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Inhibition of endothelial cell migration and angiogenesis by a vascular endothelial growth factor receptor-1 derived peptide

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ABSTRACT

Vascular endothelial growth factor receptor-1 (VEGFR-1) exists in two isoforms: a membrane-bound isoform (mVEGFR-1) and a soluble one (sVEGFR-1). mVEGFR-1 is involved in endothelial cell migration and survival supported by VEGF-A and placenta growth factor (PlGF), whereas the biologic function of sVEGFR-1 has not been fully elucidated. We previously reported that sVEGFR-1 induces endothelial cell motility and promotes endothelial cell adhesion. In this study, we tested a set of VEGFR-1-derived peptides for their ability to interfere with endothelial cell migration. Peptide B3 was found to specifically inhibit cell migration induced by sVEGFR-1 and by mVEGFR-1-specific ligands. Moreover, peptide B3 markedly hampered angiogenesis *in vitro* and *in vivo* and was found to interfere with VEGFR-1 homodimerisation. Altogether, these data demonstrate that peptide B3 might be a useful tool for the specific inhibition of VEGFR-1 function and might represent a basis for the development of new anti-angiogenic compounds.

1. Introduction

The role of VEGF-A in vasculogenesis and angiogenesis is principally mediated by two tyrosine kinase receptors: VEGFR-1 [also known as Flt-1 (VEGF receptor-1/fms-like tyrosine kinase)] and VEGFR-2 [also called KDR (VEGF receptor-2/kinase insert domain-containing receptor)]. Activation of VEGFR-2 has been shown to be associated with endothelial cell proliferation, migration, survival, and vascular permeability.[1] VEGFR-1 is the sole receptor for two other members of the VEGF family: VEGF-B and PlGF.[2] It exists in two isoforms, a membrane-bound polypeptide and a soluble one, originated by alternative splicing of the mRNA transcribed from a single gene.[3]

Membrane-bound VEGFR-1 (mVEGFR-1) contains an extracellular region structured in seven immunoglobulin-like (Ig-like) domains, a single transmembrane region and two cytoplasmic tyrosine kinase domains. It has been associated with the release of growth factors and matrix degrading enzymes by endothelial cells and with migration and survival of endothelial, haematopoietic or leukaemic cells.[1,4]

The soluble VEGFR-1 (sVEGFR-1) was initially identified as a polypeptide secreted by endothelial cells, which contains

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six of the seven extracellular Ig-like domains of mVEGFR-1 and a unique sequence of 30 amino acids at the C-terminus[3], and is able to block VEGF-mediated cell proliferation.[5] In an intent to unravel the role of this polypeptide in endothelium function, we previously demonstrated that endothelial cells deposit sVEGFR-1 into the extracellular matrix, adhere on this polypeptide through α5β1 integrin expressed on the cell surface, and migrate in response to sVEGFR-1 by a mechanism that involves this integrin.[6]

In the last years, PlGF and mVEGFR-1 have received considerable attention as novel targets to inhibit angiogenic or inflammatory disorders.[7,8] In particular, the specific involvement of mVEGFR-1 in pathological angiogenesis and its minor contribution to physiological angiogenesis suggests that inhibition of pathways in which this receptor is involved might result in less untoward effects than other anti-angiogenic therapies. In this context, VEGFR-1-derived peptides with the ability to block PlGF-stimulated angiogenic pathways would represent valuable lead compounds to design novel agents for cancer treatment or for the therapy of inflammatory diseases.

2. Materials and methods

2.1. Reagents

Endothelial Basal Medium (EBM-2) and Endothelial Growth Factor Medium (EGM-2) were from Clonetics (BioWhittaker Inc, Walkersville, MD). Fatty acid-free bovine serum albumin (BSA) was from Roche (Mannheim, Germany). VEGF-A, VEGF-B, PlGF, basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) homodimers, polyclonal antibodies against VEGF-A or PlGF, and the rhVEGFR-1/Fc, rhVEGFR-2/Fc, rat recombinant neuropilin-1/Fc (NRP-1/Fc), platelet-derived growth factor receptor β/Fc (PDGFR/Fc) and Tie-2/Fc chimeras were from R&D Systems (Abingdon, UK). Native sVEGFR-1 was from RELIATech (Braunschweig, Germany). Fibronectin and goat anti-mouse IgG (Fc specific) antibodies were from Sigma–Aldrich (St. Louis, MO). Purified human integrin α5β1 and mouse monoclonal antibody (mAb) against α5β1 integrin JBS5 were from Chemicon (Temencula, CA). Geneticin was from Invitrogen (Groningen, The Netherlands) and puromycin and other chemicals were from Sigma–Aldrich.

