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ORIGINAL RESEARCH PAPER

Soluble N-cadherin fragment promotes angiogenesis

L. Derycke · L. Morbidelli · M. Ziche · O. De Wever · M. Bracke · E. Van Aken

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Abstract Endothelial cells express two dependent intercellular adhesion molecules: vascular endothelial (VE)-cadherin, specific for endothelial cells, and N-cadherin, also present in neuronal, lens, skeletal and heart muscle cells, osteoblasts, pericytes and fibroblasts. While there exists a vast amount of evidence that VE-cadherin promotes angiogenesis, the role of N-cadherin still remains to be elucidated. We found that a soluble 90-kDa fragment N-cadherin promotes angiogenesis in the rabbit cornea assay and in the chorioallantoic assay when cleaved enzymatically from the extracellular domain of N-cadherin. Soluble N-cadherin stimulates migration of endothelial cells in the wound healing assay and stimulates phosphorylation of extracellular regulated kinase. In vitro experiments with PD173074 and knock-down of N-cadherin and fibroblast growth factor (FGF)-receptor, showed that the pro-angiogenic effect of soluble N-cadherin is N-cadherin- and FGF-receptor-dependent. Our results suggest that soluble N-cadherin stimulates migration of endothelial cells through the FGF-receptor.

L. Derycke (✉) · O. De Wever · M. Bracke
Laboratory of Experimental Cancerology, Department of Radiotherapy and Nuclear Medicine, Ghent University Hospital, De Pintelaan 185, 9000, Gent, Belgium
e-mail: lara.derycke@ugent.be

L. Morbidelli · M. Ziche
Laboratory of Angiogenesis, Section of Pharmacology, Department of Molecular Biology, University of Siena, Via Aldo Moro 2, 53100, Siena, Italy

E. Van Aken
Department of Ophthalmology, Ghent University Hospital, De Pintelaan 185, 9000, Gent, Belgium

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Introduction

Angiogenesis is the process of endothelial cells detaching from the vascular wall, invading the underlying tissues, and forming tubes that branch and organise into anastomotic networks [1]. Angiogenesis occurs in physiological (embryology, ovulation, wound healing) and in pathological situations (neoplasia, diabetic retinopathy, rheumatoid arthritis) [2]. The process is regulated by a balance between pro- and anti-angiogenic molecules, emanating from endothelial, stromal and epithelial cells [3], by comprising growth factors [4], proteinases [5] and their respective inhibitors [6], extracellular matrix molecules [7], and also by cell–cell and cell–substratum adhesion molecules [8].

In vivo angiogenesis in cornea or chorioallantoic membrane (CAM) models, induced by fibroblast growth factor (FGF)-2 depends on $\alpha_v\beta_3$ integrin, whereas angiogenesis induced by vascular endothelial growth factor type A (VEGF-A) depends on $\alpha_v\beta_5$ integrin. Antibody to $\alpha_v\beta_3$ and $\alpha_v\beta_5$ receptor for vitronectin blocked angiogenesis in the chick CAM induced by FGF-2 and VEGF-A, respectively [9–11]. Vitronectin not only binds to integrins, but also to urokinase plasminogen activator receptor (uPAR) [12], both localised at focal adhesion sites. uPAR can stimulate endothelial migration in two ways: first by plasmin formation and subsequent proteolytic degradation of the extracellular matrix [13], and second, by direct interaction with vitronectin. Vascular endothelial

(VE)-cadherin, a cell–cell adhesion molecule specific for endothelial cells serves interendothelial cell–cell adhesion and prevents endothelial apoptosis [2]. Neural (N)-cadherin is a cell adhesion molecule expressed by various cells, like neurons, fibroblasts, oocytes, spermatides, Sertoli cells, lens cells, osteoblasts and also by endothelial cells. Homophilic homotypic N-cadherin interaction in non-endothelial cells, like in cardiomyocytes serves stabilisation of the adherens junction [14]. Heterotypic N-cadherin interactions on the other hand stimulate migration. Various cancer cells invade the surrounding stroma by expressing N-cadherin aberrantly [15], and neural cells migrate on oligodendrocytes, expressing N-cadherin as well [16]. The function of N-cadherin in angiogenesis remains to be elucidated. Homophilic heterotypic interactions exists between endothelial and stromal cells [17], specifically between endothelial cells and pericytes [18, 19]. Also homotypic N-cadherin interactions were found at the intercellular junctions in endothelial cells. Luo and Radice [20] concluded that N-cadherin controls vasculogenesis upstream of VE-cadherin, because loss of N-cadherin in endothelial cells results in embryonic lethality at midgestation due to severe vascular defects. The knockdown of N-cadherin caused a significant decrease in VE-cadherin expression.

Our laboratory has provided evidence that an 80-kDa epithelial (E)-cadherin fragment (sE-CAD), released by plasmin, matrilysin or stromelysin-1, affects epithelial tissue integrity, causing loss of cell–cell adhesion and gain of invasion [21, 22]. Similar soluble fragments are released from cells expressing N-cadherin (sN-CAD), and they also exert regulatory functions, such as during neurite outgrowth in the retina of the chick embryo [23]. The aim of the present study was to identify a possible effect of sN-CAD on angiogenesis. We found that sN-CAD mediated the invasion of endothelial cells into the extracellular matrix during angiogenesis, like sE-CAD mediated the invasion of cancer cells. Therefore, we used N-cadherin expressing mouse sarcoma cells as a source of sN-CAD. We found that sN-CAD stimulated angiogenesis in vivo. In vitro studies revealed that sN-CAD is able to stimulate migration of endothelial cells through activation of the FGF-receptor.

Materials and methods

Cell lines

Human endothelial cells, PSV1, derived from umbilical veins (a gift from Véronique Fafeur, Institut Pasteur de Lille) [24]. These cells were checked after thawing for endothelial cell markers VE-cadherin, N-cadherin and factor VIII. All experiments were done with cells between passage 3 and 10. Cells were grown on 0.1% gelatin-coated dishes in RPMI 1640 (Invitrogen, Merelbeke, Belgium) supplemented with 20% fetal bovine serum, 100 IU/ml penicillin (Invitrogen), 100 μg/ml streptomycin (Invitrogen) and 2.5 μg/ml Fungizone® (Bristol-Meyers Squibb, Brussels, Belgium). S180-NCAD and S180 cells, mouse sarcoma cells (a gift from R.M. Mège, INSERM, Paris, France) [25] were grown in DMEM (Invitrogen, Merelbeke, Belgium) supplemented with 10% fetal bovine serum and penicillin, streptomycin and Fungizone (see above). The S180-NCAD cells, used as a source of sN-CAD, are S180 cells transfected with chicken cDNA encoding for N-cadherin. The cells were incubated in an 100% water-saturated atmosphere of 5% CO_2 for PSV1 and 10% CO_2 for S180-NCAD and S180 cells. All cells were routinely tested for mycoplasma contamination by staining with 4′,6-diamidino-2-phenylindole (DAPI) and found negative.

Reagents and antibodies

A 10-mer histidine–alanine–valine (HAV)-comprising peptide, identical to amino acids 235–244 in the first extracellular domain of N-cadherin (hu N-CAD^{10}, LRAHAVDING) [26] served as a peptidomimetic for the first extracellular domain (ECD1) of N-cadherin. As a control, a scrambled 10-mer peptide (scrambled N-CAD^{10}, LHDANVGRIA) (Eurogentec, Seraing, Belgium) was included. Recombinant human N-cadherin/Fc chimera was purchased from R&D Systems (Abingdon, UK). Recombinant human basic fibroblast growth factor (FGF-2) (Sigma, St Louis, MO, USA) and recombinant human vascular edothelial growth factor (VEGF-A) (R&D Systems, Abingdon, UK) were used as positive control. Cortisone acetate (Sigma, St Louis, MO, USA) was used to block the process of inflammation in the chorioallantoic assay. PD173074, a selective inhibitor of the FGF-receptor [27] was a gift from P. Doherty (King's College London, UK).

