EXPERIMENTAL CELL RESEARCH 315 (2009) 877–889

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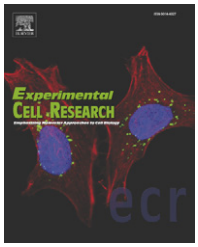

Research Article

VEGFR1 receptor tyrosine kinase localization to the Golgi apparatus is calcium-dependent

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ARTICLE INFORMATION

Article Chronology:
Received 4 August 2008
Revised version received
26 November 2008
Accepted 27 December 2008
Available online 6 January 2009

Keywords:
VEGFR1
VEGF-A
VEGFR2
Endothelial
Receptor tyrosine kinase
Golgi apparatus
Trafficking
Signaling
Calcium

ABSTRACT

Vascular endothelial growth factor receptor 1 (VEGFR1) is an essential receptor tyrosine kinase that regulates mammalian vascular development and embryogenesis but its function is not well understood. Herein, we present evidence whereby endothelial VEGFR1 is largely resident within the Golgi apparatus but translocates to the plasma membrane via a calcium-regulated process. Primary human endothelial cells reveal differing VEGFR1 and VEGFR2 intracellular distribution and dynamics. The major proportion of the full-length VEGFR1 membrane protein was resident within the Golgi apparatus in primary endothelial cells. Whereas VEGFR2 displayed down-regulation in response to VEGF-A, VEGFR1 was not significantly affected arguing for a significant intracellular pool that was inaccessible to extracellular VEGF-A. This intracellular VEGFR1 pool showed significant co-distribution with key Golgi residents. Brefeldin A caused VEGFR1 Golgi fragmentation consistent with redistribution to the endoplasmic reticulum. Metabolic labeling experiments and microscopy using domain-specific VEGFR1 antibodies indicated that the mature processed VEGFR1 species and an integral membrane protein was resident within Golgi apparatus. Cytosolic calcium ions play a key role in VEGFR1 trafficking as treatment with either VEGF-A, histamine, thrombin, thapsigargin or A23187 ionophore caused VEGFR1 redistribution from the Golgi apparatus to small punctate vesicles and plasma membrane. We thus propose a model whereby the balance of VEGFR1 and VEGFR2 plasma membrane levels dictate either negative or positive endothelial signaling to influence vascular physiology.

Introduction

The mammalian endothelium is a cell monolayer that lines all blood vessels and is essential for angiogenesis and regulation of hemostasis, thrombosis and blood pressure. An important extrinsic regulator of such processes is the vascular endothelial growth factor (VEGF) family of soluble cytokines. VEGF-A is distantly related to platelet-derived growth factor (PDGF) and is the founding member of the VEGF superfamily. VEGF-A is essential for endothelial cell proliferation, differentiation and angiogenesis during development and plays a major role in neovascularization in diverse human diseases [1, 2].

The endothelial cell monolayer that lines all blood vessels expresses two Class III receptor tyrosine kinases, VEGFR1 and

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Abbreviations: VEGF-A, Vascular endothelial growth factor A; VEGFR, Vascular endothelial growth factor receptor; PLCγ1, Phospholipase Cγ1; TGN46, trans-Golgi network protein 46 kDa; GalT, β1,4-galactosyltransferase; MannII, mannosidase II; GM130, Golgi matrix protein 130 kDa; VWF, Von Willebrand Factor; TGN, trans-Golgi network

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doi:10.1016/j.yexcr.2008.12.020

VEGFR2, that bind VEGF-A with picomole affinity [2–4]. The ability of the VEGF-A_{165} isoform (termed VEGF-A) to mediate signal transduction and gene expression is thought to be mediated primarily via VEGFR2 whereas the role of VEGFR1 in regulating endothelial function is unclear. VEGF-A can stimulate VEGFR1 tyrosine autophosphorylation, recruitment of phospholipase Cγ1 (PLCγ1) and activation of the MAP kinase pathway. However, VEGFR1 has a significantly lower tyrosine kinase activity than VEGFR2 [5, 6]. VEGFR1-deficient mice display hyperproliferation of endothelial progenitors resulting in severe vascular disorganization and embryonic lethality [7–9]. However, mice expressing a truncated VEGFR1 gene product lacking the tyrosine kinase domain develop normally [10], indicating that VEGFR1-mediated intracellular signaling is not essential for vascular development. VEGFR1 can also directly modulate VEGFR2-mediated biological responses, suggesting that VEGFR1 may have a dual function both as a 'decoy' receptor and through direct modulation of VEGFR2 tyrosine kinase activity [2]. VEGF-A can stimulate the formation of a heteromeric signaling complex composed of VEGFR1 and VEGFR2 that leads to prostacyclin synthesis and lowered blood pressure [11]. Increasingly, it is perceived that the trafficking of endothelial receptor tyrosine kinases such as VEGFR2 [12–15] plays a crucial role in the endothelial response to extracellular growth factors and cues.

