(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
7 February 2008 (07.02.2008)

PCT

(10) International Publication Number
WO 2008/015720 A2

(51) International Patent Classification:
C07K 14/71 (2006.01) *A61K 38/18* (2006.01)

(21) International Application Number:
PCT/IT2007/000551

(22) International Filing Date: 31 July 2007 (31.07.2007)

(25) Filing Language: Italian

(26) Publication Language: English

(30) Priority Data:
RM2006A000409 31 July 2006 (31.07.2006) IT

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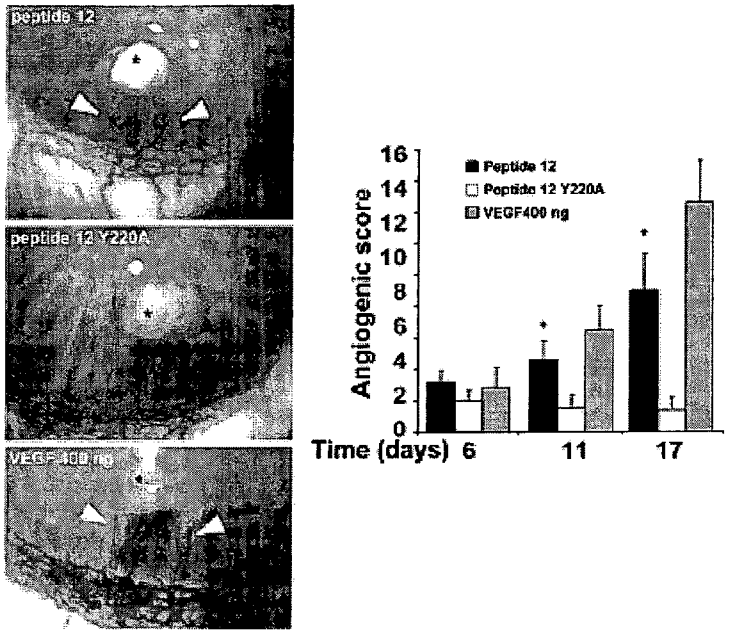
(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

[Continued on next page]

(54) Title: PEPTIDE DERIVED FROM VASCULAR ENDOTHELIAL GROWTH FACTOR RECEPTOR-1 BINDING INTEGRIN α5β1, HAVING PROANGIOGENIC ACTIVITY



(57) Abstract: Description of a sequence peptide isolated in the domain II type-immunoglobulin (Type-Ig) of the Vascular Endothelial Growth Factor receptor 1 (VEGF-1) binding integrin α5β1, usable for the preparation of pharmacological agents having proangiogenic activity.

WO 2008/015720 A2

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *without international search report and to be republished upon receipt of that report*

PEPTIDE DERIVED FROM VASCULAR ENDOTHELIAL GROWTH FACTOR RECEPTOR-1 BINDING INTEGRIN α5β1, HAVING PROANGIOGENIC ACTIVITY.

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The present invention refers to the field of molecular Biology and in particular to one peptide sequence isolated in domain II type-Ig of the VEGFR-1 able to bind and to activate the integrin α5β1. In particular, the isolated sequence peptide, object of the present invention, can favourably be employed for the preparation of pharmacologic agents having proangiogenic activities in the treatment of pathologies that benefit from the formation of new blood vessels.

Vascular endothelial growth factor receptor-1 (VEGFR-1) is a tyrosine kinase receptor for specific members of the vascular endothelial growth factor (VEGF) family. VEGFR-1 is closely related to the type III tyrosine kinase-Fms/Kit/platelet derived growth factor receptors, but it is classified, with VEGFR-2 and VEGFR-3, into a distinct class of receptors whose extracellular region is composed of seven immunoglobulin (Ig)-like domains. Ig-like domain II comprises the region involved in VEGF and placenta growth factor (PlGF) binding, whereas Ig-like domains III and IV are involved in heparin binding, and domain III is also responsible for interaction with neuropilin-1.

The VEGFR-1 is expressed mainly from the endothelial and smooth muscular cells, but also from other cellular types of the haematopoietic lineage and

from the cells of the skeletal muscle. The signal transduction in the cell from the VEGFR-1, as a result of the interaction with the growth factor, is not well known.

Gene knockout studies demonstrated that VEGFR-1 is essential for the development and differentiation of embryonic vasculature, and that its inactivation results in increased hemangioblast commitment and disorganization of blood vessels. However, mice carrying a homozygous deletion of the intracellular kinase domain show a correct blood vessel development, this result suggests that the extracellular region of VEGFR-1 is sufficient to support embryonic angiogenesis.

The tyrosine kinase activity of the VEGFR-1 is essential for the chemotattic signalling induced from VEGF and PlGF in monocyte-macrophage and in the haematopoietic stem cells.

Studies on the PlGF, a specific ligand of the VEGFR-1, have demonstrated that this receptor is been involved in the pathological angiogenesis in adults.

More recently it has been demonstrated that growth factors that bind the VEGFR-1 can induce the trans-phosphorilation of VEGFR-2 and that the stimulation of the angiogenesis can be obtained by the formation of heterodimeric complex VEGFR-1/VEGFR-2.

In addition to the transmembrane isoform of VEGFR-1, endothelial cells (EC) produce a soluble form of the receptor, arising from alternative splicing of the same gene. Soluble VEGFR-1 (sVEGFR-1) comprises the first six Ig-like domains of the extracellular region of the

receptor and a specific 30 amino acid tail at its C-terminus. Both isoforms bind VEGF with high affinity, and sVEGFR-1 acts as a potent antagonist of VEGF signalling by sequestering the growth factor and thereby inhibiting its interaction with the transmembrane receptors.

The inventors previously demonstrated that sVEGFR-1 secreted by EC in culture becomes part of the extracellular matrix and is able to mediate EC adhesion through direct binding to α5β1 integrin.

A key role of this integrin in promoting vasculogenesis and angiogenesis has been previously highlighted by the defective vascular phenotype of α5 knockout mice and by the ability of either antibodies raised against this integrin or antagonist peptides to block in vitro and in vivo angiogenesis.

Integrin binding to their ligands usually occurs through recognition of short amino acid sequences. Many integrins, including α5β1, bind to arginine-glycine-aspartic acid (RGD) containing peptides, although other motifs have also been identified.

On the basis of what was known to the state of technical, and on the assumption that the sequence of sVEGFR-1 does not contains RGD motif, the inventors of this invention focused their intention on identification of the molecular determinants responsible for its interaction with integrin α5β1.