2.2. Cells

Human umbilical vein endothelial cells (HUVEC) were isolated from freshly delivered umbilical cords as previously described[9] and cultured in EGM-2. The immortalised human endothelial cell line HUV-ST was generated and maintained in culture in EGM-2 medium supplemented with 0.4 mg/ml geneticin and 5 μg/ml puromycin, as previously described.[10]

2.3. Peptide design and synthesis

Peptides mapping on VEGFR-1 second Ig-like domain were designed based on the three-dimensional structures of this domain available from the Protein Data Bank (PDB, URL: http://www.rcsb.org/pdb)[11] using the interactive graphics software InsightII[12] and its module Biopolymer (Accelrys Inc.). The designed peptides were synthesised and HPLC purified by PRIMM (Milan, Italy), and were dissolved at 10 mg/ml as indicated by the producer.

2.4. Cell proliferation assay

HUV-ST cell proliferation in the presence or absence of peptides was evaluated in 96-well plates using the tetrazolium compound MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)2-(4-sulphophenyl)-2H-tetrazolium, inner salt] from Promega (Madison, WI), as previously described.[10]

2.5. Migration assay

Migration of endothelial cells was analysed using Boyden chambers equipped with 8 μm pore diameter polycarbonate filters (Nuclepore, Whatman Incorporated, Clifton, NJ) coated with 5 μg/ml gelatin solution, as previously described,[6] except that 1.5×10^5 cells were loaded and the assay was allowed to proceed for 18 h. Migrated cells were then counted under the microscope (×200 magnification) as described. To test the effect of VEGFR-1-derived peptides on endothelial cell migration, cells were preincubated for 30 min at room temperature in migration medium containing the peptides at the indicated concentrations and then loaded into the Boyden chambers without removing the peptides.

2.6. Binding of PlGF to VEGFR-1

Quantification of PlGF binding to VEGFR-1 was performed on Maxisorp Nunc immunoplates (Nunc, Roskilde, Denmark) coated with 10 μg/ml VEGFR-1/Fc chimera in PBS. After blocking of the plates with 3% BSA/PBS, 50 μl of PlGF (50 ng/ml in 3% BSA/PBS) was added to selected wells and incubated for 18 h. An aliquot of the cytokine was preincubated for 30 min at room temperature with 100 μg/ml of the indicated peptides or 10 μg/ml of goat anti-PlGF antibody. Detection of the amount of cytokine bound to the VEGFR-1/Fc chimera was performed with biotinylated goat anti-PlGF antibodies and streptavidin-alkaline phosphatase conjugate (1:10,000, from Roche). After the alkaline phosphatase reaction, optical density at 405 nm was measured in a Microplate reader 3550-UV (Bio-Rad, Hercules, CA). The specificity of PlGF binding to VEGFR-1 was tested in wells coated with PDGFR/Fc chimera and background was determined in 3% BSA/PBS-coated wells.

2.7. Differentiation of endothelial cells in tubules

The formation of capillary-like tubules by HUVEC was evaluated using a three-dimensional collagen type I (Roche) gel on 24-well plates, as described,[13] including peptide B3 or a scramble version of peptide B3 (scr B3) in the collagen mixture at the final concentration of 0.5 mg/ml in 0.1% BSA/serum-free basal medium and inducing tubule formation with basal medium supplemented with 100 ng/ml VEGF-A. Formation of endothelial tubules was observed and photographed 24 h later using a Canon digital camera PowerShot G5.

2.8. *In vivo matrigel assay*

The ability of peptide B3 to modulate neovascularisation was evaluated by a matrigel plug assay, using VEGF-A as *stimulus*, according to the method previously described.[14] Six hundred microlitres of matrigel (BD Biosciences, Bedford, MA) supplemented with VEGF-A (100 ng/ml) alone or containing VEGF-A plus peptide B3 or scr B3 at 200 μg/ml were injected subcutaneously into the flank of 8-week-old C57BL/6 mice (furnished by Charles River Laboratories, Calco, Italy). After five days, mice were sacrificed, the matrigel plugs were removed and the angiogenic response was evaluated by macroscopic analysis and by the spectrophotometrical measurement of the haemoglobin content in the pellet of matrigel, using the Drabkin method.[15] All procedures involving animals and care were performed in compliance with national and international guidelines (European Economy Community Council Directive 86/109, OLJ318, 1st December, 1987).

