growth factor-β (TGF-β) forward, 5'-CAGTGGAATCAGCAGGACC-3' and reverse, 5'-GGCCATGAGAAGCCAGGAAG-3'; E-cadherin forward, 5'-TGGGAGATTCGCGCTTACG-3' and reverse, 5'-CAATCAGTCAGCTGCAGCCCT-3' and α, integrin forward, 5'-GACTGTTGGAGAACATGTGCTGTA-3' and reverse, 5'-CGACTAAAGTGTAGTGGTCCGCC-3'.

**Western Blot Analysis.** Cell culture and drug levels were the same as that for the RT-PCR experiment. Total protein was extracted using radioimmunoprecipitation assay lysis buffer. Equal amounts of protein were subjected to SDS-PAGE on a 10% polyacrylamide gel for the detection of vimentin. The membrane with blotted protein was blocked for 1 h with blocking buffer containing 5% nonfat dried milk and 0.05% Tween 20 in Tris-buffered saline, followed by incubation with antivimentin antibody (1:1000, Clone V9; NeoMarkers), which was diluted in blocking buffer overnight at 4°C. Then, the membrane was washed three times with 0.05% Tween 20 in Tris-buffered saline for 30 min and incubated at room temperature for 1 h with diluted (1:2000) secondary horseradish peroxidase-labeled IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Detection was done using the enhanced chemiluminescence method.

**Short Interfering RNA Transfection.** siRNA duplexes used in this study were bought from Santa Cruz Biotechnology, Inc. to interfere with vimentin expression. Nonsilencing siRNA (5'-UAAUAGACUGUUGGCUCUUGATTT-3' and 5'-UAAGGCGCAAGUCUCAUAAATdT-3') was used as the negative controls. siRNA duplexes were transfected into HUVECs with siRNA transfection reagent (Polyplus-transfection Inc., San Marcos, CA), according to the manufacturer's instructions. After transfection for 48 h, cells were subjected to Western blot analysis to detect the efficiency of vimentin knockdown after binding of PC-3M cells.

**Surface Plasmon Resonance Studies.** All chemicals, bovine serum albumin, and dimethyl sulfoxide were purchased from Sigma-Aldrich, Biacore3000, CM5 series sensor chips, and coupling reagents [N-ethyl-N'-3-(3-dimethylaminopropyl)carbodiimide, N-hydroxysuccinimide, and ethanolamine-HCl] were purchased from BIAcore AB (Uppsala, Sweden). LMWH was dissolved directly in HEPES-buffered saline (10 mM HEPES, 150 mM NaCl, pH 7.4).

CM5 sensor chips were placed in the instrument and preconditioned with water (100 µl/min) by applying two consecutive 50 µl/min pulses of 50 mM NaOH and 1 M NaCl, then 10 mM HCl and 0.1% SDS, and finally water. Vimentin protein (Sigma-Aldrich) was immobilized onto CM5 sensor chips using amine-coupling chemistry in running buffer, which consisted of 10 mM HEPES, 150 mM NaCl, 3 mM EDTA, and 0.005% surfactant P-20, pH 7.4. Vimentin at a concentration of 50 µg/ml in 10 mM sodium acetate, pH 5.2, was injected for 7 min, resulting in immobilized densities that averaged 2000 to 4000 response units (RU). The surfaces were blocked with an 8-min injection of 1 M ethanolamine, pH 8.0. Before use, the surface coverage of vimentin on CM5 sensor chips was evaluated using antivimentin antibody (Clone V9; NeoMarkers) binding assay on CM5 sensor chips and vimentin-immobilized CM5 sensor chips.

The binding assay was performed in 10 mM HEPES, 150 mM NaCl, 3 mM EDTA, and 0.005% surfactant P-20, pH 7.4 running buffer at 25°C. LMWH was injected over the vimentin-immobilized CM5 sensor chips and reference surfaces from 0.5 to 50 µg/ml in 2-fold dilutions at a flow rate of 50 µl/min. Triplicate injections of each LMWH concentration were analyzed in random order. The LMWH-vimentin complex was allowed to associate and dissociate for 60 and 20 s, respectively, and the surfaces were washed with running buffer for 10 s between each sample injection. Buffer blanks were injected after LMWH for double referencing. Data were collected at a rate of 1 Hz.