Experiments, below detail described, was first revealed that an antibody against a peptide mapping on the domain II type-ig prevents the binding of VEGF-1 with integrin α5β1. Therefore, the experimental efforts

were focused on this domain II that supports both the accession of EC that the direct link with integrin α5β1.

Subsequently, on the basis of the information available in the experimentally determined structures of this domain II, were developed twelve peptides putatively mimicking the whole surface of the domain, which were subsequently tested for the interaction with the integrin. Of these twelve peptides one was able both to support the accession of EC, that to compete with the accession of the EC sVEGFR-1, and the migration of EC to the sVEGFR-1.

Furthermore, the peptide showed proangiogenic activity in vitro. The replacement with alanine scanning mutagenesis of the peptide has, finally, made it possible to identify the residues of the peptide responsible for its biological activity, which represent a binding motif for the integrin α5β1.

Therefore, it is object of the present invention a peptide having sequence NYLTHRQ with same proangiogenic effect as well as the peptide of sequence YLXHR in which X it can be whichever amino acid, but preferably is Thr/T.

Another object of the present invention is the use of this peptides for the preparation of a pharmacological compound, suitable for the treatment of the clinical conditions that need the induction of the angiogenesis, such as: peripheral hypertension, vascular diabetes-dependent pathologies, wound, ischemia of the muscle, the brain, the kidney, the intestine, the heart or the limbs, serious occlusive or

obstructive vascular pathologies, peripheral vascular pathologies, pericardic ischaemia, infarct whichever vascular pathologies.

Another object of the present invention is the use of peptides in order to direct molecules or particles also viral particles towards integrin α5β1.

Further features of the present invention will turn out clearer from the detailed description, equipped from the experiments, that it follows with reference to the attached figures in which:

Figure 1A shows the results of a solid-phase binding assay test of integrin α5β1 to sVEGFR-1 in the presence/absence of antibodies directed against specific regions of the sVEGFR-1 protein, the amount of bound protein was measure by colorimetric assay (absorbance at 405nm).

Figure 1B shows the results of a test of adhesion of the endothelial cells (EC) incubated on plates covered with sVEGFR-1, a protein fusion with the FC domain of human immunoglobulins (VEGFR-1 Fc), with the domain II type-Ig recombinant (d II), with fibronectin (FN) or bovine serum albumin (BSA), followed by colorimetric assay (absorbance at 540nm).

The figure 1C shows the results of an adhesion test on solid phase of integrin α5β1 on plates covered with the domain II type-Ig recombinant or with VEGFR-1 Fc, followed by colorimetric assay (absorbance at 405nm).

Figure 2A shows in the right panel the location of peptides 2, 4, 5, 7, and 12 in the structure of domain

II and in the left panel the location of peptides 1, 3, 5, 7, 9 and 11.

The figure 2B shows the results of a test of adhesion of the EC on plates covered with VEGFR-1/Fc or fibronectin, in the presence of the twelve peptides, the result is expressed as percentage of EC adhesion on the same substrates in the absence of peptides.

The figure 2C shows the results of a test of adhesion of EC on plates covered with peptides 4, 5, 7, 12, 1 and RGD, followed by colorimetric assay (absorbance at 540nm).

Figure 3A shows the results of a test of adhesion of the EC on plates covered with peptide 12 in the presence of EDTA, EGTA, an antibody against $\alpha5\beta1$, $\alpha5$, $\beta1$ and $\alpha6$, followed by colorimetric assay (absorbance at 540nm).

The figure 3B shows, in panels a-e, the results of an immunostaining for integrin $\alpha5\beta1$ of the EC incubated with magnetic beads coated with RGE peptide (panel a), RGD peptide (panel b, d) or with peptide 12 (panel c, e)

Figure 3C shows the results of a test of migration of EC induced by VEGF-1/Fc or fibronectin (FN) in the presence of the peptide 1 or 12, the result is expressed as percentage of inhibition of migration observed in the absence of peptides.

The figure 4A shows the effect of the replacement with alanine of various aminoacidic residues in peptide 12 on the cellular adhesion.

The figure 4B shows the effect of the replacement with alanine of different aminoacidic residues in the

peptide 12 in binding the integrin α5β1.

Figure 5 shows the results of a test of endothelial vessel formation in the presence or absence of peptide 12 (panels a g), Q225A (panel c), Y220A (panel d), and concentrations of VEGF (100 nM) (panels b, d), and 20 mg/ml VEGFR-1 Fc (panels e, f, g).

The figures 6A and 6B show the effect on angiogenesis of the peptide 12 or Y220 compare to VEGF at different concentrations.

Figure 7 shows the results of histological and immunohistochemical analysis of corneal angiogenesis.

1. Effect of the antibody to recognize a peptide whose sequence is present in VEGFR-1 domain II on the interaction sVEGFR-1/integrin.

It is known that the integrin α5β1 bind the VEGFR-1/Fc protein, and having previously demonstrated, the same inventors, than an antibody, able to recognize a peptide whose sequence is present in VEGFR-1 domain II, competes with the adhesion of the EC to the sVEGFR-1 mediated from the integrin α5β1; the effect of the same antibody was analyzed (able to recognize a peptide whose sequence is present in VEGFR-1 domain II) on the direct interaction sVEGFR-1/integrin.

In a binding test on solid phase, dishes covered with VEGFR-1/Fc were dealt with various antibodies anti-VEGFR-1 and incubated with the purified integrin α5β1, the amount of integrin bind was quantified by means of incubation with an antibody anti-integrin α5β1 and colorimetric identification of this last one. The capacity of the antibody to recognize a peptide whose sequence is present in domain II of VEGFR-1 (dII Ab)

completely inhibited the binding of the integrin α5β1 with the VEGFR-1. A polyclonal antibody (Flt-1 H225) directed against a proteic fragment comprising the aminoacidic residual from 1 to 225 of the VEGFR-1, did not inhibit the VEGFR-1/integrin interaction. As expected the inhibition was not obtained with an antibody (Flt-1 C17) directed against the intracellular domain of VEGFR-1 (Figure 1A).

2.Construction of a recombinant protein correspondent to VEGFR-1 domain II

Based on the domain boundaries observed in the experimentally determined structures available from the PDB database (www.rcsb.org), was designed a recombinant protein corresponding to domain II (residues 129-229). The protein was produced in cell line insect SF9 using the system of expression mediated by baculovirus and purified by affinity chromatography. The recombinant protein had the molecular weight expected on SDS-PAGE and was recognized by a polyclonal antibody anti-VEGFR-1.