The antibodies used for immunoprecipitation were rabbit polyclonal anti-human β-catenin (Sigma, St Louis, MO, USA), rabbit polyclonal anti-human FGF-receptor (FGFR1) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse monoclonal anti-pan cadherin (CH-19) (Sigma, St Louis, MO, USA) and mouse IgG isotype control (eBiosciences). The rat monoclonal antibody NCD-2 (a gift from C. Redies, University Hospital Essen, Germany) [28] was used for

immunodepletion of sN-CAD out of the medium containing sN-CAD. The primary antibodies for Western blot were: mouse monoclonal antibody GC-4, CH-19 (Sigma, St Louis, MO, USA) and 13A9 (kindly provided by M.J. Wheelock, Department of Biology, University of Toledo, Toledo, OH, USA) against N-cadherin, mouse monoclonal antibody anti-α-tubulin (Sigma, St Louis, MO, USA), rabbit polyclonal antibody anti-phospho-ERK kinase and rabbit polyclonal antibody anti-ERK kinase (Cell Signaling technology, Beverly, MA, USA). The secondary antibodies were anti-mouse or anti-rabbit antibody linked to horseradish peroxidase (Amersham Pharmacia Biotech).

Electroporation

PSV1 cells were seeded and, at 70–80% confluency, were trypsinised and collected in a Nucleofector™ certified cuvette (Amaxa GmBH, Cologne, Germany). A mixture of 100 µl Nucleofector solution and 3 µg of short interference RNA (siRNA) was added. The cells were electroporated in the Nucleofector electroporator with the A34-specific Nucleofector program. siRNAs targeting N-cadherin (GenBank/EMBL/DDBJ accession number NM-001792) were designed by Qiagen (Leusden, The Netherlands). Inhibition of N-cadherin expression was achieved by RNA interference using the following double-stranded oligoribonucleotides: siN-cad2 5′-AGUGGCAAGUGGCAGUAAA-3′ and siN-cad3 5′-GGAGUCAGCAGAAGUUGAA-3′ [29]. siRNA targeting FGFR1 (GenBank/EMBL/DDBJ accession number NM-000604) were designed by DHARMACOM (Lafayette, CO, USA). The FGFR1 siRNA represented mixtures of four distinct RNA duplexes (SmartPool). To verify specificity of the knock-down effect, we used an oligonucleotide sequence with no known mammalian target (con 5′-UUCUCCGAACGUGUCACGU) as a control.

Preparation of medium containing sN-CAD

Confluent monolayers were washed three times with phosphate buffered saline (PBS) and incubated for 2 h at 37°C with serum-free DMEM to which plasmin 1 µg/ml (Sigma, St Louis, MO, USA) was added. Our recombinant N-cadherin could also be cleaved by plasmin and releasing a 90-kDa fragment. sN-CAD was also spontaneously released by the cells: for this subconfluent monolayers were washed three times with PBS and put on serum-free medium (SFM) for 24 h, washed another three times with PBS and incubated for 48 h with Serum-free medium. The medium containing sN-CAD was harvested, centrifuged at 250*g* for 5 min followed by a centrifugation step at 2,000*g* for 15 min and filtered through a 0.22 µm filter. The medium containing sN-cad after plasmin treatment or the medium after 48 h contact with the cells was always checked by western blot for the presence of the 90-kDa N-cadherin fragment before use in functional assays (see also Fig. 2). sN-CAD was removed from the medium by immunoadsorption as follows. Medium containing sN-CAD was incubated four times for 1 h with protein G-Sepharose 4 fast flow beads (Amersham Pharmacia Biotech) coated with NCD-2 antibody against N-cadherin. Beads and supernatants were separated and the remaining supernatant was finally filtered through a 0.22 µm filter. Medium without sN-CAD (serum-free DMEM) was also incubated for 2 h at 37°C with 1 µg/ml plasmin for evaluation of the effect of plasmin on angiogenesis. Media with or without sN-CAD were concentrated twice (Amicon Ultra 50 kDa, Millipore Corp., Bedford, MA, USA) before use in angiogenesis assays in vivo and in vitro assays.

Rabbit cornea assay

In vivo angiogenesis was studied in the cornea of New Zealand rabbits (Charles River, Calco, Como, Italy) since this is an avascular and transparent tissue, where inflammatory reactions and growing capillaries can be easily monitored and changes quantitated by stereomicroscopic examination. Slow-release pellets were prepared under sterile conditions, incorporating the test substances into a casting solution of an ethynil-vinyl copolymer (Elvax-40, DuPont-De Nemours, Wilmington, DE, USA). Rabbits were anaesthetised by sodium pentothal (30 mg/kg) and in the lower half of the eyes, one or two micropockets were surgically made using an iris spatula. The pellets were implanted in the micropockets. Pellets impregnated with recombinant growth factor FGF-2 (R and D Systems, Abingdon, UK) were used as positive control [30]. Subsequent daily observations of the implants were made with a slit lamp stereomicroscope by two independent operators. Angiogenic activity is indicated by the number of implants exhibiting neovascularisation over the total implants studied, and by the angiogenic score. The angiogenic score was considered positive when budding of vessels from the limbal plexus occurred after 3–4 days and capillaries progressed to reach the implanted pellet. The angiogenic score is calculated by the number of newly formed vessels and by their growth rate (number of vessels × distance from the limbus) [31]. A density value of 1 corresponded to 0–25 vessels per cornea, 2 from 25–50, 3 from 50–75, 4 from 75–100 and 5 for > 100 vessels.

The distance from the limbus was graded with the aid of an ocular grid. The Student's *t*-test for unpaired data was used for statistics ($p < 0.05$).

CAM assay

The CAM assay was performed as described by Maragoudakis et al. [32] with some modifications. Briefly, fertilised eggs were incubated for 3 days at 37°C. On day 3, albumen was removed to detach the shell from the developing CAM. On day 4, a window was made in the eggshell, exposing the CAM, and covered with cellophane tape. The eggs were returned to the incubator until day 9, prior to application of the test compounds. Test compounds and control compound (PBS) were poured onto separate sterile discs (11 mm diameter), which were allowed to dry under sterile conditions. A solution of cortisone acetate (100 μg/disc) was poured onto all discs to prevent an inflammatory response. Test discs probed with the 165 amino acid isoform of VEGF-A served as positive control. On each CAM, the disc containing control compound and the disc containing test compound were placed at a distance of 1 cm. The windows were covered and the eggs were incubated until day 11, before assessment of angiogenesis. Therefore, the eggs were flooded with 10% buffered formalin, the discs were removed, and the eggs were kept at room temperature for at least 2 h. A large area around the discs was cut out and placed on a glass slide, and the vascular density index was measured by the method of Harris-Hooker et al. [33]. Briefly, a grid containing three concentric circles of 6-, 8- and 10-mm diameter was positioned on the surface of the CAM previously covered by the disc. All vessels intersecting the circles were counted. The angiogenic index = $(t–c)/c$, with t the number of intersections in the area covered by the test disc and c the number of intersections in the area covered by the control disc in the same egg. All experiments were performed at least twice, and the Mann–Whitney *U*-test was used for statistics ($p < 0.05$).

Wound healing migration assay

Cells were grown in 6-well tissue culture dishes until confluent. Medium was removed and the monolayers were wounded with a plastic tip. Wounded monolayers were washed three times with Ca^{2+}- and Mg^{2+}-containing PBS pH 7.4 to remove dead cells. Cell migration occurred in 1 ml serum-free medium. Wounds were marked and measured at time points zero and 16 h with an inverted microscope. The migration distance of the untreated cells was put at 100% and compared to the treated cultures. The Student's *t*-test was used for statistics ($p < 0.05$).

F-actin staining

Cells were grown on glass coverslips in 24-well culture dishes until islands of cells were formed. Cells were washed with PBS and serum-starved overnight, followed by treatment or left untreated for 6 h in serum-free medium. Cells were fixed in 3% paraformaldehyde, blocked in 50 mM NH_4Cl in PBS, permeabilised in 0.2% Triton-X-100 in PBS and stained with phalloidin-FITC (Sigma, St Louis, MO, USA).

