METHODS AND MATERIAL

Beta3 integrin promotes long-lasting activation and polarization of Vascular Endothelial Growth Factor Receptor 2 by immobilized ligand

C. Ravelli et al.

Cell cultures

Human umbilical vein endothelial cells (HUVECs) were grown in M199 medium (Gibco, Life Technologies, Grand Island, NY) supplemented with 20% foetal calf serum (FCS, Gibco, Life Technologies), endothelial cell growth factor (100 μg/mL) (Sigma Chemical Co., St. Louis, MO) and porcine heparin (Sigma) (100 μg/mL). HUVECs were used at early passages (I-IV) and grown on plastic surface coated with porcine gelatin (Sigma). Foetal bovine aortic endothelial GM7373 cells [1] were grown in Dulbecco's modified Eagle medium (DMEM, Gibco, Life Technologies) containing 10% FCS, vitamins, essential and non-essential amino acids. GM7373 cells were transfected with a pcDNA3.1 expression vector harbouring the mouse VEGFR2 cDNA (provided by G. Breier, Max Planck Institute, Bad Nauhein, Germany) to generate stable GM7373-VEGFR2 transfectants [2]. Also, GM7373 cells were co-transfected with a pcDNA3/Enhanced Yellow Fluorescent Protein (EYFP) vector harbouring the extracellular domain of human VEGFR2 (ECD-VEGFR2) cDNA (provided by K. Ballmer-Hofer, PSI, Villigen, Swiss) and with the pcDNA3/β_3-ECFP vector to generate β_3-ECFP/ECD-VEGFR2-EYFP GM7373 cells. β_3-Enhanced Green Fluorescent Protein-overexpressing (β_3-EGFP) GM7373 cells were obtained as described [3]. Finally, GM7373 cells were also transfected with the expression vectors pCDNA 3.1 FRNK or pCDNA 3.1 FRNK-Ser1034 encoding for the FAK C- terminal domain (FRNK) or the inactive FRNKL1034S mutant, respectively [4]. Mock and gremlin-expressing VEGF$^{-/-}$ fibroblasts (provided by K. Alitalo, Haartman Institute, Helsinki, Finland) were grown in DMEM containing 10% FCS, vitamins, essential and nonessential amino acids.

Analysis of gremlin expression in human tumor xenografts

Human endometrial adenocarcinoma HEC-1-B-derived Tet-FGF2 cells [5] were injected s.c. in nude mice. After 8 weeks, animals were sacrificed and tumors (400-500 mg) were snap-frozen in liquid nitrogen. Frozen sections were incubated overnight with anti-murine PECAM1 antibody MEC 13.3 (kindly provided by A. Vecchi, Istituto Humanitas, Milano) and anti-murine gremlin antibody (R&D Systems, Minneapolis, MN, USA) followed by 1 hour incubation with biotin-conjugated rabbit anti-rat secondary antibody (DAKO, Glostrup, Denmark) and Alexa-fluor 488 rabbit anti-goat secondary antibody (Molecular Probes, Life Technologies) followed by 45 minutes incubation with Texas red Avidin D (Vector, Burlingame, CA).

Murine Matrigel plug assay

C57BL/6 mice (Charles River, Calco, Italy) were injected subcutaneously with 400 μL of growth factor reduced Matrigel (Cultrex BME, Gaithersburd, MD) containing 400 ng of gremlin, VEGF-A or FGF2 [6]. After 7 days, plugs were collected after whole animal perfusion fixation [7]. Upon appropriate antigen retrieval (boiling in 1 mmol/L EDTA pH 8 followed by 15 minutes at sub-boiling temperature), 7 μm-paraffin embedded sections were incubated overnight with rabbit anti-VEGFR2 (Cell Signaling Technology) and goat anti-PECAM1 (Santa Cruz Biotechnology) or goat anti-Podocalyxin (R&D Systems) antibodies followed by 1 hour incubation with AlexaFluor 594-conjugated anti-goat IgG and AlexaFluor 488-conjugated anti-rabbit IgG. Finally, sections were incubated with 0.1% Sudan Black in 70% ethanol for 30 minutes at room temperature and washed 8 times with PBS. Samples were photographed under a Zeiss Axiovert 200M epifluorescence microscope equipped with a Plan-Apochromat 63x/1.4 NA oil objective; Z-stack images were acquired using ApoTome imaging system and elaborated through AxioVision Extended Focus module (Carl Zeiss). Quantification of VEGFR2 fluorescence within each apical and basal region of interest belonging to the same EC was carried on acquired images. Values obtained were divided by the total fluorescence to give a percentage of distribution between apical and basal aspects (n=4, 2 vessels per experiment).