The full cDNA of VEGFR-1 used for the production of the domain II recombinant was kindly provided by Dr. Shibuya (Institute of medical science, University of Tokyo, Japan). The cDNA was modified in the laboratory of Dr. Ballmer-Hofer (Paul-Scherrer Institut, Villigen-PSI, Switzerland) to introduce the site KPN I followed by a Kozak sequence, a start codon to the 5' end, a site BSI WI followed by an HA tags and a stop codon at the 3' end. The polylinker of the vector pFastBac 1 of Baculovirus (Invitrogen-Life Technologies) was replaced with a linker containing a site KPN I and a BSI WI

together with a His tag.

Firstly, the full cDNA of VEGF-1 was cloned in pFastBac 1 using the sites KPN I and the BSI WI. To build the domain II Recombinant, the signal sequence of VEGFR-1 was amplified by PCR using the oligonucleotides:

5'- GCGCGGATCCGGTACCGC-3' and

5 ' - GTGATGCGTACGTGAACCTGAACTAGATCCTGTG-3'

and cloned into PFastBac previously modified.

Then the domain II was amplified with the oligonucleotides:

5'-GTGATGCGTACGAGTGATACAGGTAGACCTTTC-3' and

5'-GTGATGCGTACGTCCGATTGTATTGGTTTGTCGATG-3'.

The PCR fragment was cloned into the modified pFastBac vector carrying the VEGFR-1 signal sequence. Recombinant Baculovirus was obtained using the Bac-to-Bac system (Invitrogen-Life Technologies). SF9 (Spodoptera frugiperda) cell line was purchased from Invitrogen, and maintained in culture at 27°C in TC100 medium (Invitrogen-Life Technologies) containing 10% heat inactivated fetal calf serum. Infected SF9 cells were cultured in serum free SF-900 medium (Invitrogen-Life Technologies). For protein expression, SF9 cells were grown up to 60% confluence and infected with recombinant virus. Forty-eight hours post-infection cells were collected, and the recombinant protein was purified by affinity chromatography using the B-PER His Fusion Protein purification kit (PIERCE, Rockford, IL).

3. Ability of domain II to support the adhesion of the EC

The endothelial cells derived from human umbilical

vein (HUVEC) were used to examine ability of the domain II to support the adhesion of EC in plates covered with the recombinant protein.

The HUVEC were incubated on plates covered with VEGFR-1 FC, with the domain II (dII) recombinant, with fibronectin (FN) or bovine serum albumin (BSA). The protein VEGFR-1/Fc, and the fibronectin were used as positive controls and BSA as negative control.

The cells assayed were coloured and the absorbance was measured.

The dII recombinant supported the adhesion of the HUVEC (figure 1B) in analogous way to the VEGFR-1/Fc.

The direct bind of the integrin α5β1 to the domain had been determined also with binding test on solid phase. The purified integrin α5β1 was added to plates covered with dII or with VEGFR-1/Fc like positive control and the integrin bound was measured using an antibody anti-integrin α5β1 and a colorimetric test. As shown in figure 1C a similar amount of integrin interacted with the VEGFR-1/Fc and the dII recombinant.

4. Chosen of peptides on VEGFR-1 domain II.

In order to identify a region of domain II potentially interacting with the integrin α5β1, were employed the determined structures of the domain with X-ray crystallography available in the data bank PDB. The three-dimensional structures were examined in order to identify the contiguous aminoacidic sequences on the surface of the protein that could mimic all the exposed region of domain II.

Twelve peptides (table I), were elaborate based on the following criteria:

1) they are on the protein surface and have high overall solvent accessible surface area;

2) they correspond to different regions of the domain surface, covering it almost entirely;

3) they are shorter than 15 residues;

4) they contain an high percentage of polar residues and, whenever possible, residues charged at physiological pH values (Asp, Glu, Lys and Arg);

5) they contain a low percentage of hydrophobic residues (i.e. Leu, Val, Ile, Met, Phe and Trp), to minimize solubility problems;

6) they do not contain Cys residues, which might lead to formation of dimeric peptides.

Table I.

P1	GRPFVEMYSE	132-141
P2	YSEIPEIIH	139-147
P3	ETTHMTEGR	144-152
P4	TEGRELVIPARVT	149-161
P5	NITVTLKKFPL	164-174
P6	KKFPLD	170-175
P7	KFPLDTLIPDG	171-181
P8	DTLIPDGKRII	175-185
P9	DSRKGFIISNAT	187-198
P10	TYKEIGL	198-204
P11	EATVNGHLYKT	208-218
P12	NYLTHRQ	219-225
sP12	LTQNYRH	-

To satisfy the requirement 6, VEGFR-1 residue Cys 158 was replaced by Ala in peptide 4. Additionally, to explore whether the presence of two branched hydrophobic residues next to each other might cause solubility problems, the adjacent Ile 145 and Ile 146 were both replaced by Thr in peptide 3. The sequence of

peptide 5 has been previously published and reported to act as an angiogenesis inhibitor without binding to VEGF or inhibiting VEGF binding to its receptors.

5. Test on the ability to twelve peptides to inhibit the adhesion mediated from α5β1 of the EC to VEGFR-1

In order to test the ability of the twelve chosen peptides to inhibit the adhesion of the EC to VEGFR-1 mediated from the integrin α5β1, every peptide was added to one suspension of HUVEC before plated on dishes covered with VEGFR-1/Fc or fibronectin (FN). The data were indicated as percentage of adhesion, calculated to the number of cells adhered on the substrate itself in absence of peptides.

Like shown in figure 2B peptides 4, 5, 7, and 12 effectively blocked the adhesion of the EC to VEGFR-1/Fc. Moreover, peptides 4, 7 and 12 were able to partially inhibit the adhesion of the EC to the fibronectin, being suggested some interference with the binding site of the integrin α5β1.

Localization of peptides 4, 7 and 12 on the structure of VEGFR-1 domain II is shown in figure 2A (ribbon representation of the track of carbon determined by x-ray crystallography): peptide 4 nearly overlaps entire to the chain B inside of the first (ABED) beta sheet; peptide 12 is localized on the opposite beta sheet inside of the chain G to the C terminal of the domain; peptide 7 comprises part of chain C, chain C', and loop C' D, that connects the two beta sheets one to the other.

6. Effects of peptides on the adhesion of the EC

To establish whether the four peptides, were able to inhibit the adhesion of the EC to VEGFR-1, by supporting directly the adhesion of the EC, the peptides were linked covalently to plates and the HUVEC were incubated on plates for 1 hour. The peptide 1 was used as negative control and the peptide RGD as the positive control. Unspecific adhesion was measured by absorbance in the plates covered with BSA. In this test only peptide 12 induced the adhesion of EC (Figure 2C).