All sensograms were processed by first subtracting the binding response recorded from the control surface (center reference spot), followed by subtracting an average of the buffer blank injections from the reaction spot.

**Detection of Fluorescently Labeled Heparin Inside HUVECs.** To identify whether heparin could enter HUVECs through the cell membrane, we incubated these cells with fluorescently labeled heparin (Invitrogen) for 24 and 48 h. Then cells were washed three times with PBS to remove excess heparin. Next, an antivimentin antibody (Clone V9; NeoMarkers) binding assay on CM5 sensor chips and vimentin-immobilized CM5 sensor chips was performed by first subtracting the binding response recorded from the control surface (center reference spot), followed by subtracting an average of the buffer blank injections from the reaction spot.

**Statistical Analysis.** Each of these experiments was conducted in triplicate. Data are expressed as mean ± S.E.M. Statistical significance of differences between means was determined by one-way analysis of variance followed by Dunnett’s test (SPSS 10.0 software; SPSS Inc., Chicago, IL). A p value of <0.05 was considered to be statistically significant.
Results

Inhibitory Effect of LMWH on PC-3M Adhesion to HUVECs. CFDA was used to label PC-3M cells to differentiate PC-3M cells from HUVECs under a 492-nm excitation wavelength. The viability of CFDA-labeled PC-3M cells was more than 95%. LMWH used at a concentration between 0.5 and 500 μg/ml reduced the number of tumor cells that adhered to the HUVEC monolayer. The numbers of PC-3M cells found in the 50 and 500 μg/ml LMWH groups (32 ± 1.6 and 30 ± 1.9, respectively) were significantly different (p < 0.01) compared with that of the untreated control group (43 ± 4.9) (Fig. 1).

Under SEM, HUVECs were seen to form a monolayer with gaps of 10 to 20 nm between cells (Fig. 2A). Most tumor cells were seen to adhere to the boundary of the HUVECs, with fewer cells sticking to the HUVEC surface. The addition of PC-3M cells caused shrinkage of the HUVECs such that the gaps between HUVECs widened (Fig. 2B). LMWH prevented HUVEC shrinkage when added at a concentration of 50 μg/ml (Fig. 2C).

Under SEM high magnification, the surfaces of normal HUVECs were seen to be smooth (Fig. 2D), but when PC-3M cells adhered to the surface of these cells breaks in the membranes could be seen with resulting leakage of cytoplasm granules (Fig. 2E). In contrast, when HUVECs were treated with 50 μg/ml LMWH for 24 h their membrane surfaces were smoother than those of the untreated controls after PC-3M cell adhesion (Fig. 2F).

Inhibitory Effect of LMWH on PC-3M Cell Transendothelial Migration. Transwell chambers were used to asssay the effect of LMWH on PC-3M cell transendothelial migration after tumor cell adhesion to HUVEC monolayers. Fibronectin was added into the basolateral chamber to function as a chemoattractant. PC-3M cells were cocultured with HUVECs for 18 h; cells that migrated to the lower face of the filter all were shown to be PC-3M cells using factor VIII staining (data not shown). The pretreatment of HUVECs with LMWH for 24 h led to a dose-dependent decrease in the transendothelial migration of PC-3M cells across the HUVEC monolayers (Fig. 3). The number of PC-3M cells in the 50 and 500 μg/ml LMWH groups (8700 ± 399 and 7768 ± 328 cells, respectively) that migrated across the epithelium was greatly reduced (p < 0.01) compared with that found for the control group (14,249 ± 1204 cells).

Effect of LMWH on Viability of HUVECs. LMWH concentration did not influence HUVEC cell growth significantly after 24-h treatment (p > 0.05; data not shown).