Western blotting

24 hours and 10 times concentrated conditioned media, 1.5 mol/L NaCl washes and cell lysates from mock and gremlin-overexpressing VEGF$^{-/-}$ fibroblasts were probed with anti-gremlin antibody (R&D System) in a Western blot.

Confluent HUVECs were detached from culture plates, resuspended in M199 medium containing 5% FCS, allowed to adhere on immobilized gremlin or FG for the indicated time in the absence or in the presence of 10 μg/mL of BV4 antibody (Immunological Sciences, Rome, Italy). Cells were then lysed in lysis buffer [50 mmol/L Tris-HCl buffer (pH 7.4) containing 150 mmol/L NaCl, 1% Triton X-100, 1 mmol/L Na_3VO_4, and protease and phosphatase inhibitors (Sigma)]. Next, 50 μg of total cell lysate were separated by SDS–PAGE and probed with anti-phospho-VEGFR2 antibody (pTyr1175, Cell Signaling Technology, Beverly, MA), anti-FAK antibody (Santa Cruz Biotechnology), anti-VEGFR2 antibody (Santa Cruz Biotechnology) or anti-phospho-β_3 integrin (pTyr759, Santa Cruz Biotechnology) in a Western blot.

Boyden chamber haptotactic assay

Mock and gremlin-overexpressing VEGF$^{-/-}$ fibroblasts were seeded on the lower face of gelatin-coated PVP-free polycarbonate filters (8 μm pore size, Costar, Cambridge, MA). Filters were incubated for 48 hours in DMEM 10% FCS to allow attached cells to deposit their own ECM and inserted in the Boyden chamber. Then, ECD-VEGFR2-EYFP-overexpressing GM7373 cells were seeded in the upper compartment of the Boyden chamber at 1×10^6 cells/mL. After 1 hour of incubation at 37°C, cells adherent to both sides of the filter were fixed and immunostained for β_3 integrin as described below. Then, cells were analysed using a LSM510 Meta confocal microscope equipped with Plan-Apochromat 63x/1.4 NA oil objective (Carl Zeiss). Z-Stack sections and orthogonal z reconstitution for VEGFR2 were analysed by confocal microscopy, while 3D reconstruction images were obtained through AxioVision Inside 4D module (Carl Zeiss).

Immobilization of proteins to tissue culture plastic or glass coverslip

Aliquots (100 μL) of sterile PBS containing 2 μg/mL of gremlin or FG were added to polystyrene tissue culture plates or glass coverslips. After 16 hours of incubation at 4°C, the solution was removed and wells were washed 3 times with cold PBS. Uncovered plastic/glass was blocked with 1 mg/mL bovine serum albumin (BSA) for 1 hour at room temperature. Under these conditions gremlin binds to tissue culture plastic in a dose-dependent manner, with maximal binding at coating concentrations ≥ 2 μg/mL, as assessed by ELISA. Also, substratum-immobilized gremlin is resistant to high molar salt (2 mol/L NaCl) and detergent (0.2% Triton X-100) washes (Figure S3).