Immunoprecipitation and Western blotting

All cell lysates were made from cell cultures at approximately 70% confluence. All cells were washed three times with PBS, serum-starved over-night, washed again three times and treated for the indicated times. Cells were lysed with PBS containing 1% Triton X-100 and 1% Nonidet P-40 and the following protease- and phosphatase inhibitors: aprotinin (10 μg/ml), leupeptin (10 μg/ml), phenylmethylsulphonyl fluoride (1.72 mM), NaF (10 mM), $NaVO_3$ (1 mM) and $Na_4P_2O_7$ (1 mM) (Sigma, St Louis, MO, USA). For the co-immunoprecipitation of FGF-receptor and N-cadherin the following lysis buffer was used: 50 mM Tris–HCl pH of 7.5, 150 mM NaCl, 1% Nonidet P-40 and the same protease- and phosphatase inhibitors as described above. The protein concentration was measured using Rc Dc protein assay (Bio-Rad), and samples were prepared at equal protein concentrations. For immunoprecipitation, equal amounts of proteins were first incubated with protein A or G-Sepharose beads (Amersham Pharmacia Biotech) for 30 min. After discarding the beads, the supernatant was incubated with primary antibody for 3 h at 4 °C, followed by incubation with protein A or G-Sepharose beads for 1 h. Sample buffer (Laemmli) with 5% 2-mercaptoethanol and 0.012% bromophenolblue was added, followed by boiling for 5 min and separated on 8% SDS-PAGE and transferred on a nitrocellulose membrane (Amersham Pharmacia Biotech). Quenching and immunostaining were done in 5% non-fat dry milk in PBS containing 0.5% Tween 20, except for anti-phospho-ERK antibody, where 4% bovine serum albumin in PBS containing 0.2% Tween 20 was used instead. The membranes were quenched for 1 h, incubated with primary antibody for 1 h, washed four times for 10 min, incubated with horseradish peroxidase-conjugated secondary antibody for 45 min, and washed six times for 10 min. Detection was carried out using enhanced chemiluminescence reagent

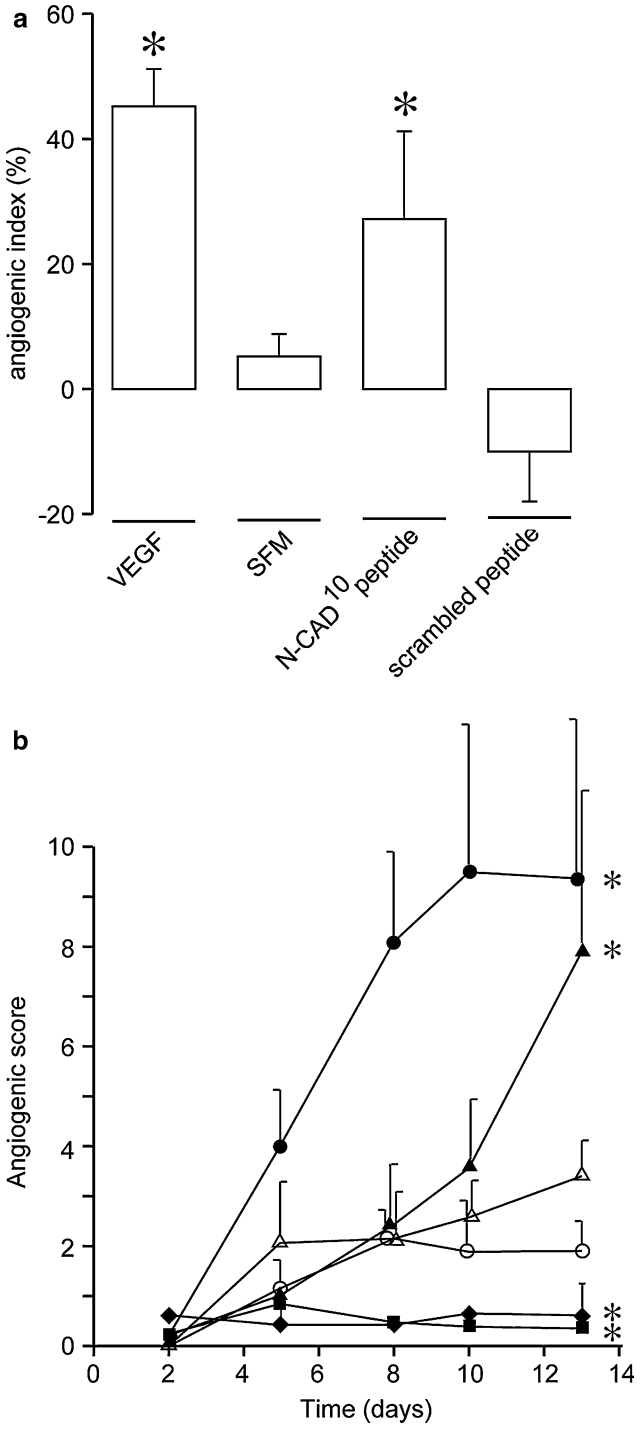


Fig. 1 hu N-CAD^{10} peptide induces angiogenesis. **a** hu N-CAD^{10} peptide induces angiogenesis in the CAM. *Bars* indicate angiogenic indices of CAM probed with VEGF-A (1 μg/ml), serum-free medium without or with hu N-CAD^{10} peptide 2 μg/μl, or with a scrambled N-CAD^{10} peptide 2 μg/μl. Each value (mean + standard deviation) is the result of three experiments. In each experiment, five eggs were tested per condition. *Statistically different from the mean angiogenic index of CAMs probed with scrambled peptide (Mann–Whitney *U*-test, $p < 0.05$). **b** hu N-CAD^{10} peptide induces angiogenesis in the rabbit cornea. Angiogenic scores of rabbit corneas probed with pellets impregnated with hu N-CAD^{10} peptide 200 ng (*closed diamonds*, $n = 4$), hu N-CAD^{10} peptide 500 ng (*closed squares*, $n = 4$), hu N-CAD^{10} peptide 10 μg (*closed triangles*, $n = 3$), hu N-CAD^{10} peptide 50 μg (*closed circles*, $n = 3$), scrambled N-CAD^{10} peptide 10 μg (*open triangles*, $n = 3$) and scrambled N-CAD^{10} peptide 50 μg (*open circles*, $n = 3$). *Symbols* represent the mean + standard deviation of angiogenic scores of *n* number of rabbit corneas tested. *Statistically different from the mean angiogenic score of rabbit corneas probed with scrambled peptide 10 μg (Student's *t*-test, $p < 0.05$)

(Amersham Pharmacia Biotech) as a substrate. To control for equal loading of total lysates, immunostaining with anti-tubulin antibody was performed. Quantification of the bands was done using Quantity-One software (Bio-Rad).

Results

HAV-comprising N-CAD^{10} peptide promotes angiogenesis in vivo

The effect of hu N-CAD^{10} peptide, comprising the HAV motif of the first extracellular domain of N-cadherin, was examined in the CAM (chorioallantoic assay) (Fig. 1a). VEGF-A (1 μg/ml), Serum-free medium without and with hu N-CAD^{10} peptide (2 μg/μl) or with scrambled peptide (2 μg/μl) were tested. VEGF-A and hu N-CAD^{10} peptide induced angiogenesis, 45 and 27.7%, respectively, with angiogenic indices that were statistically different from the angiogenic index of the scrambled peptide. Serum-free medium with or without scrambled peptide did not induce angiogenesis.