7. Interference of the divalent cations on the adhesion of the EC to peptide 12.

The binding of proteins of the extracellular matrix to the integrins is generally dependent on the presence of the divalent cations, then it was investigated the interference of divalent cations with the adhesion of the EC to peptide 12. The peptide 12 or the BSA, as non-specific adhesion control, were linked on plates and the HUVEC added in the presence of chelating agents (EDTA or EGTA).

As shown in Figure 3A treatment with EDTA OR EGTA completely abolished adhesion of EC indicating that the adhesion of the EC to peptide 12 requires divalent cations.

In order to demonstrate that the integrin α5β1 was the protein that mediated the adhesion of the EC to peptide 12, anti-integrin antibody was tested. The HUVEC were pre-incubated with an antibody against the integrin α5β1 or versus the integrinic subunit α6 and left to adhere on plates covered with peptide 12 or BSA. The pre-incubation of the HUVEC with the antibody against the integrin α5β1 inhibited the adhesion of the

EC to peptide 12, while the antibody against α6 was ineffective (Figure 3A).

8. Induction of integrin α5β1 aggregation upon binding to peptide 12.

The integrins not linked are widely distributed on cellular membrane, but after the ligand binding and the activation merged in distinct structures called focal contacts and ECM contact.

It was examined whether the peptide 12 was able to induce aggregation of integrin α5β1 after the binding. EC adherent cells were incubated with magnetic beads coated with peptide 12, or peptide RGD or peptide RGE respectively as positive and negative controls, and immunostained with an antibody against integrin α5β1.

A separate colouring for integrin α5β1 was observed around the beads covered with the peptide 12(figure 3B, panels c, e) similar to that observed for the beads covered with the peptide RGD (figure 3B, panels b, d). No beads covered with the peptide RGE was attached with HUVEC in this test (Figure 3B, panel a).

9. Effects of peptide 12 on migration and cellular proliferation

In order to investigate the ability to peptide 12 to influence the migration of the EC induced from VEGFR-1/Fc, a process mediated from the integrin α5β1, the HUVEC were pre-incubated with peptide 12 and peptide 1 and placed in Boyden chambers in order to analyze the migration induced from VEGFR-1/Fc or fibronectin (FN). Data represent the average of 14 microscopic fields and are referred to as percentage of inhibition compared to the migration observed in the

absence of peptides. As shown in Figure 3C the peptide 12 completely annulled the migratory response induced by VEGFR-1 Fc while the incubation of cells with a control peptide was completely ineffective. The inhibitory effect of the peptide 12 on cell migration was specific to the VEGFR-1, while no significant inhibition was observed on migration induced by fibronectin.

10. Effects of peptide 12 on the formation of vascular structures.

It was tested the ability of the peptide 12 to induce the formation of tubules of EC. HUVEC cells were plated on 24 wells plates previously covered with a mixture of gel Type I collagen, in the absence or presence of 500 µg/ml peptide 12 or peptide 1. A second layer of gel Type I collagen was placed on the adherent cells. As shown, in the absence or presence of peptides, were added, in the culture medium, different concentrations of VEGF (40 or 100 nM), or 20 µg/ml of protein VEGFR-1/ Fc. The capacity of the peptide 12 to induce the formation of tubules in EC on the collagen I matrix was found similar to that of VEGF (Figure 5). Furthermore, when the VEGF was used to low concentration (40nM) the simultaneous treatment with the peptide 12 increased the formation of induced VEGF tubules. On the contrary, the peptide 1 did not induce the formation of tubules neither increased the effect of VEGF. The addition of VEGFR-1 Fc, that negatively regulate the function of VEGF through the sequestration of growth factor and the consequent inhibition of its interaction with the tyrosine kinase isoform of the

receptor, was used as negative control and led to a strong inhibition of the constitution of tubules.

To address the angiogenic role, in vivo, pellets releasing peptide 12 were implanted into rabbit corneal tissue and neovascularization was monitored for 17 days. As shown in Figure 6A and Figure 7 peptide 12 strongly promoted neoangiogenesis.

In presence of VEGFR-1/Fc, peptide 12 partially maintained its ability to induce the formation of vascular structures suggesting that its mechanism of action is independent from that of VEGF (data not shown).

11. Identification of the residues of the peptide 12 involved in the binding with integrin.

To determine which residues of the peptide 12 had a role in the adhesion with integrin $\alpha 5\beta 1$, were synthesized ten different peptides, each having a single or double replacement with alanine (ALA). These peptides were tested for their ability to directly support the cellular adhesion. The ten peptides were covalently linked to plates, and were also tested, as negative controls, a peptide "scrambled" in which the amino acid sequence of the peptide 12 was modified (see Table 1) (sP12) and BSA. The HUVEC were plated and allow to adhere. The results were expressed as percentage of the cellular adhesion observed on plates covered with the peptide 12.

As shown in Figure 4A, the replacement of only 3 residues with ALA influence the capacity of the peptide 12 to support the adhesion of EC: the replacement of Tyr 220 with ALA completely abolished cellular adhesion

to peptide, the mutation of Leu 221 in ALA resulted in a partial reduction in the cellular adhesion, comparable to that observed in His 223 peptide mutant in Ala. These results demonstrated that the residues which are important in mediate the binding of the cells are Tyr 220 Leu 221 and His 223. Was also assessed the direct binding to integrin α5β1 with mutant peptides by a binding test on solid phase. The integrin α5β1 purified was added to plates covered with the various peptides and the amount bound was measured using an antibody anti-integrin α5β1 with a colorimetric method. The data were expressed as percentage of protein linked observed on plates covered with the original peptide 12.

As shown in Figure 4B, the Tyr 220 is the residue more critical to the integrin binding and Leu 221 that His 223 are important in supporting the protein/protein interaction. Differently from cellular adhesion, Arg 224 also plays a role in mediating the binding of integrin with the peptide 12.

The peptide 12 is located at the C terminus of the G chain of Domain II (Figure 2A). Its residues are accessible to solvent, with the exception of Tyr 222 whose side chain was close in the central core of the domain. The side chain of Tyr 220 is partially protected from the solvent by the carboxylate group of the Glu 144 in the side chain. The last residue is, however, highly flexible, as demonstrated by the high values of B factor in all crystalline structures of domains available, and from the conformational variability observed in the NMR structures.

Therefore, it is assumed that Tyr 220 was available for the binding with the ligand.