Effect of LMWH on PC-3M Cell-Induced [Ca^{2+}]_i Elevation in HUVECs. Other studies have found that endothelial cell intracellular Ca^{2+} concentration is increased after tumor cell contact and this rise mediates tumor cell transendothelial migration (Lewalle et al., 1998). We measured the Ca^{2+} concentration in HUVECs that had been pretreated with LMWH for 24 h to determine whether this treatment affected the level of intracellular calcium ions in HUVECs after PC-3M tumor cell adhesion. PC-3M cell-induced [Ca^{2+}]_i change in HUVECs was expressed as relative fluorescence density (F/F_0), in which F_0 is the initial fluorescence density of each cell, and F is the real-time fluorescence density of the same cell after the addition of tumor cells. When quiescent, HUVEC monolayers had a weak Ca^{2+} signal that was detected in the cytoplasm and remained stable for several hours in the assay buffer (Fig. 4A). The addition of PC-3M to the HUVEC monolayers induced an immediate and marked elevation in HUVEC [Ca^{2+}]_i with a maximal increase of 220% of relative fluorescence intensity that was reached within 20 s after PC-3M contact with HUVECs (Fig. 4B). When we used PC-3M-conditioned medium, no elevation in HUVEC [Ca^{2+}]_i was observed (data not shown). The [Ca^{2+}]_i levels then decreased slowly to the basal level within 200 to 220 s (Fig. 4G). In HUVECs that were pretreated with 50 and 500 μg/ml LMWH, before PC-3M addition there is a weaker Ca^{2+} signal similar to that of carrier-treated group (Fig. 4, C and E); the addition of PC-3M to the HUVEC monolayers induced a slow and delayed elevation in [Ca^{2+}]_i (Fig. 4, D and F). For HUVECs pretreated with 50 and 500 μg/ml LMWH, respectively, this elevation in [Ca^{2+}]_i, almost completely abated, and the maximal increase of relative fluorescence intensity of HUVEC [Ca^{2+}]_i was approximately 170 and 140%, respectively (p < 0.01 compared with the carrier group; Fig. 4G).

Comparative Proteomic Analysis in HUVECs with or without LMWH Treatment. Schematic representations of both control and treated 2D electrophoresis protein patterns are shown in Fig. 5A, which shows more than 150 Coomassie
Blue-stained protein spots, with most spots in the pH 4 to 7 region. The protein profile of carrier-treated and LMWH-treated HUVECs was compared using computer-assisted analysis of the Coomassie-stained spot patterns; 43 protein spots were found to alter their expression level in response to LMWH exposure.

Four spots that were significantly affected by LMWH treatment were subjected to tryptic digestion and MALDI-TOF-MS analysis. Of these four spots, two marked as C111 and C125 had a decrease in intensity; the other two protein spots, marked as L38 and L141, increased in intensity after LMWH treatment. The data obtained from MALDI-TOF-MS (Fig. 5, B–E) were applied to the protein database, and searches were performed to determine the identity of the four proteins or peptides. Our results showed that protein spots C111 and C125 might be vimentin, protein components of class III intermediate filaments; protein spot L38 might be a C-type lectin superfamily member 1 precursor (cartilage-derived C-type lectin) fragment; and protein spot L141 might be serine/threonine PP1-B catalytic subunit fragment. We confirmed this change using immunofluorescence chemistry, Western blot, and RT-PCR analysis and investigated the function of vimentin in LMWH’s antiadhesion effect on HUVECs and PC-3M cells using vimentin siRNA.

Effects of LMWH on Vimentin Redistribution in HUVECs and PC-3M Cell Adhesion. We decided to look at changes in vimentin expression and redistribution in HUVECs and on PC-3M cell adhesion after 30 min to inves-
tigate the mechanisms of the LMWH effects on endothelium and PC-3M cell adhesion and migration.

Carrier-treated HUVECs presented a finely stained intracellular vimentin network that radiated homogeneously from the perinuclear area to the cell periphery (Fig. 6, A and C). LMWH-treated HUVECs presented no dramatically different distribution pattern compared with the control, with only a slight change in vimentin filaments (Fig. 6B).

When PC-3M cells were added to the HUVECs, the vimentin in the HUVECs showed significant filament bundling and collapsed intermediate filaments (Fig. 6D). LMWH-treated HUVECs could alleviate this change and presented a more normal radial pattern (Fig. 6E).

**Effects of LMWH on Vimentin Protein and Vimentin-Related Gene Expression in HUVECs and PC-3M Cell Adhesion.** After incubation of HUVECs with LMWH (50 and 500 μg/ml) for 24 h, there was an increase tendency in E-cadherin and TGF-β gene expression and a decrease in PP-1B and αv integrin gene expression. LMWH at the concentration of 500 μg/ml could significantly inhibit the mRNA expression of αv integrin (p < 0.05). In contrast, expression of vimentin only decreased by a small amount, and this was observed only at the higher dose (500 μg/ml) (Fig. 6F). For HUVECs with bound PC-3M cells, LMWH at the 50 μg/ml concentration could inhibit the expression of PP-1B and αv integrin significantly (p < 0.05; Fig. 6G).