Different concentrations of hu N-CAD^{10} peptide and scrambled peptide were tested in the rabbit cornea assay (Fig. 1b). Mean angiogenic scores of hu N-CAD^{10} peptide were concentration-dependent. Low doses of hu N-CAD^{10} peptide (200 and 500 ng/pellet) were devoid of any angiogenic capacity (0/4 rabbit corneas were positive for both concentrations). Pellets impregnated with 10 or with 50 μg hu N-CAD^{10} peptide induced angiogenesis in, respectively, 2/3 and 3/3 rabbit corneas. Pellets impregnated with 10 or 50 μg scrambled N-CAD^{10} peptide induced angiogenesis in only 1 rabbit cornea (1/3 rabbit corneas positive for both concentrations). The mean angiogenic score of rabbit corneas probed with pellets impregnated with hu N-CAD^{10} peptide in any concentrations was statistically different from the angiogenic score of rabbit corneas probed with pellets impregnated with

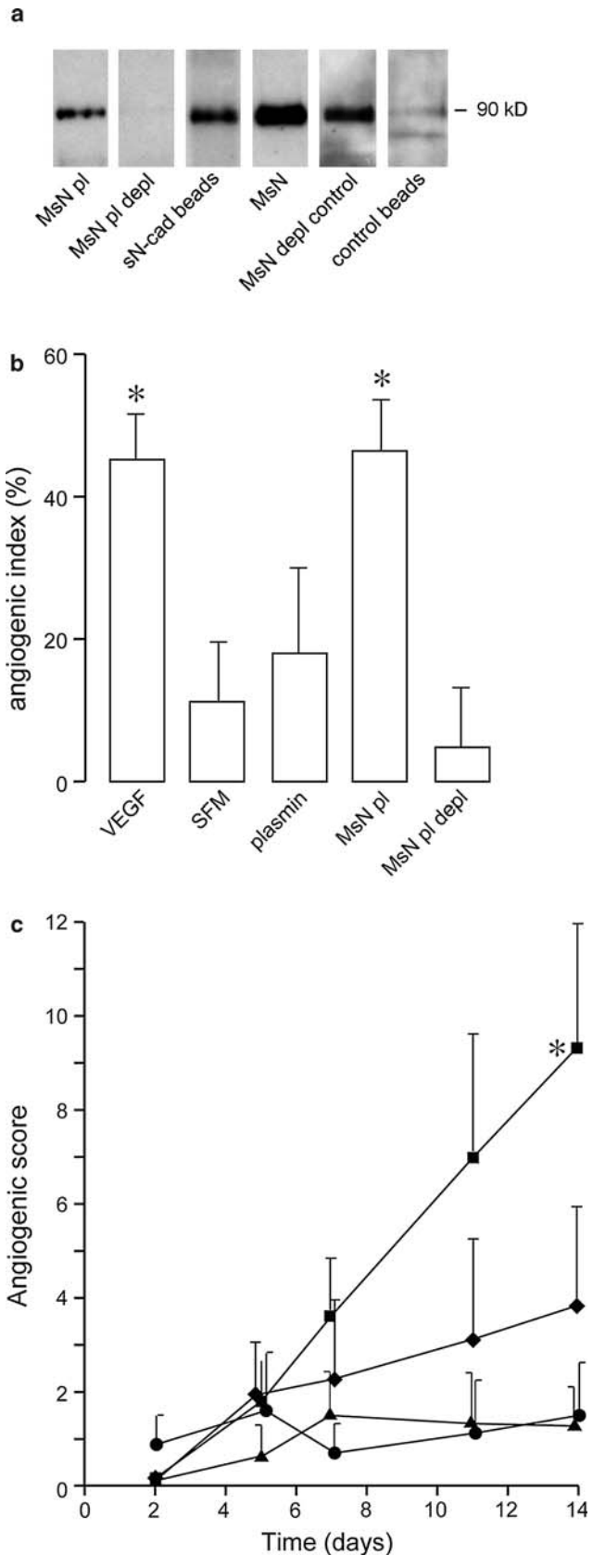


Fig. 2 Detection of sN-CAD and induction of angiogenesis. **a** Immunodepletion of medium containing sN-CAD is performed by immunoadsorption with NCD-2 antibody-coated beads removing sN-CAD. *Lanes* represent medium containing sN-CAD, immunodepleted medium and sN-CAD linked to NCD-2 antibody-coated beads used for immunodepletion of medium containing sN-CAD. As a control for sN-CAD containing medium, spontaneously released sN-CAD was used. For this purpose, S180-NCAD cells were incubated with serum-free medium (Serum-free medium) and medium was harvested after 48 h. As control for the sN-CAD depletion we used an isotype control antibody. Media and beads were separately dissolved in sample buffer, and proteins were separated by SDS-PAGE, blotted and immunostained with NCD-2 antibody. **b** *Bars* indicate angiogenic indices of CAMs probed with VEGF-A 1 μg/ml, Serum-free medium, Serum-free medium with plasmin 1 μg/ml, medium containing sN-CAD or sN-CAD immunodepleted medium. Each value (mean + standard deviation) is the result of three experiments. In each experiment, five eggs were tested per condition. *Statistically different from the mean angiogenic index of CAMs probed with serum-free medium (Mann–Whitney *U*-test, $p < 0.05$). **c** Angiogenic scores of rabbit corneas, probed with pellets impregnated with Serum-free medium (*diamonds*, $n = 4$), Serum-free medium with plasmin (*triangles*, $n = 5$), medium containing sN-CAD (*squares*, $n = 6$), or sN-CAD immunodepleted medium (*circles*, $n = 3$). *Symbols* represent the mean of angiogenic scores of n number of rabbit corneas tested. Flags represent standard deviations. *Statistically different from the mean angiogenic score of rabbit corneas probed with pellets impregnated with Serum-free medium (Student's *t*-test, $p < 0.05$)

scrambled peptide. No inflammatory effect was microscopically observed at any of the concentrations tested.

In these in vivo experiments we could prove that the HAV-comprising N-cadherin peptide induced angiogenesis.

sN-CAD promotes angiogenesis in vivo

To approach the physiological situation, we used for all further experiments soluble N-cadherin, which is released from the mature N-cadherin after enzymatical cleavage. Different enzymes, like matrix metalloproteinases (MMP), plasmin, ADAM10, are able to shed the 90-kDa N-cadherin fragment. sN-CAD is also present in different body fluids of the patients, like the serum [34].

Medium from S180-NCAD cells treated with 1 μg/ml of plasmin was collected after 2 h (MsN pl). This medium was used in the CAM assay because we presumed it contained less growth factors than the 48 h medium containing sN-CAD. Medium harvested from S180-NCAD cells after a 48 h incubation-period with Serum-free medium contained spontaneously released sN-CAD (MsN) (Fig. 2a). The immunosignal for sN-CAD was present at 90 kDa in medium containing sN-CAD, but not when sN-CAD was immun-

odepleted by immunoadsorption. NCD-2 antibody-treated beads, used for immunoadsorption of sN-CAD, also showed an immunosignal for sN-CAD. When the medium containing sN-CAD was immunodepleted with an isotype control antibody, there was no change in MsN and no immunosignal appeared in isotype antibody-treated beads.

The effect of sN-CAD on angiogenesis was examined in the CAM assay (Fig. 2b). VEGF-A, Serum-free medium, plasmin, medium containing sN-CAD and sN-CAD immunodepleted medium were tested. Only VEGF-A and medium containing sN-CAD induced angiogenesis statistically different from Serum-free medium ($p < 0.05$). Results with plasmin alone were not statistically different from those with Serum-free medium. sN-CAD immunodepleted medium did not induce angiogenesis compared to Serum-free medium while sN-CAD medium depleted with isotype control antibody gave the same angiogenesis response as the non-depleted medium.