12. Specificity of the residues YLXHR

In order to verify the specificity of residual YLXHR (X = whichever residual one) in extracellular proteins, the annotated human proteins from the UniProtKB/Swissprot consortium had been searched for its recurrence. The motif was found in 20 (0,2%) of 8,687 analyzed proteins, nobody of which was extracellular, suggesting that this sequence of binding for the integrin α5β1 is peculiar of the sVEGFR-1. On the contrary, the motif RGD was found again in 7,9% of all human proteins and was more frequent in extracellular proteins (9,7%).

As stated in the experiments described detail here, the research have led to many important results, the following are summarised:

- the two isoforms, derived from alternative splicing of the VEGF receptor-1 play a different biological role during angiogenesis. In particular, the transmembrane isoform of the receptor is a tyrosine kinase, while the soluble form acts as molecule in extracellular matrix, involved in adhesion and cell migration, that as negative regulator of the activity of VEGF signal.

- an antibody against a peptide whose sequence is present in the domain II of VEGFR-1 inhibits cellular adhesion on areas covered with sVEGFR-1 and blocks the interaction with integrin α5β1.

- a fragment of VEGFR-1, corresponding to the domain II, directly bind integrin α5β1 and is

sufficient to support the adhesion of the endothelial cells (EC).

- of the twelve peptides, that mimic selected regions of the surface of the receptor, four peptides (4, 5, 7 and 12) inhibit cellular adhesion to sVEGFR-1.

- the peptide 12 is the only that directly supports the adhesion of EC, and therefore interacts directly with integrin α5β1. In fact, the binding EC/peptide 12 is sensitive to divalent cations and is inhibited by antibodies against integrin α5β1.

- the direct interaction between the peptide 12 and integrin activate integrin.

- Peptide 12 inhibits the induced cellular migration from VEGFR-1, process that is mediated by the integrin α5β1.

- Peptide 12 is able to induce in vitro the formation of vascular structures (tubules), suggesting that sVEGFR-1, the whose domain II contains sequence of peptide 12 is involved in the formation of blood vessels, adhesion and migration of the endothelial cells and is a positive modulator of blood vessels morphogenesis.

- three residues of the peptide 12, Tyr 220, Leu 221 and His 223 if mutated, significantly reduce the capacity of the peptide support cellular adhesion.

- the motif having sequence YLXHR, represents the region, in the domain II of the receptor VEGFR-1, strictly necessary for the interaction with integrin.

- This sequence does not interact with growth factors, VEGF and PLGF, therefore, although the residue responsible for the binding with the growth factors and

integrin are close, the two interactions may be mutually exclusive.

Finally the biological effects of VEGF-1 on the endothelial cells and the identification of molecules interfering the different functions of this receptor could be the basis for the development of more effective therapeutic products.

In fact, the gene sequence YLXHR could be effectively used as a proangiogenic agent, in the treatment of clinical conditions that require triggering of cell angiogenesis.

These clinical conditions are included in group consisting of:

Hypertension, peripheral vascular pathologies diabetes-dependent, wounded, ischaemia of : muscle, brain, kidney, gut, heart or the arts; vascular pathologies occlusive or obstructive serious, peripherals vascular pathologies, pericardial ischaemia, myocardial infarction, diseases of the coronary arteries, cerebral vascular pathologies, visceral vascular pathologies.

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CLAIMS

1) Peptide sequence of YLXHR having proangiogenic effect characterized by the fact that X is selected in the group consisting of: Ala/A, Arg/R, Asn/N, Asp/D, Cys/C, Glu/E, Gln/Q, Gly/G, Ile/I, Leu/L, Lys/K, Met/M, Phe/F, Pro/P, Ser/S, Thr/T, Trp/W, Tyr/Y, Val/V.

2) Peptide sequence of YLXHR having proangiogenic effect according to the claim 1 characterized by the fact that X is Thr/T.

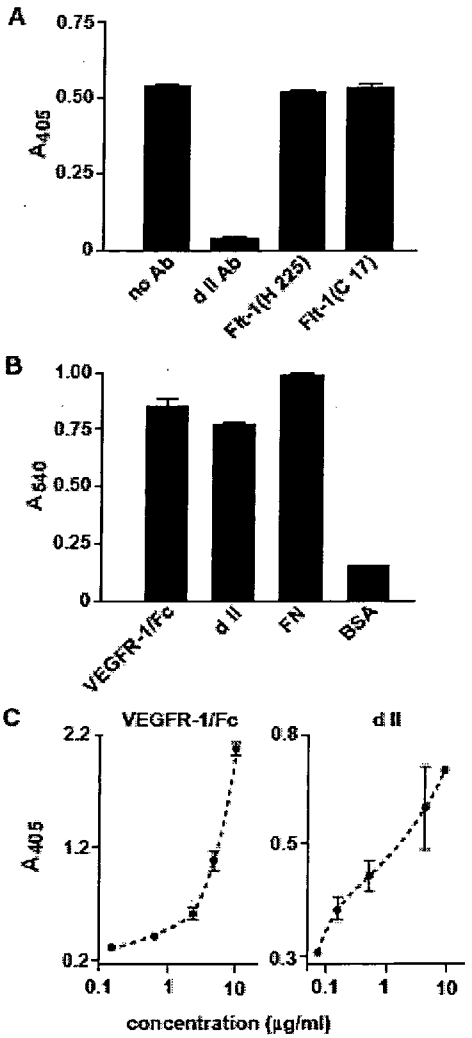
3) Peptide sequence of NYLTHRQ having proangiogenic effect.

4) Use of the peptide by any of the claims from 1 to 3 for the preparation of a pharmacological compound.

5) Use of the peptide by any of the claims from 1 to 3 for the preparation of a pharmacological compound usable for the treatment of clinical conditions that require induction of angiogenesis.

6) Use according to the claim 5 characterized by the fact that the clinical condition requiring angiogenesis are choices in the group consisting of: hypertension, peripheral vascular pathologies diabetes-dependent, wounded, ischemia of the muscle, ischemia of the brain, ischemia of the kidney, ischemia of the gut, ischemia of the heart or ischemia of the the arts, vascular pathologies occlusive or serious obstructive, peripherals vascular pathologies, pericardial ischaemia, myocardial infarction, diseases of the coronary arteries, cerebral vascular pathologies, visceral vascular pathologies.