**Vimentin-Targeted siRNA in HUVECs Reduced PC-3M Cell Adhesion to Endothelium.** When HUVECs were transfected with siRNA against the vimentin gene for 48 h, the expression of vimentin in HUVECs was partially reduced by 25% in comparison with the controls (p < 0.05; Fig. 7A), and the adhesion of PC-3M cells to HUVECs was reduced to approximately 16% of the value of the control, that is the number of adherent tumor cells to HUVECs decreased from 67 ± 5 in controls to 56 ± 4 in vimentin-targeted siRNA (p < 0.05; Fig. 7B).

---

**Fig. 4.** Inhibitory effect of LMWH on PC-3M cell-induced [Ca²⁺] elevation in HUVECs. A to F, HUVEC monolayers pretreated with carrier (A and B), 50 μg/ml LMWH (C and D), or 500 μg/ml LMWH (E and F) were loaded with Fluo-3/AM. Changes in [Ca²⁺] were recorded in individual endothelial cells after the addition of PC-3M cells for 5 min. [Ca²⁺] levels in endothelial cells after the addition of PC-3M cells are shown in B, D, and F. G, the addition of PC-3M cells is indicated by the arrow. The PC-3M cell-induced [Ca²⁺] changes in HUVECs are expressed as relative fluorescence density (Ft/F₀), in which F₀ is the initial fluorescence density of each cell, and Ft is the real-time fluorescence density of the same cell after the addition of tumor cells. Each experiment was repeated in triplicate. Mean ± S.E.M. (n = 3). *, p < 0.05 and **, p < 0.01 compared with carrier group.
Fig. 5. Two-dimensional electrophoresis of HUVEC proteins before and after LMWH treatment with marked spots selected for MALDI-TOF-MS. A, representative two-dimensional electrophoresis maps of proteins in HUVECs without or with LMWH treatment. Approximately 55 μg of total protein was focused on linear IPG strips (pH 3–10, 7 cm) before separation by 10% SDS-PAGE. Coomassie Blue staining was used to detect protein spots. Experiments were conducted in triplicate. B and C, MALDI-TOF-MS analysis of protein C111 and C125 mass spectrometry of in-gel trypsin digests of this protein resulted in the identification of vimentin. D, MALDI-TOF-MS analysis of protein L38 mass spectrometry of in-gel trypsin digests of this protein resulted in the identification of C-type lectin superfamily member 1 precursor (cartilage-derived C-type lectin) fragment. E, MALDI-TOF-MS analysis of protein L141 mass spectrometry of in-gel trypsin digests of this protein resulted in the identification of serine/threonine PP1-γ catalytic subunit fragment.
Binding of LMWH to Vimentin by SPR. SPR biosensor technology was used to detect the association and dissociation of LMWH to vimentin in real time. Sensorgrams were processed using automatic correction for nonspecific bulk refractive index effects. Figure 8A shows a typical sensorgram obtained by using LMWH concentrations of 0.5 to 50 μg/ml, which had been corrected for the response caused by refractive index differences between running buffer and sample solution by subtracting the respective curves from a reference channel. We found that LMWH treatment resulted in...
a significant and dose-dependent increase in SPR RU. The LMWH concentration series were fitted to a one-to-one binding with a mass-transfer model encoded in the BIAcore 3000 evaluation software for binding affinity determination.

**Drug Distribution of Fluorescently Labeled Heparin in HUVECs.** When fluorescently labeled heparin was added to HUVEC-conditioned medium for 24 h, confocal imaging showed us that most of this heparin was located around the cell membrane with only a small amount inside the cells. When this incubation was extended to 48 h, confocal imaging showed that several heparin particles had entered some cells and was found mainly in the cytoplasm (Fig. 8B).