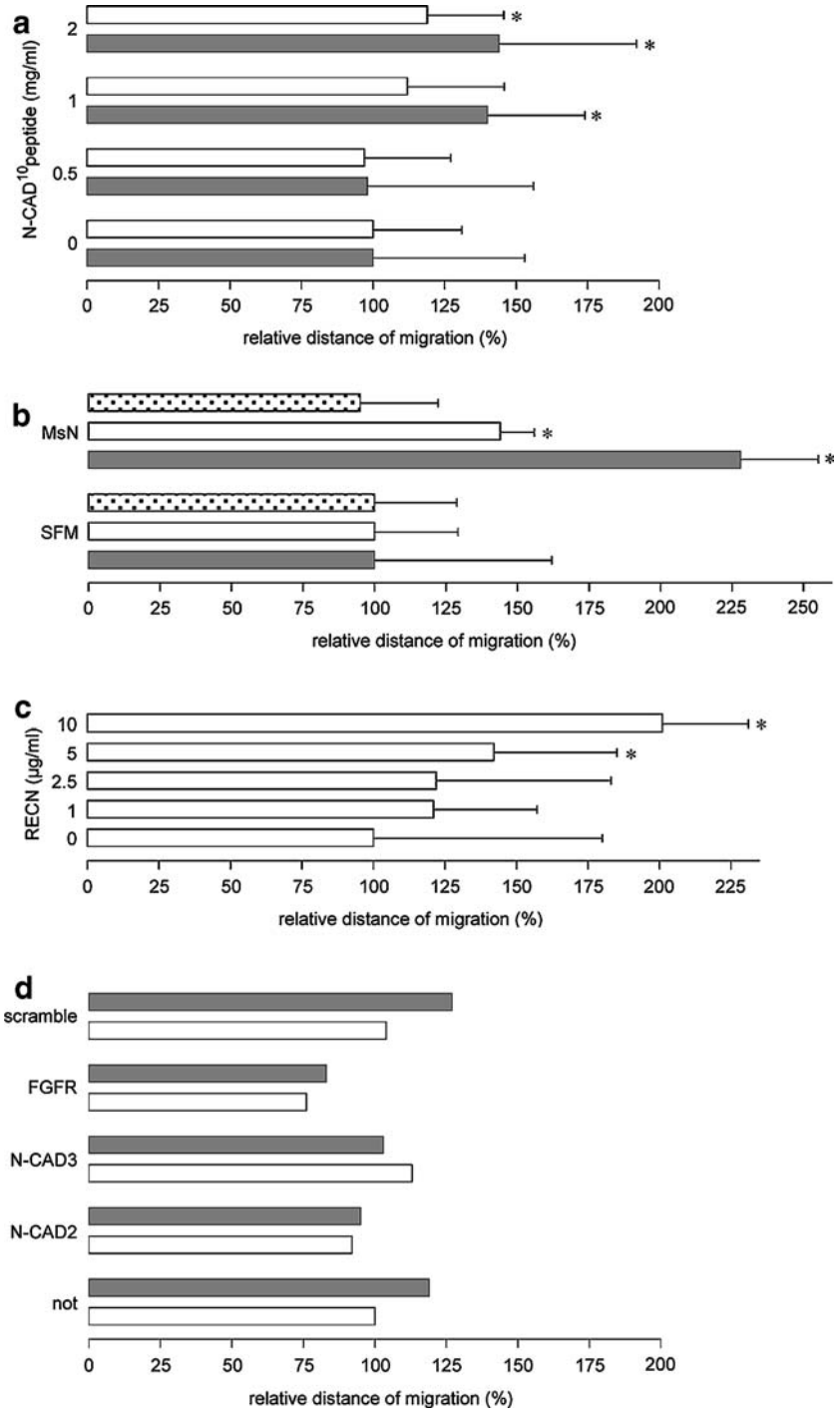
Medium containing sN-CAD, sN-CAD immunodepleted medium and Serum-free medium with and without plasmin were also tested in the rabbit cornea assay (Fig. 2c). The mean angiogenic score of rabbit corneas probed with pellets impregnated with medium containing sN-CAD, was 7 and statistically different from the mean angiogenic score of rabbit corneas probed with pellets impregnated with Serum-free medium. Six out of six rabbit corneas probed with pellets impregnated with medium containing sN-CAD were positive. Pellets impregnated with sN-CAD immunodepleted medium or with Serum-free medium with plasmin induced angiogenesis in, respectively, 1/3 and 1/5 rabbit corneas. The mean angiogenic score of rabbit corneas probed with pellets impregnated with sN-CAD immunodepleted medium or with Serum-free medium with plasmin did not differ statistically from the mean angiogenic score of rabbit corneas probed with pellets impregnated with Serum-free medium. sN-CAD induced angiogenesis the CAM and in the rabbit cornea assay.

sN-CAD-stimulated migration in vitro is N-cadherin-dependent

Since angiogenesis is dependent on migration of endothelial cells we were interested whether sN-CAD modulates the migration of endothelial cells in the wound healing migration assay in vitro. Confluent PSV1 cultures were serum-starved for a minimum of 24 h to establish quiescence such that the presence of cells in the wounded area was owed to cell motility rather than cell proliferation. After wounding the monolayer, PSV1 cells were treated with different concentrations (0.5–2 mg/ml) of the hu N-CAD^{10} peptide (Fig. 3a). Endothelial cells migrated perpendicularly to the wound in a irregular shaped front and there was a statistically difference in migration between hu N-CAD^{10} peptide treated cells (144%) and serum-free treated cells (100%). Next, we treated wounded PSV1 monolayers with Serum-free medium containing sN-CAD (MsN) or with Serum-free medium (Fig. 3b). Medium containing sN-CAD induced migration that was significantly faster (228%) than cells treated with Serum-free medium (100%). Medium from S180, not expressing N-cadherin, was also tested in the wound healing assay but had no stimulatory effect on the PSV1, S180-NCAD or S180 cells. Moreover, recombinant N-cadherin (RECN), which consist of the extracellular domain of N-cadherin linked to the Fc fragment of human IgG1, stimulated migration of PSV1 cells in a dose response manner (0–10 μg/ml) (Fig. 3c). Untreated PSV1 cells migrated slower compared to PSV1 cells treated with 10 μg/ml of RECN (100% versus 201%). Other recombinant cadherins like E- or P-cadherin did not stimulate the migration of the endothelial cells. For all migration assays, knock-down and immunocytochemistry experiments we used RECN at a concentration of 5 μg/ml. Furthermore, we use this in vitro assay to analyse the molecular mechanism of sN-CAD-stimulated migration.

We found the presence of full-length N-cadherin to be a prerequisite for the stimulatory effect of sN-CAD. Migration of S180-NCAD cells, transfected with full-length N-cadherin and their parental S180 cells devoid of N-cadherin, were compared (Fig. 3b). S180-NCAD cells treated with their own medium containing sN-CAD (MsN) migrated significantly faster then S180-NCAD cells treated with Serum-free medium (144 and 100%, respectively). Migration of S180 cells (100%) was not stimulated by adding MsN (95%).

To confirm the role of N-cadherin in sN-CAD-stimulated migration, we used the siRNA knock-down approach. N-cadherin expression was silenced using siRNA. For this, endothelial cells were electroporated with two double-stranded oligonucleotides derived from different regions of N-cadherin cDNA. PSV1 cells electroporated either with control oligonucleotide or without were used as controls. As revealed by Western blot analysis 72 h after transfection, siN-cadherin suppresses N-cadherin protein expression by 93% (see Fig. 5). After 48 h, confluent monolayers of electroporated PSV1 cells were wounded and treated with or without RECN (5 μg/ml) (Fig. 3d). Control cells were stimulated by RECN (5 μg/ml) in the wound healing assay (not: Serum-free



medium 100% and RECN 119% and scramble: Serum-free medium 104% and RECN 127%). RECN-stimulated migration of N-cadherin silenced cells was hampered in comparison with not or control transfected cells (siNCAD2 Serum-free medium 92% and RECN 95% and siNCAD3 Serum-free medium 113% and RECN 103%).