2.9. *Binding of peptide B3 to VEGFRs or integrin α5β1*

Analysis was performed using Reacti-Bind microplates (PIERCE, Rockford, IL) coated with the peptide or with native sVEGFR-1 and incubated with VEGFRs or integrin α5β1, as previously described.[16] Background values were evaluated as the absorbance in peptide- or in sVEGFR-1-coated wells in which no chimeras or integrin was added. The influence of peptide B3 on the binding of the different VEGFRs or integrin α5β1 to sVEGFR-1 was analysed by incubating the sVEGFR-1-coated plates with the polypeptides for 30 min at 37 °C in a 100-fold molar excess of the peptide.

2.10. *Statistical analysis*

When data are expressed as percentage of inhibition, each value corresponds to the mean of three independent experiments performed with triplicate samples and bars indicate standard error of the mean. Percentages were subjected to angular transformation in order to obtain normally distributed data and to perform standard error calculation and Student's *t*-test statistics.

3. Results

3.1. *A peptide derived from VEGFR-1 second Ig-like domain inhibits endothelial cell migration*

We previously reported that the second Ig-like domain of VEGFR-1, which is involved in the interaction of the receptor with VEGF-A and PlGF, plays a role also in sVEGFR-1-mediated migration and adhesion of endothelial cells through the interaction with integrin α5β1.[6,16]

On the basis of the crystallographic structure of this domain in complex with VEGF-A or PlGF (PDB IDs: 1FLT, 1RV6), we designed seven peptide sequences (A1–A7, Fig. 1) with the potential ability to block sVEGFR-1-induced migration of endothelial cells. Peptides satisfy the following criteria: (i) high solvent accessible surface area; (ii) length shorter than 15 residues to avoid synthesis problems; (iii) presence of residues charged at physiological pH, low percentage of hydrophobic residues and no branched hydrophobic residues next to one another, to minimise solubility problems; (iv) absence of Cys residues, which might form disulphide bridges (residue Cys158 has been replaced by alanine in peptide A4).

The ability of the peptides to affect endothelial cell migration induced by sVEGFR-1 was assessed using an immortalised human endothelial cell line (HUV-ST), previously generated in our laboratories.[10] Cells were preincubated for 30 min with the peptides (30 μM), that were thereafter maintained during the migration assay. Peptide A4 completely abrogated the migratory response of endothelial cells whereas the other peptides were less efficient, inhibiting migration by 14–44% (Fig. 1). Therefore, peptide A4 was selected for further studies. It should be noted that the sequence of peptide A7 (44% inhibition in our experimental conditions) exactly matches the sequence of a previously described peptide[17] (Flt2-11 peptide), which showed anti-angiogenic properties in the chick chorioallantoic membrane assay and inhibited VEGF-A-induced vascular permeability without directly binding to VEGF-A.

It was then assessed whether peptide A4 specifically inhibited sVEGFR-1-dependent migration and whether it might also modulate activation of mVEGFR-1. For this purpose, cell motility was evaluated in the presence of different *stimuli*, in particular other α5β1 integrin binding proteins such as fibronectin, or mVEGFR-1 ligands such as VEGF-A and PlGF.

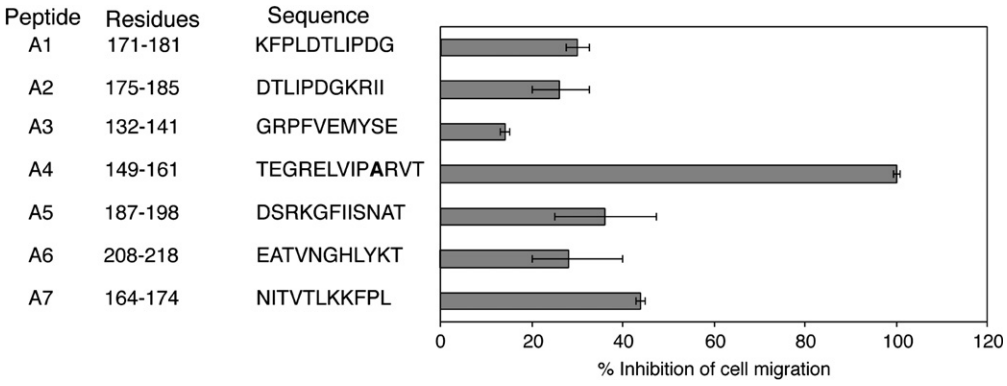

Fig. 1 – Peptide A4 inhibits endothelial cell migration mediated by sVEGFR-1. Effect of peptides A1–A7 (30 μM) on HUV-ST cell migration induced by 5 μg/ml sVEGFR-1.

As illustrated in Fig. 2A, in addition to the abrogation of sVEGFR-1-induced migration, peptide A4 provoked almost 100% inhibition of PlGF-induced response. A peptide corresponding to a scramble sequence of peptide A4 had no effect on endothelial cell migration under any of the experimental conditions tested (Fig. 2A). Finally, the inhibitory activity of peptide A4 was not due to an effect on cell viability, since treatment of HUV-ST cells with 30 μM peptide A4 for three days did not significantly affect cell proliferation (not shown).