**Discussion**

The intact endothelium can serve as a “defensive barrier” to the extravasation of tumor cells (Lewalle et al., 1997; Lee et al., 2003). In the present study, we incubated HUVECs with LMWH for 24 h before the addition of PC-3M cells. We found that both adhesion of PC-3M cells to HUVECs and tumor cell transendothelial migration in vitro were inhibited by the incubation of HUVECs with LMWH. We therefore proposed that LMWH protected the HUVECs from damage caused by PC-3M cells and enhanced the role of endothelium as a barrier against the extravasation of tumor cells (Lewalle et al., 1997; Lee et al., 2003). We found that LMWH-treated HUVECs adhered to by PC-3M cells had enhanced endothelial integrity under SEM. Furthermore, the incubation with LMWH could reduce the level of HUVEC stimulation by inhibiting the rise in Ca\(^{2+}\) ions that occurred when PC-3M tumor cells bound to the HUVEC cell surface.

Malignant tumor cells can transiently increase the calcium concentration of endothelial cells in the contact area and stimulate endothelial cell retraction by disrupting the intercellular junctions and promoting the transendothelial migration of tumor cells (Lewalle et al., 1998; Lee et al., 2003). This rapid increase of [Ca\(^{2+}\)]\(_i\) in HUVECs stimulated by PC-3M cells was inhibited when HUVEC monolayers alone were treated with LMWH. Intracellular Ca\(^{2+}\) movement is part of a signal transduction pathway that is initiated by the contact between tumor cells and endothelial cells. Transient changes in endothelial [Ca\(^{2+}\)]\(_i\) may have persistent effects and mediate multiple steps in the transendothelial migration of tumor cells. The effect of these on interendothelial cohesion and levels of cytosolic free Ca\(^{2+}\) may modulate the presence of various ligands on the endothelial cell surface, including lectins, intercellular adhesion molecules, and integrins (Pili et al., 1993). Furthermore, Ca\(^{2+}\) may stimulate the secretion of chemoattractant and/or motility factors, which then stimulate tumor cell transendothelial migration. Timar et al. (1992) found that calcium channel blockers (nifedipine, verapamil, and diltiazem) could inhibit the interaction between tumor cells and platelets; they postulated that tumor cell metastasis is caused not only by the effects of the Ca\(^{2+}\) channel blockers on platelets but also by their effect on the tumor cell cytoskeleton, including vimentin intermediate filaments.
ments. This results in the inhibition of the mobility and function of the αIIb β3 receptor (Timar et al., 1992).

Furthermore, Ca$^{2+}$ signals activate Ca$^{2+}$/calmodulin-dependent protein kinase II, which has been reported to be one of the kinases that phosphorylate vimentin in vitro. Thus, the amplitude and duration of an extracellular stimulation are likely to coordinate the spatially organized phosphorylation of vimentin filaments through the Ca$^{2+}$/calmodulin/calmodulin-dependent protein kinase II/vimentin signaling pathway (Inagaki et al., 1997). The structural organization of vimentin was strongly disrupted in the presence of the calcium channel blocker, and increased concentrations of calcium channel blocker could reduce the levels and assembly of vimentin phosphate. Therefore, we deduced that the inhibition of [Ca$^{2+}$], rise was involved during the modulation of vimentin morphology by LMWH in HUVECs (Retrosen and Gallin, 1986; Ehringer et al., 1996).

Vimentin is a protein that is altered by the effects of LMWH on HUVECs, which we detected with the use of the 2D electrophoresis methodology. Using IFC, we found that the structure of vimentin on HUVECs was altered greatly by the addition of PC-3M cells; vimentin in the HUVECs showed significant filament bundling and collapsed intermediate filaments; however, the resulting fusion of the cytoskeleton vimentin intermediate filament could be prevented greatly by a prior incubation of the HUVECs with LMWH for 24 h.