The pro migratory effect of sN-cad on PSV1 cells in the wound healing assay was dependent on N-cadherin.

sN-CAD-stimulated migration in vitro is FGF-receptor-dependent

We investigated a possible association between full-length N-cadherin and the FGF-receptor (Fig. 4a). S180-NCAD cells of approximately 70% confluency were lysed and co-immunoprecipitation was performed using an antibody against the C-terminus of N-cadherin or the C-terminus of the FGF-receptor. After gel elec-

◀ **Fig. 3** sN-CAD stimulates migration. **a** Confluent monolayers of S180-NCAD (*white bars*) and PSV1 (*grey bars*) cells were wounded with a plastic tip, and treated with hu $NCAD^{10}$ peptide in a concentration range of 0.5–2 mg/ml or with serum-free medium (SFM). The distance of migration of Serum-free medium-treated cultures was set at 100%. Each value (mean + standard deviation) is the result of two experiments. *Statistically different from the mean relative distance of Serum-free medium-treated cultures (Student's *t*-test, $p < 0.05$). **b** Confluent monolayers of S180 (*dotted bars*), S180-NCAD (*white bars*) and PSV1 (*grey bars*) cells were wounded with a plastic tip, and treated with medium containing sN-CAD (*MsN*) or Serum-free medium. Each value (mean + standard deviation) is the result of three experiments. *Statistically different from the mean relative distance of Serum-free medium-treated cultures (Student's *t*-test, $p < 0.05$). **c** Confluent monolayers of PSV1 cells were wounded with a plastic tip, and treated with different concentrations of recombinant N-cadherin (*RECN*, 1–10 μg/ml). Wounds were marked and measured at time points zero and after 16 h. *Bars* represent mean values of at least three independent experiments and flags indicate standard deviation. *Statistically different from the mean migration of Serum-free medium-treated cultures (Student's *t*-test, $p < 0.05$). **d** Confluent monolayers of siRNA-transfected PSV1 cells were wounded after 48 h with a plastic tip and subsequently treated with Serum-free medium (*white bars*) or RECN (5 μg/ml) (*grey bars*). Wounds were marked and scored at time zero and after 16 h. *Bars* represent mean value of one experiment performed in triplet

trophoresis, proteins were blotted and immunostained using an antibody against N-cadherin and the FGF-receptor. N-cadherin co-immunoprecipitated with the FGF-receptor suggesting a direct or indirect interaction between both molecules (as has been demonstrated before in cell lines by Suyama et al. [35]).

We then examined the effect of sN-CAD on the N-cadherin/FGF-receptor complex. S180-NCAD cells were used at 70% confluency, followed by serum starvation overnight. Cells were treated for 30 min with Serum-free medium, medium containing sN-CAD (MsN) or RECN. Cells were lysed and co-immunoprecipitation was performed. In Serum-free medium-treated cells N-cadherin still interacted with the FGF-receptor. In sN-CAD-treated cells however, N-cadherin was dissociated from the FGF-receptor (MsN 71% and RECN 50%) (Fig. 4b).

We then examined the effect of PD173074, a specific FGF-receptor inhibitor [27], on the migration of PSV1 and S180N-CAD cells. Wounded monolayers were stimulated to migrate by treatment with RECN or FGF-2 (12.5 ng/ml + heparin 5 μg/ml) (Fig. 4c). PSV1 and S180-N-CAD cells that were treated with Serum-free medium migrated slower then treatment with RECN stimulated migration up to 134 and 127%. Addition of 500 nM PD173074 to RECN-treated cultures counteracted the pro-migratory effect of sN-CAD both in PSV1 and in S180-NCAD cells. By contrast, PD173074 alone had no effect on migration of cells.

Next, the FGF-receptor was knocked down in PSV1 cells using siRNA. For this, endothelial cells were electroporated with a pool of four double-stranded oligonucleotides derived from different regions of FGF-receptor cDNA. PSV1 cells electroporated either with control oligonucleotide or without were used as controls. As revealed by Western blot analysis 72 h after transfection, siFGF-receptor suppresses FGF-receptor protein expression by 74% (Fig. 5b). After 48 h, confluent monolayers of electroporated PSV1 cells were wounded and treated with or without RECN (5 μg/ml) (Fig. 3d). Control cells were stimulated by RECN (5 μg/ml) in the wound-healing assay (not: Serum-free medium 100% and RECN 199% and scramble: Serum-free medium 104 and 127%). RECN-stimulated migration of FGF-receptor silenced cells was hampered in comparison with control (siFGFR Serum-free medium 76% and RECN 83%). We conclude that FGF-receptor expression and activity is necessary to observe the pro-migratory effect of sN-CAD in the wound-healing assay.

sN-CAD phosphorylates ERK

The FGF-receptor signals through the ERK pathway to stimulate migration [11]. We therefore examined the possible contribution of ERK as signalling component in sN-CAD-stimulated migration of endothelial cells. As shown in Fig. 5a, PSV1 and S180-NCAD cells were grown until 70% confluency, followed by serum starvation overnight. Cells were treated for 30 min with Serum-free medium, medium containing sN-CAD (MsN), RECN (5 μg/ml) or FGF-2 (12.5 ng/ml), as a positive control, in the presence or absence of PD173074. ERK was hardly phosphorylated when cultures were treated with Serum-free medium, but ERK was strongly phosphorylated in PSV1 and S180-NCAD cell lines treated with MsN, RECN or FGF-2 (Fig. 5a). Staining with the anti-ERK1/2 antibody and anti-tubulin was used as control and remained the same in all conditions. The relative intensity was calculated by measuring the intensity of the p-ERK bands compared to intensity of the total ERK bands.

PSV1 cells were knocked down for N-cadherin and FGFR, using oligonucleotides, and were serum-starved after 60 h and treated for 30 min in absence or presence of RECN. As revealed by Western blot analysis, siRNA efficiently reduced N-cadherin and FGF-receptor expression (Fig. 5b). Tubulin expression was used as control for equal protein loading. Again, phosphorylation of ERK was checked (Fig. 5b). By knocking down the expression of N-cadherin and FGF-receptor phosphorylation of ERK was strongly