To investigate whether down-modulation of PlGF response induced by peptide A4 was due to interference of the peptide with PlGF binding to mVEGFR-1, the influence of peptide A4 and of the scramble peptide on PlGF binding to the extracellular region of the receptor was assessed by a solid-phase binding assay. The results indicated that the binding of PlGF to the extracellular domain of VEGFR-1 was not affected by peptide A4, whilst it was completely abrogated by a PlGF-neutralising antibody (Fig. 2B).

3.2. *Identification of peptide A4 region responsible for its inhibitory activity*

A second series of shorter peptides (seven amino acid-long) were designed, each one corresponding to a part of peptide A4 sequence. Ser162 (not present in peptide A4) was included at the carboxy-terminus of peptide B3 in order to maintain a fixed number of seven amino acids for each peptide and Cys at the position 158 was substituted with an Ala in peptides B2 and B3 to avoid formation of disulphide bridges (Fig. 3). The effect of these peptides on endothelial cell migration in response to sVEGFR-1 or fibronectin was assessed. Peptide B2 showed a significant effect on endothelial cell response to sVEGFR-1 and peptide B3 resulted to be the most efficient inhibitor of sVEGFR-1-induced migration (Fig. 3). The peptides had no effect or only slightly reduced fibronectin-induced migration. Interestingly, peptide B3 resulted to be a specific inhibitor for ligands that exclusively bind to mVEGFR-1 (i.e. PlGF and VEGF-B), whilst it showed a limited effect on migration induced by VEGF-A, which may interact with both VEGFR-1 and VEGFR-2 (Fig. 4). Moreover, peptide B3 did not affect stimulation of endothelial cell migration induced by other unrelated *stimuli* (EGF and bFGF) (Fig. 4). A peptide containing a scramble sequence of B3 (scr B3) had no effect on endothelial cell migration.

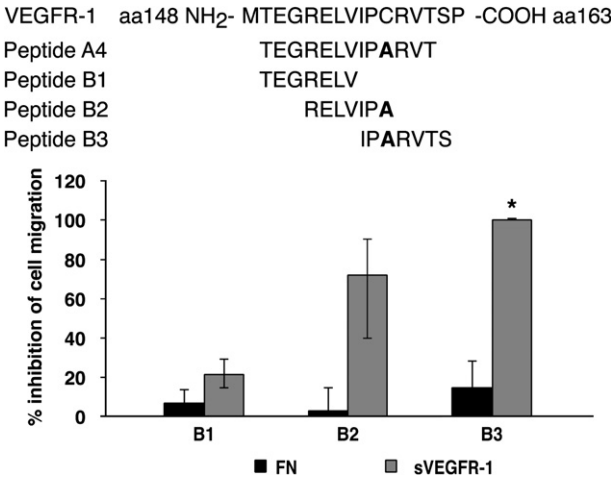

Fig. 3 – Identification of the region of peptide A4 endowed with inhibitory activity on endothelial cell migration triggered by sVEGFR-1. Amino acid sequence of A4-derived peptides and their effect (30 μM) on HUV-ST cell migration induced by 1 μg/ml fibronectin (FN) or 5 μg/ml sVEGFR-1. *$p < 0.05$ comparing the percentages of peptide B3-treated cells with those of peptide B2-treated cells for sVEGFR-1 stimulation.

The inhibitory activity of peptide B3 was further evaluated by analysing the effect of graded concentrations of the peptide on endothelial cell migration stimulated by sVEGFR-1 or PlGF, and was expressed in terms of IC_{50} (i.e. peptide concen-