Immobilized gremlin ELISA

Ninety-six-well tissue culture plates were coated for 16 hours at 4°C with 0.2, 2 or 10 μg/mL of gremlin as described above. Then, plastic-immobilized gremlin was washed with 2 mol/L NaCl or with 0.2% Triton X-100 for 15 minutes at 37°C. An anti-gremlin goat polyclonal antibody (R&D Systems) diluted in PBS containing 0.1% BSA, 5 mmol/L EDTA, 0.004% Tween 20 (PBET buffer) was added to the wells at 500 ng/mL and incubated for 2 hours at room temperature. Finally, wells were incubated for 1 hour at room temperature with a secondary anti-goat horseradish peroxidase (HRP)-conjugated antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

Ventral plasma membrane (VPM) preparation

VPMs were prepared by osmotic shock using a modification of the squirting lysis technique [8]. Briefly, cells were washed twice with ice-cold water; after 1 minute cells were squirted over by using a jet of ice-cold water and immediately fixed for immunocytochemistry analysis. In all the experiments, the absence of DAPI staining and the persistence of actin filaments were used to unequivocally identify the VPM remnants bound to the substratum.

Glass coverslips were coated with gremlin, FG or FGF2 (2 μg/mL) as described above. Mock cells (75.000/cm^2 in cell culture medium containing 1% FCS) or cells expressing FRNK and FRNKL1034S mutant and were allowed to adhere to the coverslips for 4 hours in absence or presence of different inhibitors used in pre-treatment or not: VEGFR2 inhibitor SU5416 (5 μmol/L,

Calbiochem, La Jolla, CA), RhoA inhibitor exoenzyme C3 from Clostridium botulinum (1 μg/mL, Calbiochem), β_3 integrin inhibitor BV4 monoclonal antibody (10 μg/mL, Immunological Sciences). For lipid rafts disruption experiments, cells were incubated in suspension (1 hour at 37°C) with 2,6-di-O-methyl-β-ciclodextrin (MβCD, 10 mmol/L, Sigma). VPMs were acquired under an Axiovert 200 fluorescence microscope equipped with a Plan-Apochromat 63x/1.4 NA oil objective and ApoTome system (Carl Zeiss). VEGFR2-positive areas and total VPM areas, defined by actin staining, were quantified using Image-Pro Plus software.

Time-lapse adhesion assay

β_3-ECFP and ECD-VEGFR2-EYFP co-expressing GM7373 cells were seeded and cultured on coverslips for 24 hours in FCS-free Endothelial Cell Basal Medium (Lonza, Basel, Swiss). Coverslips were then flipped on immobilized gremlin or FG coated μslides (IBIDI). Z-stack images in time-lapse were recorded for 120 minutes using a Zeiss Axiovert 200M epifluorescence microscope equipped with a Plan-Apochromat 63x/1.4 NA oil objective and ApoTome system. Collected images were then used to quantify the fluorescence of ECD-VEGFR2-EYFP and β_3-ECFP normalized to the background.

Fluorescence resonance energy transfer (FRET) analysis

FRET experiments were performed as previously described [3]. Briefly, β_3-ECFP/ECD-VEGFR2-EYFP GM7373 cells were seeded on immobilized gremlin or FG (both at 2 μg/mL) and allowed to adhere for 4 hours. After adhesion, VPMs were isolated, fixed with 4% paraformaldehyde and subjected to FRET analysis at LSM510 Meta confocal microscope (Carl Zeiss). To this purpose, a region of interest was selected and photobleached by applying 100% intensity of a 514 nm laser. FRET efficiency was calculated using the formula: FRET = $(D_{post} - D_{pre})/D_{post}$, where D_{post} and D_{pre} represent the donor (ECFP) emission intensities before and after photobleaching, respectively. FRET efficiency was also measured in a non-photobleached region of the same cell as an *in situ* control. In all experiments, cells transfected with ECFP-EYFP fusion protein were used as FRET positive controls [9].

Cell motility assay and lipid raft disruption experiments

EC motility was assessed by time-lapse video-microscopy. To this purpose, ECs were seeded on immobilized gremlin or FG (both at 2 μg/mL) at 150 cells per mm^2 in 24 well plates in the absence or in the presence of SU5416 (5μmol/L, Calbiochem), BV4 antibody (10 μg/mL, Immunological Sciences) or exoenzyme C3 (1 μg/mL, Calbiochem). For lipid rafts disruption experiments, cells were incubated in suspension (1 hour at 37°C) with MβCD (10 mM, Sigma) followed by 1 hour incubation with cholesterol (chol, 400 mg/mL, Sigma). Constant temperature (37°C) and 5% CO_2 were maintained throughout the experimental period by means of a heatable stage and climate chamber. Cells were observed under an inverted photomicroscope (Zeiss Axiovert 200M) and phase-contrast snap photographs (one every 10 minutes) were digitally recorded for 8 hours. Cell paths (40-50 cells per experimental point) were generated from centroid positions and migration parameters were analysed with the "Chemotaxis and Migration Tool" of ImageJ Software (http://rsbweb.nih.gov/ij).