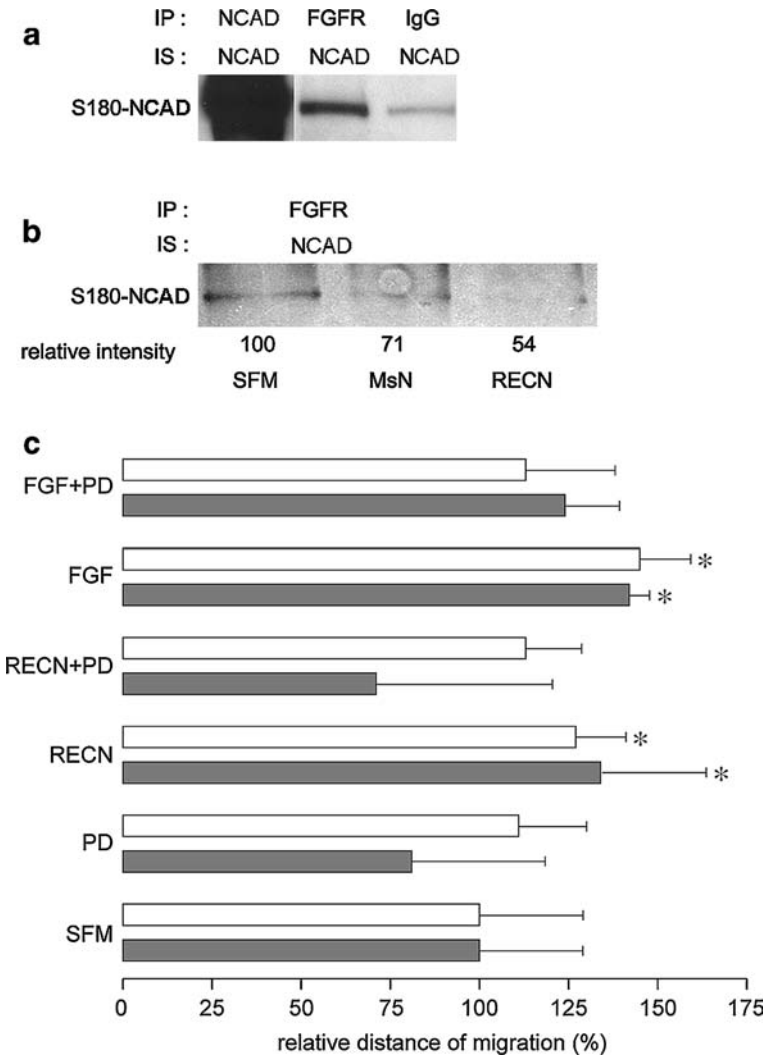


Fig. 4 sN-cad activates the FGF-receptor. **a** Subconfluent monolayers of S180-NCAD were solubilised in low detergent lysis buffer and immunoprecipitation was performed with an antibody against N-cadherin or the FGF-receptor. The precipitated proteins were resolved by SDS-PAGE, and immunoblots were stained with antibody against N-cadherin. **b** sN-CAD dissociates the N-cadherin/FGF-receptor interaction. Serum starved S180-NCAD cells were treated 30 min with serum-free medium (SFM) (without sN-CAD), medium containing sN-CAD (MsN) or RECN (5 μg/ml), followed by solubilisation in low detergent lysis buffer. Equal amounts of protein were immunoprecipitated with an antibody against the FGF-receptor, the precipitated proteins were resolved by SDS-PAGE. Immunoblots were stained for N-cadherin. The bands in the immunoblot stained with anti-FGFR remained the same. All imunoblots were quantified with the Quantity-One software and the relative intensity of the N-cadherin/FGFR bands are showed in the figure and this figure is representative for at least three independent experiments performed. **c** Confluent monolayers of S180-NCAD (*white bars*) and PSV1 (*grey bars*) cells were wounded with a plastic tip and subsequently treated with Serum-free medium, PD173074 (PD, 500 nM), RECN (5 μg/ml), RECN with PD, FGF-2 (12.5 ng/ml) or FGF-2 with PD. Wounds were marked and measured at time points zero and after 16 h. *Bars* represent mean values of at least three independent experiments and flags indicate standard deviation. *Statistically different from the mean migration of Serum-free medium-treated cultures (Student's *t*-test, $p < 0.05$)

diminished in RECN-treated cell cultures. As control these cells were also treated with FGF-2, in the FGF-receptor silenced cells ERK could not be phosphorylated (data not shown).

Our experiments suggest that sN-CAD-stimulated ERK activation is dependent on expression of N-cadherin and FGF-receptor, and FGF-receptor activity.

sN-CAD induces cytoskeleton reorganisation

PSV1 and S180-NCAD were seeded at low density on glass coverslips. Cells were put on Serum-free medium and cells were treated further with Serum-free medium, or with medium containing sN-CAD (MsN), RECN, RECN with PD173074 or PD173074 alone for

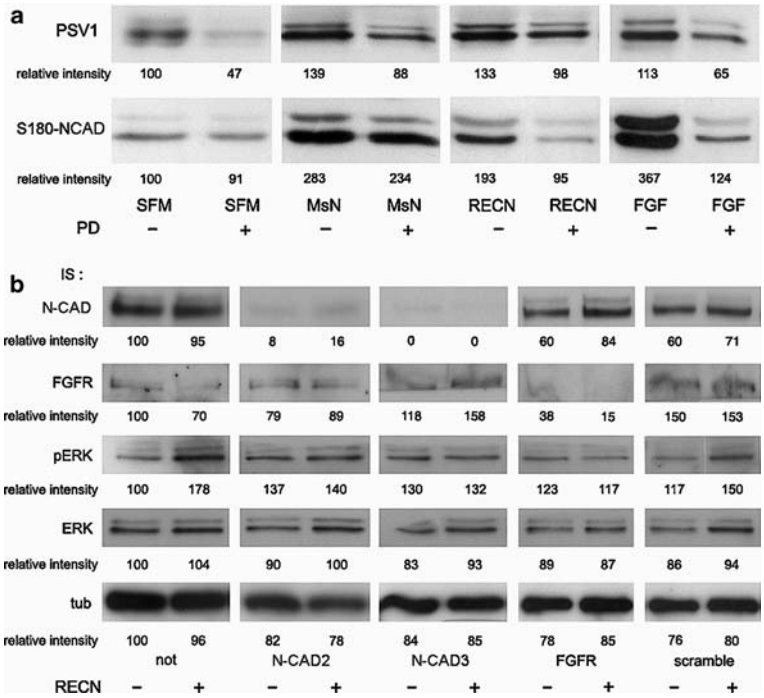


Fig. 5 sN-CAD stimulates ERK phosphorylation **a** sN-CAD induces ERK phosphorylation. PSV1 and S180-NCAD were serum-starved and treated for 30 min with serum-free medium (*SFM*), medium containing sN-CAD (*MsN*), RECN (5 μg/ml) or FGF-2 (12.5 ng/ml) without or with PD173074 (*PD*). Equal amounts of protein were loaded on SDS-PAGE and stained with an antibody against phospho-ERK and with an antibody for total ERK. Immunoblots from phospho-ERK and total ERK were quantified with a Quantity-One software (Bio-Rad) and the relative intensity is the value of p-ERK corrected for the amount of total ERK present. This result is representative for at least three independent experiments performed. **b** PSV1 cells, knocked down with oligonucleotides against N-cadherin, the FGF-receptor or a non-mammalian target, were serum starved after 60 h and after 72 h treated with serum-free medium (SFM) or RECN (5 μg/ml) during 30 min. Cells were lysed and equal amounts of protein were loaded on SDS-PAGE. Western blot was stained for N-cadherin, the FGF-receptor, phospho-ERK and total ERK and tubulin. All imunoblots were quantified with the Quantity-One software. This experiment was done twice and gave the same result

6 h (Fig. 6). MsN and RECN treatment of PSV1 and S180-NCAD cells induced cytoskeleton reorganisation of cells: loss of stress fibres, and more filopodia-like extensions were formed and cells were more elongated, compared to cells treated with Serum-free medium. Cytoskeleton reorganisation induced by sN-CAD was counteracted by adding PD173074.

Discussion

We present here evidence that a hu HAV N-CAD[10] peptide (LRAHAVDING) induces angiogenesis in the CAM and in the rabbit cornea assay dose-dependently. Several experiments have been published with substratum bound N-cadherin peptides containing HAV-sequence dimeric versions of the N-CAD peptides promote neuronal cell survival and neurite outgrowth, while cyclic peptides containing the HAV-sequence of extracellular domain 1 induce FGF-receptor-mediated apoptosis in endothelial cells [36] and inhibit neurite outgrowth [37]. When presented as soluble molecules, dimeric peptides stimulate neurite outgrowth in a manner similar to native N-cadherin [38, 39].

As a better approach of the physiological situation we tested the complete ectodomain of N-cadherin (90-kDa extracellular N-cadherin fragment, sN-CAD) in two angiogenesis models in vivo, where sN-CAD stimulated angiogenesis in both assays. Different proteases have already been described which are able to cleave N-cadherin extracellularly, like MMP [23] and ADAM10 (protein with a disintegrin and a metalloprotease domain) [40], giving rise to a 90-kDa sN-CAD fragment. Other proteases like presenilin/γ-secretase [41] and caspase-3 [42] are able to cleave N-cadherin intracellularly. In tumours sN-CAD can originate from different cell types, like endothelial, fibroblast, cancer cells,...because N-cadherin on the cell membrane can be cleaved by multiple proteases present in the

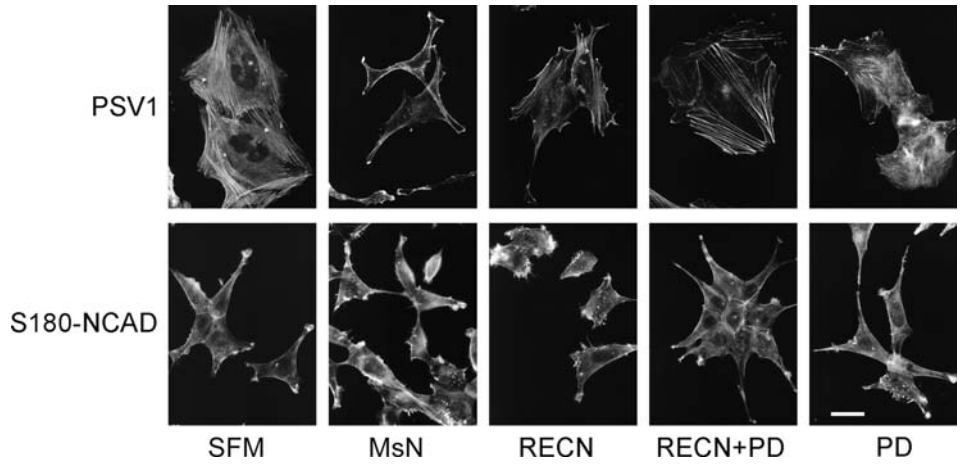


Fig. 6 sN-CAD induces filopodia formation. PSV1 and S180-NCAD were sparsely seeded on glass coverslips and grown until islands of cells were formed. Cells were put on serum-free medium (SFM) overnight and treated with SFM, medium containing sN-CAD (*MsN*), RECN (5 μg/ml), RECN with PD173074 or PD173074 (500 nM) alone for 6 h. Actin filaments were visualised with FITC-phalloidin. Scale bar, 50 μm

micro-environment We used plasmin, a serine protease, to cleave N-cadherin in its extracellular domain, in order to release a 90-kDa sN-CAD in culture medium of the cells.