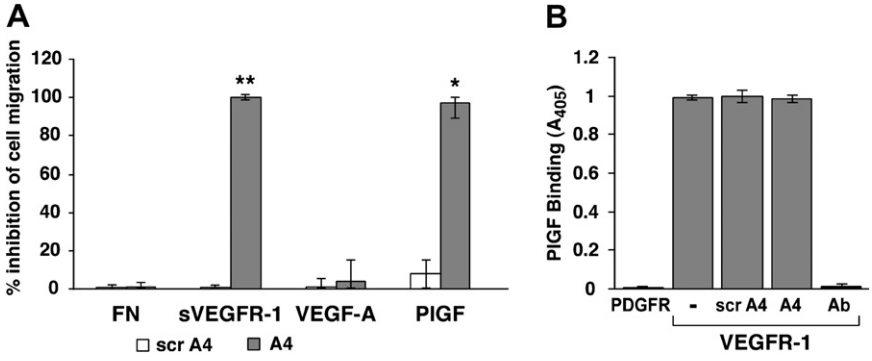

Fig. 2 – Peptide A4 inhibits endothelial cell migration induced by PlGF without affecting its binding to VEGFR-1. (A) Effect of peptide A4 on endothelial cell migration induced by different *stimuli*. HUV-ST cells were preincubated with 30 μM peptide A4 or with a peptide containing a scramble sequence of peptide A4 (scr A4, ICRVGTPELETVR) before testing cell migration in response to 1 μg/ml fibronectin (FN), 5 μg/ml sVEGFR-1, 50 ng/ml VEGF-A or PlGF. *$p < 0.05$ and **$p < 0.001$, comparing the percentages of peptide A4-treated cells with those of peptide scr A4-treated cells. (B) Effect of peptide A4 on PlGF binding to VEGFR-1. Binding of the growth factor to VEGFR-1 was measured on 96-well plates coated with sVEGFR-1/Fc chimera. Control wells were coated with a chimera containing the extracellular region of the PDGFRβ. PlGF binding to VEGFR-1 was analysed in the presence of peptides A4 or scr A4, neutralising anti-PlGF antibodies (Ab) or just buffer (–). Histograms represent the mean (±S.E.) of the absorbance values at 405 nm obtained from a representative experiment performed in triplicate.

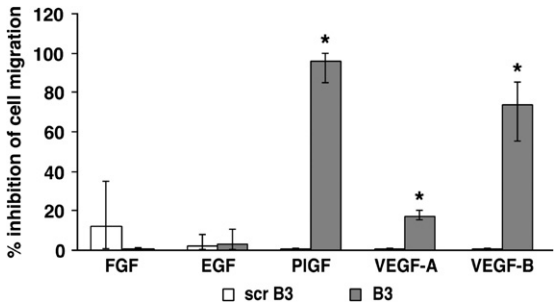

Fig. 4 – Peptide B3 inhibits endothelial cell migration induced by PlGF and VEGF-B. HUV-ST cells were exposed to 30 μM peptide B3 or scramble B3 (scr B3, IARVSTP), and migration in response to different *stimuli* (100 ng/ml VEGF-A, 50 ng/ml PlGF, 50 ng/ml VEGF-B, 5 ng/ml bFGF or 100 ng/ml EGF) was assessed. $^{*}p < 0.05$ comparing the percentages of peptide B3-treated cells with those of peptide scr B3-treated cells.

tration producing 50% inhibition of cell migration induced by the different *stimuli*, calculated on the regression line in which the percentage of migrated cells with respect to untreated samples was plotted against the logarithm of the peptide concentration). The results showed an IC_{50} of 2.81 ± 0.81 μM for sVEGFR-1 stimulation and of 7.88 ± 2.62 nM for PlGF stimulation.

Similarly to what was found for peptide A4, exposure to peptide B3 did not affect HUV-ST cell proliferation (results not shown).

3.3. *Peptide B3 impairs the ability of HUVEC to differentiate in tubule-like structures in vitro and inhibits angiogenesis in vivo*

The effect of peptide B3 on endothelial cell migration strongly suggested that this peptide could represent a valuable tool to inhibit the angiogenic process. This possibility was tested using two angiogenesis assays: *in vitro* formation of capillary-like structures and *in vivo* matrigel plug assay.

Differentiation of HUVEC in capillary-like structures was tested using collagen gels embedded with peptide B3 or scr B3 or not containing the peptides. VEGF-A induced the formation of tubule-like structures by cells maintained in scr B3/collagen or collagen alone, whereas tubules were not observed when cells exposed to VEGF-A were maintained in gels containing peptide B3 (Fig. 5).

In vivo matrigel plug assay showed a potent induction of angiogenesis five days after injection of the plugs containing VEGF-A as *stimulus* in the flank of C57BL/6 mice. The angiogenic response was observed macroscopically, both in the absence of peptides and in the presence of peptide scr B3 (Fig. 6). By contrast, macroscopic appearance of the plugs that included peptide B3 showed the absence of newly formed blood vessels as in those plugs in which VEGF-A was not included (Fig. 6). The results observed by macroscopic analysis were confirmed by the quantitative measurement of haemoglobin content in the excised matrigel plugs (Fig. 6).