7) Use of peptide according to any of the claims from 1 to 3 to direct molecules or particles, also viral, to integrin α5β1 in vitro and in vivo.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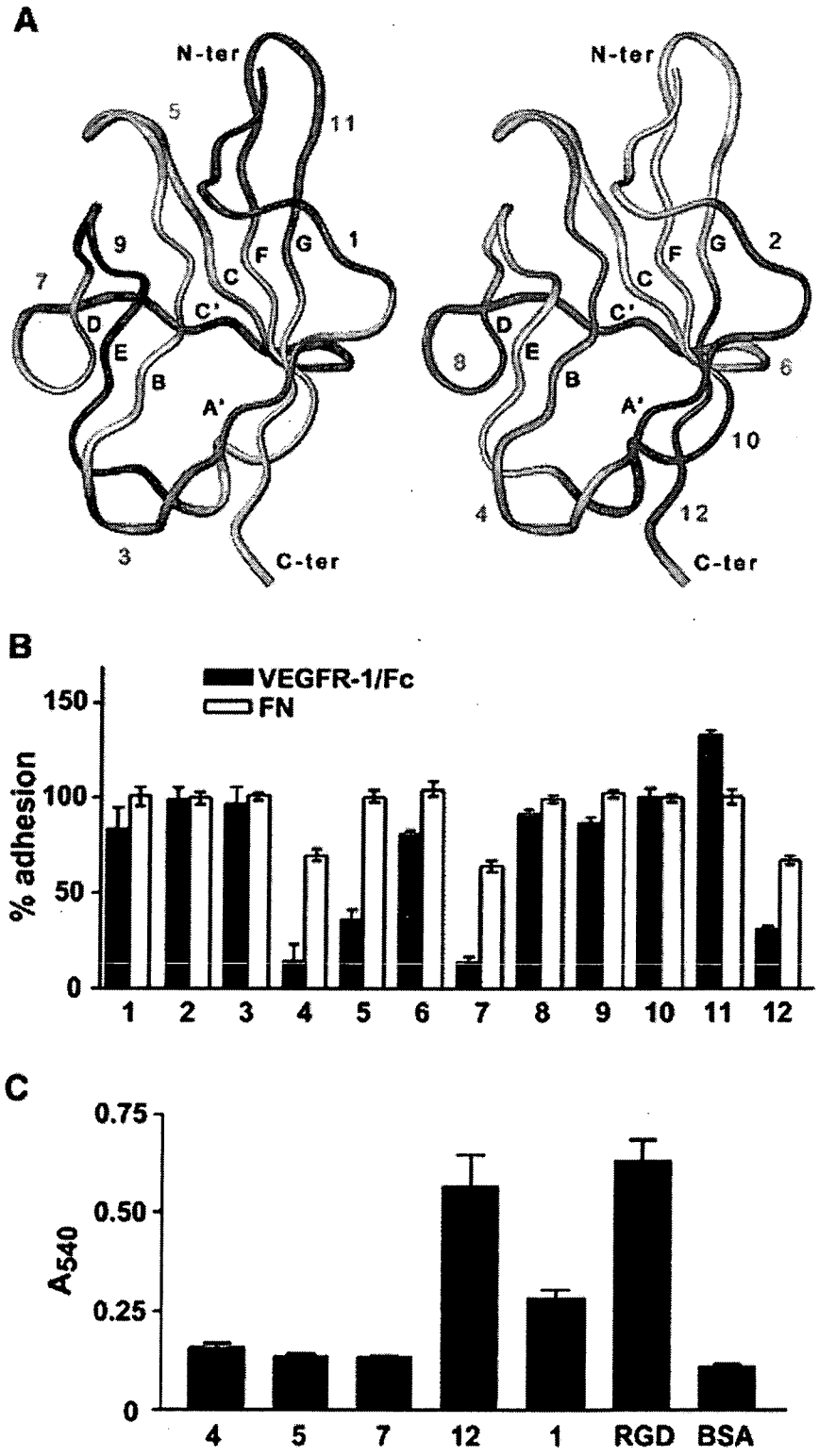
0.50
0.25
0
A405
no Ab
d II Ab
Flt-1(H 225)
Flt-1(C 17)
B
1.00
0.75
0.50
0.25
0
A 540
VEGFR-1/Fc
d II
FN
BSA
C
VEGFR-1/Fc
d II
2.2
1.2
0.2
0.8
0.5
0.3
A 405
0.1
1
10
concentration (µg/ml)

Fig. 1


A
N-ter
5
11
9
1
7
F
G
C
C'
D
E
B
A'
3
C-ter
N-ter
F
G
2
C
C'
D
E
8
B
6
A'
10
4
12
C-ter
B
VEGFR-1/Fc
FN
150
100
50
0
% adhesion
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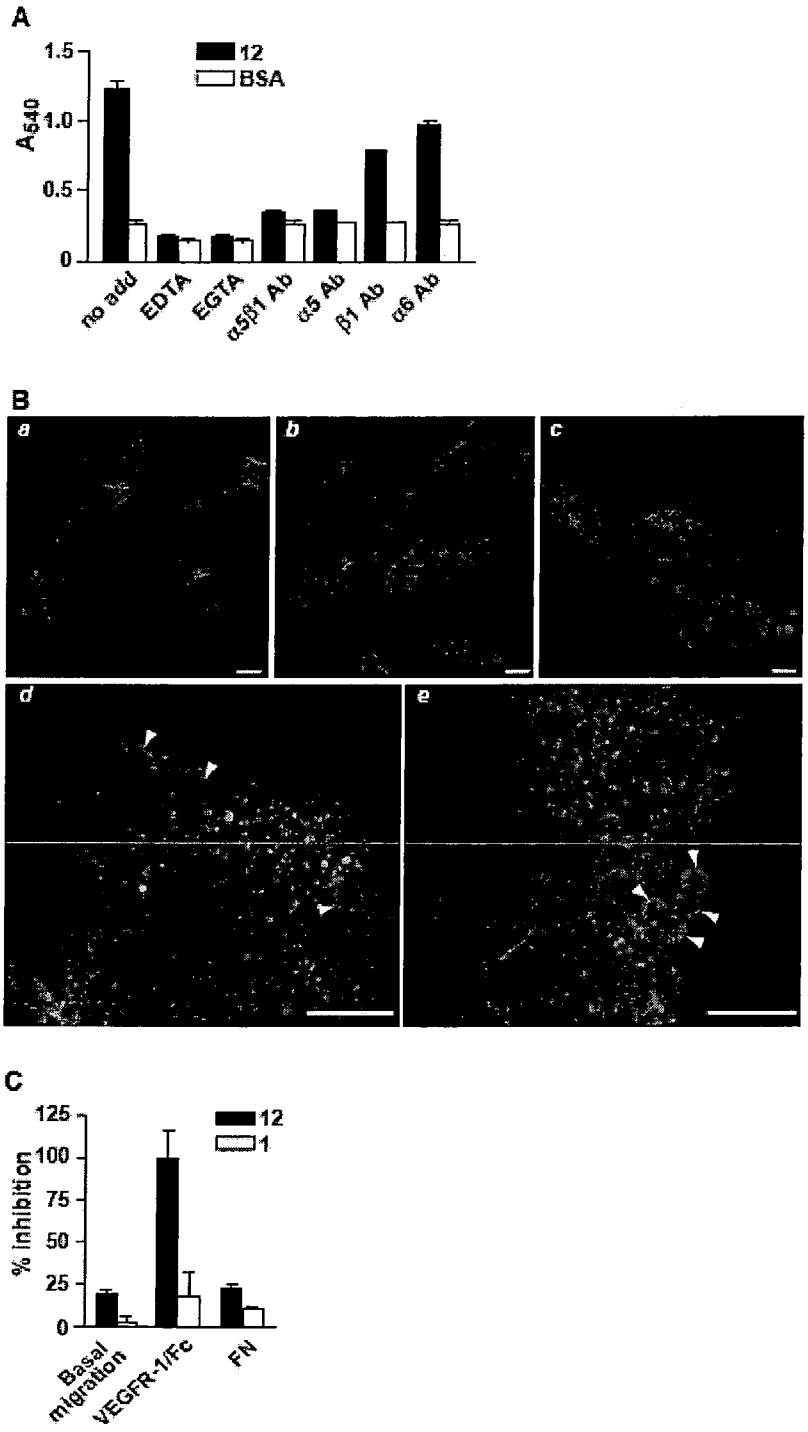
0.50
0.25
0
A540
4 5 7 12 1 RGD BSA