Immunocytochemistry

Fixed whole cells or VPMs were permeabilized with 0.5% Triton-X100 and saturated with 3% BSA in PBS. Adherent cells, migrating cells or VPMs underwent the following staining procedures: i) actin staining: samples were incubated for 30 minutes with TRITC-phalloidin (0.9 mg/mL in PBS, Sigma); ii) GM1 ganglioside staining: samples were incubated for 30 minutes with AlexaFluor 594-conjugated Cholera Toxin B subunit (CTB, Sigma); iii) paxillin immunostaining: samples were incubated overnight with a monoclonal anti-paxillin antibody (Transduction Laboratories, Lexington, KY) followed by a 45 minutes incubation with AlexaFluor 488-conjugated anti-mouse IgG (Molecular Probes, Life Technologies); iv) total VEGFR2 immunostaining: samples were incubated overnight with a rabbit polyclonal anti-VEGFR2 antibody (Santa Cruz Biotechnology) followed by 1 hour incubation with AlexaFluor 594 and AlexaFluor 488-conjugated anti-rabbit IgG or with a goat polyclonal anti-VEGFR2 antibody (Santa Cruz Biotechnology) followed by 1 hour incubation with

Alexa594-conjugated anti-goat IgG (Molecular Probes, Life Technologies); v) phospho-VEGFR2 immunostaining: samples were incubated overnight with a rabbit monoclonal anti-phospho-VEGFR2 antibody (pTyr1175, Cell Signaling Technology) followed by 1 hour incubation with AlexaFluor 488-conjugated anti-rabbit IgG; vi) β_3 integrin immunostaining: samples were incubated overnight with a polyclonal anti-β_3 integrin antibody (Immunological Sciences) followed by 1 hour incubation with Cy5-conjugated anti-rabbit IgG; vii) phospho-p85 immunostaining: samples were incubated overnight with a polyclonal anti-phospho-p85 antibody (Santa Cruz Biotechnology) followed by 1 hour incubation with AlexaFluor 594-conjugated anti-goat IgG. viii) Caveolin 1 immunostaining: samples were incubated overnight with a mouse monoclonal anti-Caveolin 1 antibody (BD Transduction Laboratories, Franklin Lakes, New Jersey) followed by 1 hour incubation with AlexaFluor 488-conjugated anti-mouse IgG. ix) Phospho-β_3 integrin immunostaining: samples were incubated overnight with a rabbit polyclonal anti-phospho-β_3 integrin (pTyr759, Santa Cruz Biotechnology) followed by 1 hour incubation with AlexaFluor 594-conjugated anti-rabbit IgG. All antibody dilutions were in PBS containing 3% BSA. Cells and VPMs were photographed under a Zeiss Axiovert 200M epifluorescence microscope equipped with a Plan-Apochromat 63x/1.4 NA oil objective. In some experiments the extent of antigen immunoreactivity was quantified by computerized image analysis (Image-Pro Plus; Media Cybernetics, Rockville MD). When indicated cells were photographed using a Zeiss LSM510 Meta confocal microscope equipped with a Plan-Apochromat 63x/1.4 NA oil objective. VEGFR2 and Caveolin 1 co-localization analysis was performed using AxioVision Co-localization module (Zeiss) while VEGFR2, β_3 integrin and GM1 triple co-localization was analyzed using BlobProb ImageJ plugin [10,11].

Data representation

Data are expressed as mean ± SD or mean ± SEM. Statistical analyses were performed using the Student's *t*-test.

SUPPLEMENTAL REFERENCES

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