3.4. *Interaction of peptide B3 with VEGFRs or integrin α5β1*

To clarify the mechanism by which peptide B3 negatively modulates migration induced by VEGFR-1 activation and the angiogenic process, the interaction of this peptide with the extracellular region of different VEGFRs (VEGFR-1, VEGFR-2 and neuropilin-1) or with the integrin α5β1 was investigated. sVEGFR-1, B3 or scr B3 peptide immobilised on a solid support were used. Incubation of the different VEGFRs or of the integrin α5β1 on sVEGFR-1-coated plates showed the interaction of sVEGFR-1 with the chimeras VEGFR-1/Fc, VEGFR-2/Fc, neuropilin-1/Fc and with the integrin α5β1 (Fig. 7A), as already published.[6,18,5,19] Conversely, the analysis performed on peptide B3-coated plates demonstrated that only the extracellular region of VEGFR-1 binds to this peptide (Fig. 7A). When sVEGFR-1 was allowed to interact with the extracellular region of VEGFR-1 itself, VEGFR-2, neuropilin-1 or integrin α5β1 in the presence of peptide B3, binding of this peptide to sVEGFR-1 interfered exclusively with the homologous interaction VEGFR-1/VEGFR-1 (Fig. 7B).

4. Discussion

We previously reported that an antibody raised against a peptide mapping to the second Ig-like domain of VEGFR-1 was able to inhibit endothelial cell response to sVEGFR-1.[6] This result prompted us to identify peptidic sequences within this domain that might be involved in the migration-inducing

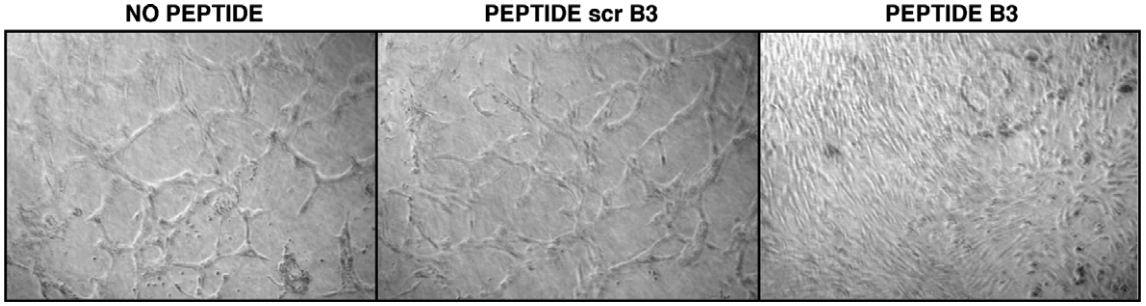

Fig. 5 – Peptide B3 hampers the ability of HUVEC to differentiate in tubule-like structures on collagen gels. Cells were cultured into collagen I gels containing peptide scr B3 or peptide B3 (both at 300 μM) or without peptide and then incubated in basal medium supplemented with 50 ng/ml VEGF-A for 24 h before photographing (×40 magnification).

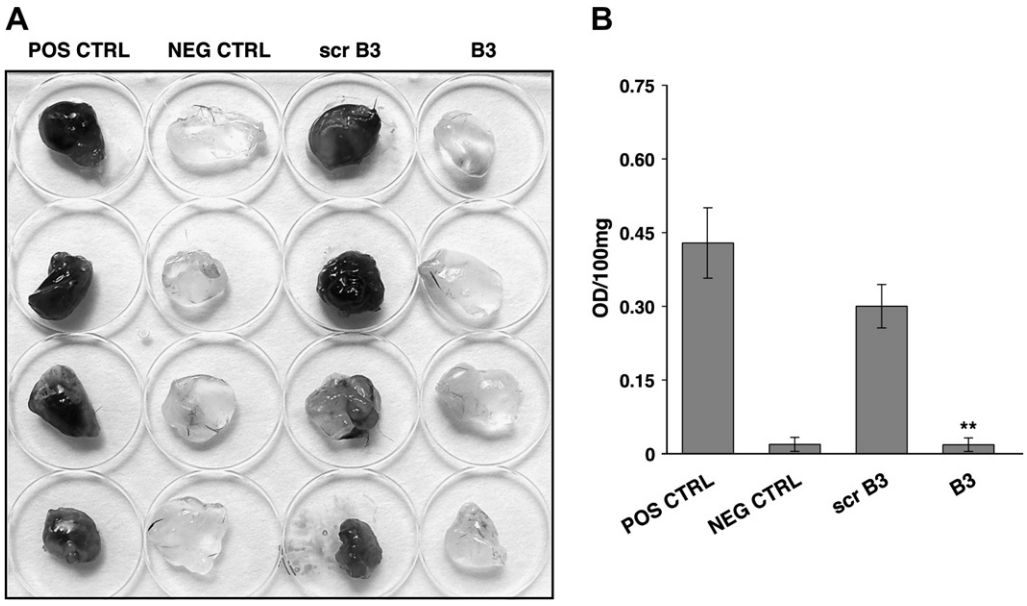

Fig. 6 – Peptide B3 avoids *in vivo* neovascularisation evaluated in matrigel plugs. The photograph shows the matrigel plugs containing (POS CTRL) or not (NEG CTRL) the angiogenic *stimulus* VEGF-A. Plugs indicated as B3 and scr B3 correspond to the samples containing the peptides (250 μM) in addition to VEGF-A. Histograms represent the quantification of the haemoglobin content in the matrigel pellets from groups of 6 plugs per experimental condition. The values are expressed as the mean (±SE) optical density (OD)/100 mg of matrigel. $^{**}p < 0.001$ comparing the values of peptide B3-containing plugs with those of the positive control plugs.