Fig. 2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BSA
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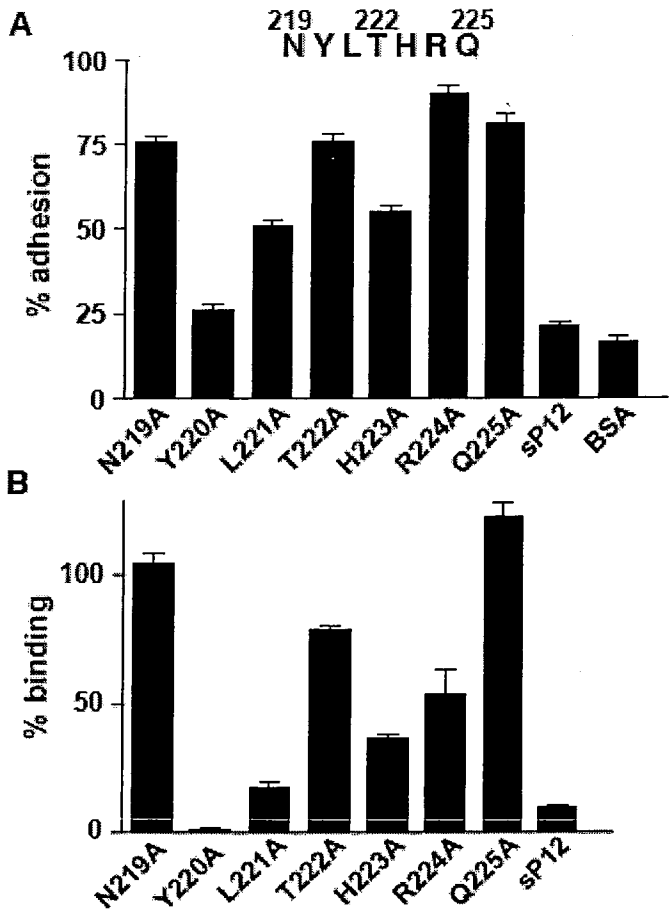
1.0
0.5
0
no add
EDTA
EGTA
α5β1 Ab
α5 Ab
β1 Ab
α6 Ab
B
a
b
c
d
e
C
12
1
% Inhibition
125
100
75
50
25
0
Basal migration
VEGFR-1/Fc
FN

Fig. 3


A
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100
75
50
25
0
% adhesion
N219A
Y220A
L221A
T222A
H223A
R224A
Q225A
sP12
BSA
B
100
50
0
% binding
N219A
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L221A
T222A
H223A
R224A
Q225A
sP12