Primary human endothelial cells were used to investigate VEGFR1 localization as a function of ligand and pharmacological-mediated cell stimulation. We discovered a major pool of endothelial VEGFR1 maintained within the Golgi apparatus until stimulation by ligand or agonist. Translocation of VEGFR1 from the Golgi to cytoplasmic vesicles and to the plasma membrane required elevation of cytosolic calcium ion levels. We have therefore discovered a potential negative feedback mechanism whereby extracellular VEGF-A binding to VEGFR2 leads to trafficking of VEGFR1 to the plasma membrane and can modulate VEGF-A effects on endothelial function.

Methods

Chemicals and reagents

Chemicals were purchased from Sigma (Poole, UK) and Merck-BDH (Lutterworth, UK) unless otherwise stated. HUVECs were prepared from human umbilical cords as described [16]. Media was from Promocell (Heidelberg, Germany) and Invitrogen (Amsterdam, Netherlands). Tissue culture plastic was from Nunc (Roskilde, Denmark). Recombinant VEGF-A_{165} (termed VEGF-A) was from Merck Biosciences (Nottingham, UK).

Antibodies

Sheep antibodies to human TGN46, mouse anti-GalT (β1,4-galactosyltransferase) and mouse antibodies to the human transferrin receptor (OKT9) have been previously described [17]. Mouse anti-ERGIC-53 was from H. Hauri (Basel Biocenter, Switzerland). Rabbit anti-MannII was from AbD-Serotec (Oxford, UK) and rabbit anti-alkaline phosphatase was from A. Booth (University of Leeds, UK). Rabbit and mouse anti-VWF were from Dako (Copenhagen, Denmark) and mouse anti-PECAM-1 from Ancell (Bayport, USA). Sheep antibodies to human GM130 were from F. Barr (University of Liverpool, UK). Mouse, rabbit and goat anti-VEGFR1 were from Sigma-Aldrich (Poole, UK), Abcam (Cambridge, UK), Santa Cruz Biotech (Santa Cruz, USA) and R & D Systems (Abingdon, UK). Secondary antibody conjugates were from Jackson ImmunoResearch (Pennsylvania, USA) and Invitrogen (Amsterdam, Holland).

Cell culture

All work with human tissues and material conformed to UK rules and regulations and was approved by the Leeds General Infirmary NHS Trust Local Ethics Committee. Umbilical cords from Cesarean births were processed as described previously [16]. Briefly, endothelial cells were detached from the major umbilical vein by digestion with 0.1% (w/v) type I collagenase and resuspended in media M199 (Invitrogen) containing 10% (v/v) fetal calf serum with recombinant EGF, basic FGF and VEGF-A (Prospec-Tany Technogene, Tel Aviv, Israel). HUVECs were plated out into tissue culture plastic or glass coverslips coated with 0.1% (w/v) pig skin gelatin and fresh media was replenished every other day over a 7–10 day period. Confluent HUVEC monolayers were passaged weekly at 1:3 using trypsin detachment. HUVECs were maintained for 3–4 passages in media containing various supplements including EGF, basic FGF and VEGF-A. Endothelial cells were characterized by antibody labeling for VWF and PECAM-1 [16]. All experiments were carried out on confluent HUVEC monolayers. Primary human coronary artery endothelial (HCAECs) cells were from Promocell (Heidelberg, Germany).

Protein analysis

HUVECs were grown to confluence on gelatin-coated plastic surfaces and lysed in buffer containing 2% (w/v) SDS in PBS, 1 mM PMSF, 1 μg/ml each of leupeptin, aprotinin, pepstatin A, benzamidine, chymostatin and sheared using 15–30 passes through a 25 gauge needle. For analysis of protein levels in response to VEGF-A using immunoblotting, cells were pre-treated for 1–2 h with cycloheximide (20 μg/ml) to block new protein synthesis before VEGF-A addition. Media used for cell stimulation also contained cycloheximide. For proteinase K protection experiments, confluent monolayers were treated with proteinase K (1 μg/ml) for 30 min on ice, rinsed with PBS containing 1 mM PMSF and lysed as described. Lysates were centrifuged at 16000 *g* at 4 °C for 10 min and protein concentrations determined using the BCA assay (Perbio, Illinois, USA). Samples of total cell lysate (50 μg) were loaded on 6% SDS-PAGE gels run at 30 mA/gel and blotted onto reinforced nitrocellulose membranes (Schleicher and Schuell, Germany) at 100 V for 1–2 h. Membranes were probed as described previously [13] using the West Pico ECL system (Perbio, USA) and data collected using a Fuji LAS-3000 (FujiFilm, Japan) CCD camera-based imaging system [18]. Images were analyzed using AIDA software (FujiFilm, Japan) and exported into MS-Powerpoint. For quantification, data were analyzed using MS-Excel and histograms imported into MS-Powerpoint.

FACS analysis

Cells were processed largely as previously described [19]. Confluent HUVEC monolayers were rinsed with PBS, gently detached from the substratum using a scraper, rinsed and fixed in 1% (w/v)