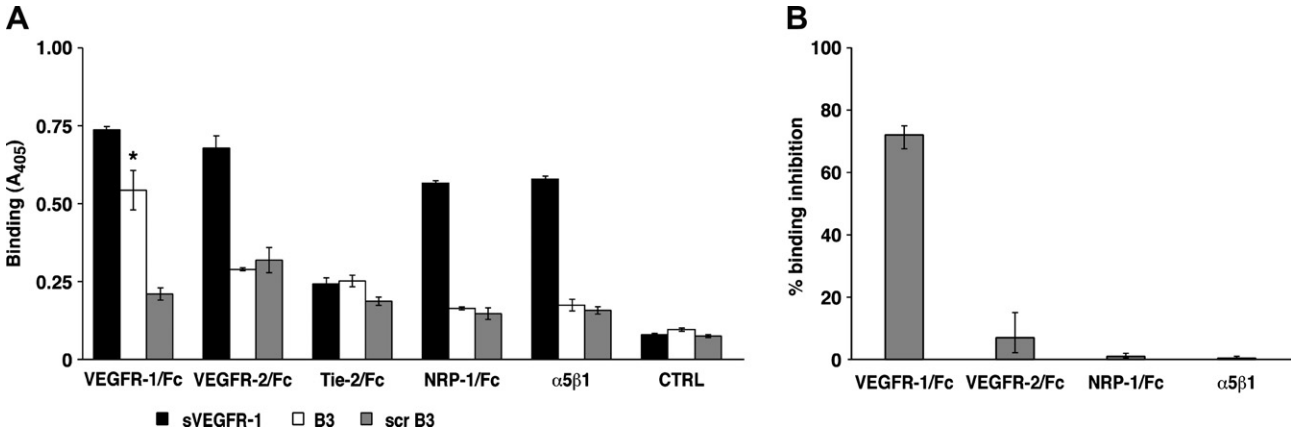

Fig. 7 – Peptide B3 binds to the extracellular region of VEGFR-1 and inhibits its homodimerisation. (A) Chimeric polypeptides containing the extracellular region of the different VEGFRs or the purified α5β1 integrin were allowed to bind on microtiter plates previously coated with sVEGFR-1 or with the peptides B3 or scr B3. Tie-2/Fc chimera was included to evaluate non-specific binding. Background values were evaluated by the absorbance of samples in wells coated with peptide or with sVEGFR-1 in which no chimeras or integrin were added (CTRL). Data correspond to the mean value (±SE) of a representative experiment performed in triplicate. $^{*}p < 0.05$ comparing the absorbance values of VEGFR-1/Fc binding to peptide B3 with those of control samples (CTRL). (B) The influence of peptide B3 on the homologous or heterologous interactions of VEGFR-1 was evaluated in Microtiter plates coated with sVEGFR-1 protein. Chimeric polypeptides containing the extracellular region of the different VEGFRs or the purified α5β1 integrin were allowed to bind the protein in the presence or absence of peptide B3. Histograms represent the mean values (±SE) of the percentage inhibition for the binding of sVEGFR-1 to the different polypeptides. Data are from a representative experiment performed in triplicate.

effects of sVEGFR-1 and to test them in *in vitro* endothelial cell migration assays. The studies herein presented allowed us to find a thirteen-amino acid sequence (peptide A4) capable of blocking endothelial cell migration induced by sVEGFR-1. This peptide strongly inhibited also PlGF-promoted migration, whilst it did not significantly affect cell migration induced by VEGF-A, which is capable of interacting with both VEGFR-1 and -2, or by fibronectin, a ligand of α5β1 integrin. Down-modulation of PlGF-induced migration by peptide A4 was not due to the blockage of PlGF/VEGFR-1 interaction.