Fig. 4

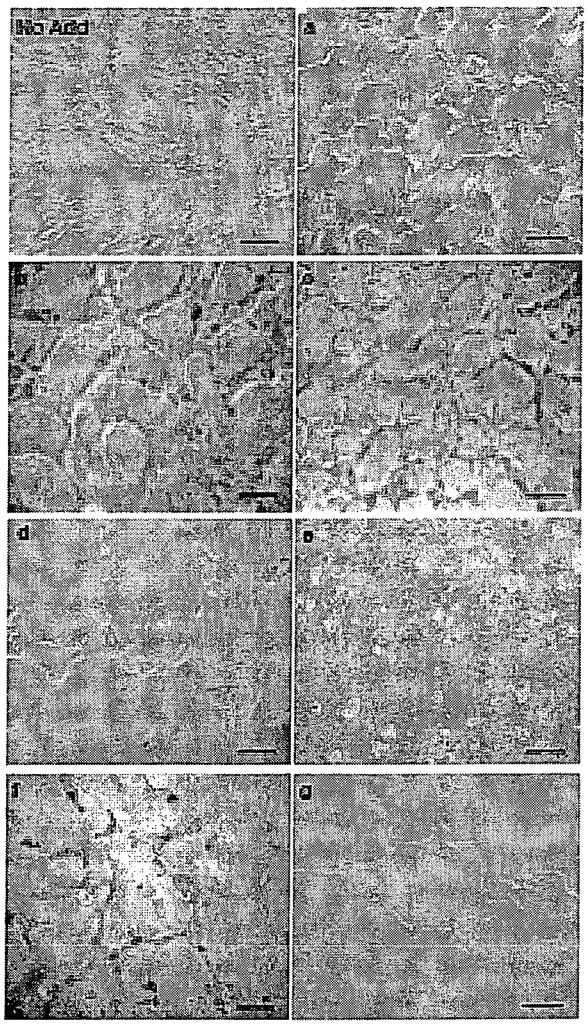
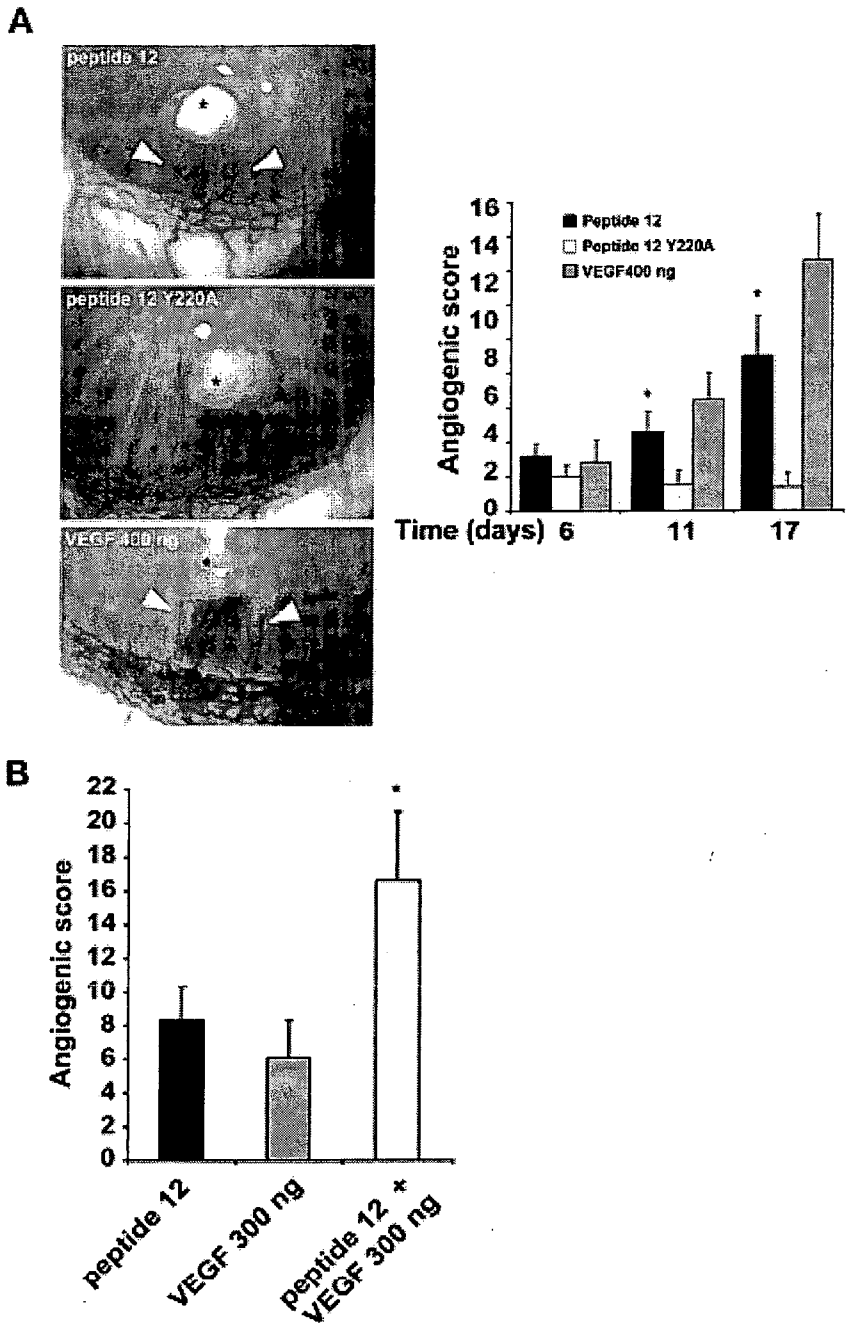


Fig.5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peptide 12
peptide 12 Y220A
VEGF 300 ng
Angiogenic score
Peptide 12
Peptide 12 Y220A
VEGF400 ng
Time (days) 6
11
17
B
Angiogenic score
peptide 12
VEGF 300 ng
peptide 12 +
VEGF 300 ng

Fig. 6

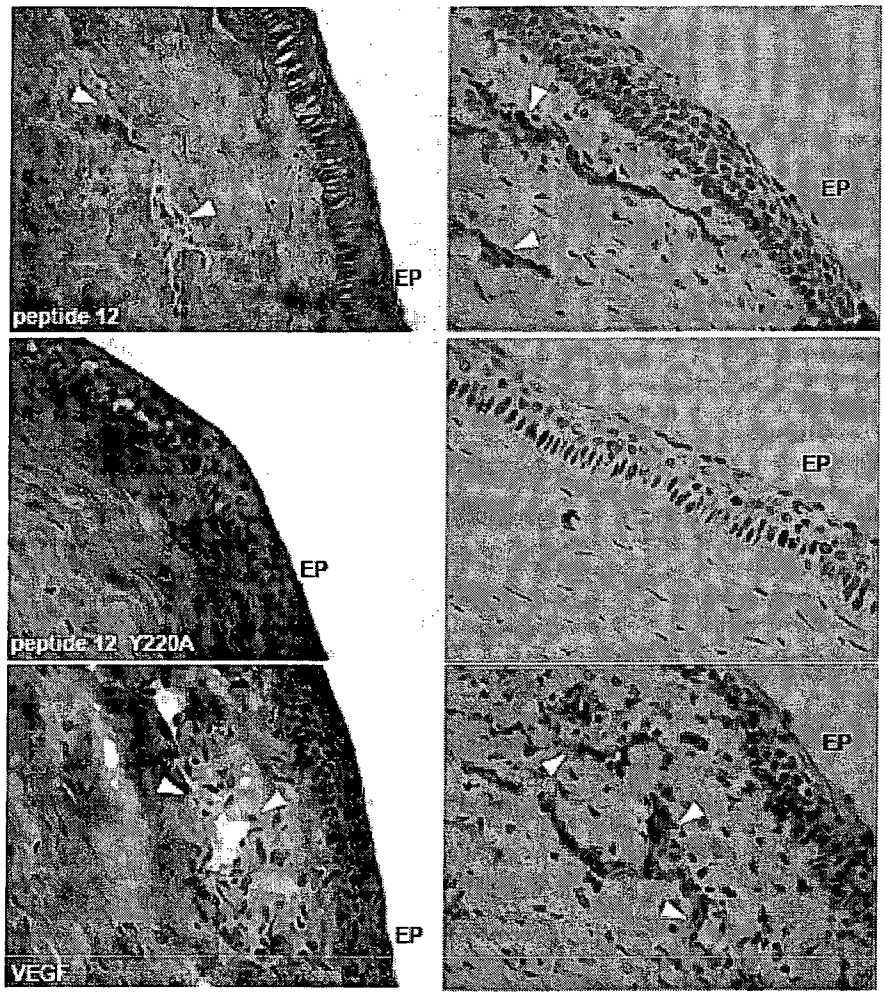

Fig. 7