Vimentin intermediate filaments, in addition to their potential interactions with microfilaments and microtubules, participate in many other specialized cell functions. These include functioning as the cytoplasmic organizer, a supracellular mechanical scaffold, intercellular transporter, and signal transducer (Tang et al., 1993; Asaga et al., 1998). Several groups have reported that, in endothelial cells, vimentin controls directly or indirectly the cell-cell contact area and stabilizes cell-matrix adhesion (Tsuruta and Jones, 2003). Vimentin has a key role in adhesion through the regulation of integrin functions. It has been reported that vimentin colocalizes with adhesion plaques to stabilize the plaque. Adhesion plaque formation therefore influences cell-cell adhesion (Bershadsky et al., 1987; Burridge et al., 1988). In our present experiment, the structural organization of vimentin in endothelial cells by LMWH could therefore play an important role in the antiadhesion and antitransendothelial migration functions of LMWH. Our results of vimentin-targeted siRNA showed that partial knockdown of vimentin in HUVECs could reduce the ability of PC-3M cells to bind to HUVECs. We also ascertained that α, integrin alone was down-regulated in HUVECs preincubated with LMWH and in HUVECs after the addition of PC-3M cells. Therefore, we concluded that vimentin and α, integrin is involved in the molecular mechanism of LMWH inhibition on PC-3M cell adhesion to and migration through the endothelium.

It is noteworthy that SPR studies, which are used to detect molecular binding patterns, have shown that, as well as the regulation of the expression and structure of vimentin by LMWH, there is direct interaction between LMWH and vimentin when purified vimentin protein is used. Binding by the multihydroxyl molecule heparin may be the reason that it affects cellular vimentin, because previous findings have shown that heparin could bind to heparin sulfate on cell membranes and then has multiple biological activities. Does this direct binding between these molecules take place in cells? Therefore, we used fluorescently labeled heparin to determine whether heparin could exist in cells and binding with cytosolic vimentin. Incubation of HUVECs with fluorescently labeled heparin for 24 h showed that most of the heparin was not present inside the cell membrane. Therefore, we concluded that direct binding of heparin with vimentin was low or nonexistent for cells that underwent a 24-h incubation with fluorescently labeled heparin. However, LMWH can regulate vimentin, possibly via cell membrane molecules, such as tyrosine kinases, heparin binding proteins, or even inositol phosphate 3 receptors, rather than by direct intracellular binding with vimentin.

In addition to vimentin, we reconfirmed that the levels of PP-1B decreased with the 2D electrophoresis experiment, whereas TGF-β and E-cadherin gene expression levels increased. PP-1B is another protein whose gene expression was found to be influenced by LMWH in HUVECs. PP-1 is a major phosphatase that can directly dephosphorylate proteins, such as AKT, to modulate their activation and regulate the expression of downstream genes. It may be related with the inhibitory effect of heparin on tumor growth as other studies have reported; PP-1 can promote cell survival and modulate cell migration (Xiao et al., 2010). TGF-β has profound effects on all cell types that comprise the vasculature, including endothelial cells. TGF-β inhibits both the proliferation and migration of endothelial cells in monolayer cultures in vitro (Roberts and Sporn, 1989). E-cadherin, which is one of the most widely studied tumor suppressors in many kinds of cancers, belongs to a family of calcium-dependent cell adhesion molecules. The loss of E-cadherin expression has been reported to induce epithelial-mesenchymal transition in several cancers (Baranwal and Alahari, 2009). We deduced that the increase of E-cadherin in HUVECs after incubation with LMWH was beneficial for endothelial cells in terms of its antiadhesive and antitransendothelial migration properties.

In conclusion, in this study we have demonstrated that LMWH effectively inhibits PC-3M cell binding to, and migration across, endothelium by regulating endothelial calcium ions and vimentin and by the further down-regulation of integrins.

Authorship Contributions
Participated in research design: Y. Pan, Zhang, Xiao, Yu, and Li.
Conducted experiments: Y. Pan, Lei, Han, and Wang.
Contributed new reagents or analytic tools: Y. Pan, Lei, Teng, Liu, Yu, and Ren.
Performed data analysis: Y. Pan, Teng, Zhang, Xiao, X. Pan, and Wang.
Wrote or contributed to the writing of the manuscript: Y. Pan, Teng, An, and Li.

References
Bershadsky AD, Tint IS, and Svitkina TM (1987) Association of intermediate fila-
ments with vinculin-containing adhesion plaques of fibroblasts. Cell Motil Cyto-
skeleton 8:274–283.
Douglas GC, Thirkill TL, and Blankenship TN (1999) Vitronectin receptors are expressed by macaque trophoblast cells and play a role in migration and adhesion to endothelium. Biochim Biophys Acta 1452:36–45.

Address correspondence to: Xuejun Li, Department of Pharmacology, School of Basic Medical Sciences, Peking University, Beijing 100191, China.
E-mail: xjl@bjmu.edu.cn