A seven amino acid peptide (peptide B3) containing part of the A4 sequence was shown to retain the same inhibitory activity of A4 towards sVEGFR-1 and to interfere markedly with *stimuli* that activate cell migration exclusively through mVEGFR-1 (i.e. PlGF and VEGF-B). Nevertheless, using an *in vitro* assay on collagen gels, peptide B3 was found to inhibit differentiation of endothelial cells in capillary-like structures in response to VEGF-A. Noteworthy, the anti-angiogenic properties of peptide B3 were further confirmed in a matrigel plug assay of *in vivo* angiogenesis, which uses VEGF-A as *stimulus*. It is possible to hypothesise that although the peptide only moderately affected endothelial cell migration in response to VEGF-A, VEGFR-1 stimulation by this angiogenic factor might be important during the process of tubule assembling. Moreover, it has been previously demonstrated that VEGF-A induction of capillary-like structures is potentiated by the simultaneous presence of PlGF in the medium,[20,21] suggesting a role for mVEGFR-1 in this process. Since endothelial cells secrete high levels of PlGF when cultured *in vitro* it is possible that the inhibition of tubule formation observed after treatment with VEGF-A in combination with peptide B3 might be the result of a compromised response of endothelial cells to PlGF due to a reduced mVEGFR-1 functionality.

mVEGFR-1 is known to be involved in autologous or heterologous interactions on the cell membrane.[6,18,5,19] Stimulation by PlGF or VEGF-B results in the homodimerisation of this receptor, which consequently becomes fully activated, and specific sites on the fourth Ig-like domain are essential to stabilise this autologous interaction.[18] Conversely, VEGF-A or VEGF-A/PlGF heterodimer stimulation of endothelial cells results in the formation of both mVEGFR-1 homodimers and mVEGFR-1/VEGFR-2 heterodimers[5] that activate different signal transduction pathways.[21] Heterodimer formation involves the extracellular regions of both receptors through still unknown receptor sequences. mVEGFR-1 may also interact with neuropilin-1[19], a receptor that binds specific VEGF-A or PlGF isoforms, by the third and the fourth Ig-like domains. It has been suggested that this interaction might result in the down-regulation of neuropilin-1 activity as VEGFR-2 co-receptor, negatively modulating angiogenesis.[19] Finally, we described the interaction of sVEGFR-1 with integrin $\alpha5\beta1$, mediated by the second Ig-like domain of the receptor.[6,16]

In this context, binding experiments indicate that B3 directly interferes with VEGFR-1 homodimer formation, suggesting a key role for the second Ig-like domain of the receptor in the mechanism underlying this homologous interaction. On the other hand, the peptide does not affect the binding of mVEGFR-1 to other polypeptides known to interact with it (i.e. VEGFR-2, NRP-1 and integrin $\alpha5\beta1$). Therefore, the inhibition of endothelial cell migration in response to PlGF-induced by peptide B3 might be due to a reduced formation of VEGFR-1 homodimers. Moreover, the fact that peptide B3 was also able to block sVEGFR-1 stimulation of endothelial cells suggests that this polypeptide needs to be in its homodimeric form to be able to interact with the cell surface components that trigger the chemotactic response. Finally, based on the ability of peptide B3 to affect angiogenesis triggered by VEGF-A, we suggest that certain angiogenic properties of this growth factor might also require as a first step VEGFR-1 homodimerisation. However, the elucidation of the mechanism responsible for the inhibitory effects of peptide B3 on VEGF-A-induced angiogenesis requires further investigation.

Peptide B3 is expected to represent an important tool in the determination of the mechanism of endothelial cell migration stimulated through mVEGFR-1 or by sVEGFR-1. Moreover, B3 is a short peptide whose structure is expected to be easily modified to improve bioavailability and *in vivo* activity. The identification of the amino acid residues essential for B3 activity might allow the design of new and more efficient compounds with anti-angiogenic properties derived from this peptide.

In the last years, several anti-angiogenic agents have entered clinical trials.[22] Amongst them, Bevacizumab, a humanised monoclonal antibody against VEGF-A, recently approved by the FDA for the treatment of colorectal carcinoma in combination with chemotherapy, appears to be the most promising agent.[23,24] Inhibitors of VEGFR-2 tyrosine kinase activity, most of which interfere also with the activity of other angiogenic receptors, have also shown encouraging results in combination with chemotherapy.[25,26] Although VEGFR-1 appears to be a very promising target for anti-angiogenic therapy, no molecules directed selectively against this receptor, with the exception of the ribozyme Angiozyme,[27] are presently under clinical investigation. Compared to other VEGFR-1 antagonists, such as anti-VEGFR-1 monoclonal antibodies[28] or peptides,[29,30] peptide B3 does not directly target a specific ligand-receptor binding, but acts downstream, blocking the interactions required for the formation of receptor homodimers. In this way, peptide B3 might interfere with every pathway that needs VEGFR-1 homodimerisation, showing a broad range of action.

Conflict of interest statement

None declared.

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