To elucidate the possible working mechanism we tested both the medium containing sN-CAD and the dimeric recombinant N-cadherin/Fc chimera (termed both as sN-CAD) in vitro, which consist of the extracellular part of N-cadherin linked to the Fc fragment of the human IgG1 antibody. N-cadherin is known as a cell–cell adhesion molecule, but it is also a pro-migratory factor, since transfection of epithelial cells with N-cadherin induces the motile phenotype [43, 44]. The domain implicated in migration was restricted to 69 amino acids in extracellular domain 4 [45]. Although the role of N-cadherin in endothelial cells is not yet completely clear, it is important for its interaction with surrounding pericytes in the micro-environment [19]. Recent knock-down experiments of N-cadherin in endothelial cells showed a role of N-cadherin during vasculogenesis [20]. Furthermore, recombinant N-cadherin/Fc chimera was shown to stimulate neurite outgrowth in an FGF-receptor-dependent manner [46], but nothing has been reported to date about its function in migration and invasion of endothelial cells. We found that sN-CAD stimulates the migration of endothelial cells and this event requires the presence of N-cadherin on the acceptor cells, because silencing of N-cadherin by siRNA in the endothelial cells strongly reduced the sN-CAD pro-migratory effect and S180 (N-cadherin negative, parent) cells cannot be stimulated by sN-CAD containing medium nor by recombinant N-cadherin. We observed no differences in the cell–cell adhesion of endothelial cells when they were treated with sN-CAD or HAV N-CAD[10] peptide (data not shown). So, the sN-CAD-stimulated migration is not due to alterations in cell–cell adhesion.

We also investigated the role of the FGF-receptor in the pro-migratory effect of sN-CAD, because we could demonstrate that N-cadherin co-immunoprecipitates with the FGF-receptor. It was shown in literature that in neuronal cells N-cadherin interacts directly with the FGF-receptor via the HAV-binding region present in extracellular domain 4 of N-cadherin [47], and by this prolongs the activation of the FGF-receptor by stabilisation of the receptor on the membrane [35]. In pancreatic tumour cells as well as in neurons N-cadherin can trigger FGF-receptor signalling independently from FGF [48, 49]. Indeed we were able to reduce sN-CAD-mediated migration of endothelial cells in two ways. First, by using a specific inhibitor PD173074, which binds to the ATP pocket of the FGF-receptor [27], and second by knocking down the FGF-receptor by siRNA. FGF-stimulated chemotaxis and/or chemokinesis in endothelial cells requires the activation of the ERK signalling pathway [11]. We confirmed that both sN-CAD containing medium and recombinant N-cadherin stimulated phosphorylation of ERK, and this was abolished by addition of the FGF-receptor inhibitor, PD173074. However, the stimulated phosphorylation of ERK induced by sN-CAD containing medium could not be blocked by the FGF-receptor inhibitor, presumably because of the presence of other growth factors.

Cytoskeletal reorganisation is essential for migration of endothelial cells and therefore the formation of new vessels. sN-CAD stimulates the loss of stress fibres and the formation of filopodia and cells become elongated. Again these effects are N-cadherin- and FGF-receptor-dependent as evidenced by using siRNA in endothelial cells and N-cadherin-deficient S180 cells. It is noteworthy that sN-CAD stimulates the activation of Cdc42 which usually held responsible for the formation

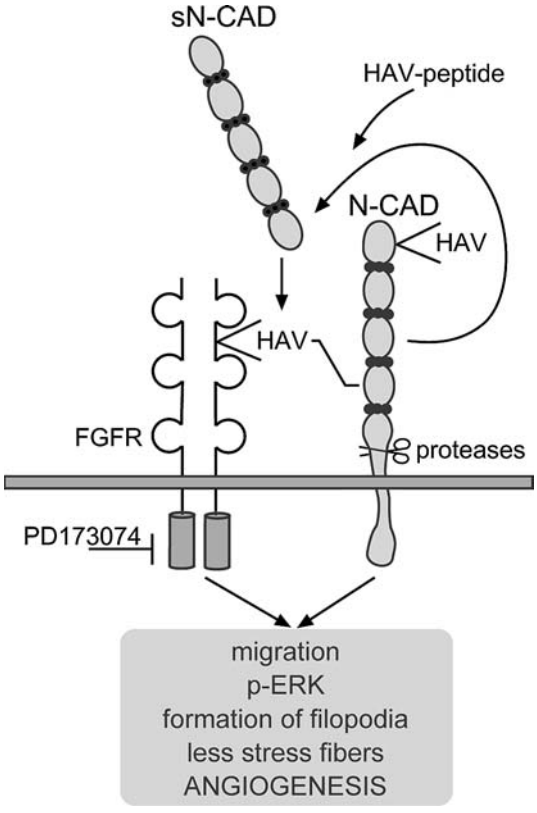


Fig. 7 Hypothetical model of the sN-cadherin-mediated angiogenesis pathway. N-cadherin (*N-CAD*) contains an HAV-sequence in its first extracellular domain and an HAV-binding motif in extracellular domain 4. In quiescent endothelial cells, N-cadherin is directly linked to the HAV-sequence present on the FGF-receptor (*FGFR*). In the presence of proteases, endothelial cells are activated by soluble N-cadherin (*sN-CAD*). sN-CAD, a 90-kDa fragment, is directly released by proteases. sN-CAD can directly or indirectly interact with the FGF-receptor. sN-CAD stimulates the migration of the endothelial cells, sN-CAD phosphorylates extracellular regulated kinase (*p-ERK*), which can be blocked by adding PD173074, it stimulates the formation of filopodia and activates Cdc42. All these cell activities promote sN-CAD or the 10-mer HAV peptide (LRAHAVDING) mediated angiogenesis

of filopodia, and diminishes the activation of RhoA (data not shown).

Comparable results were published with another neural cell adhesion molecule, L1. Plasmin is responsible for the posttranslational cleavage of L1 in fibronectin domain 3 of the molecule with the release of a 150-kDa fragment in the medium [50], and ADAM10 can also cleave L1 extracellularly with the shedding of a 200-kDa fragment. Both L1 extracellular fragments can stimulate cell migration [51]. Furthermore, pro-migratory effects were observed also with other soluble cadherins, like sE-cadherin [22].

In ''quiescent'' endothelial cells N-cadherin is responsible for the adhesion with other endothelial cells and with stromal cells like pericytes (Fig. 7). However in the micro-environment of tumours and in inflammatory processes, numerous proteases activate endothelial cells to form new blood vessels. We hypothesise that sN-CAD plays an important role in this process. Proteases cleave the extracellular fragment of N-cadherin from stromal cells, endothelial cells or N-cadherin-expressing tumour cells. sN-CAD will on its turn interact with the N-cadherin/FGF-receptor complex present on endothelial cells and stimulate the migration of endothelial cells in an FGF-receptor-dependent manner. sN-CAD activates the ERK pathway, leading to upregulation of protease expression, like plasmin and MMP, via the zinc-finger transcription factor Ets-1 [52], which has indeed been shown to induce angiogenesis [53]. By this an autocrine loop is formed: newly expressed proteases on their turn are responsible for the formation of sN-CAD which again induces migration of the endothelial cells.

Our results indicate that sN-CAD stimulates angiogenesis in vivo and migration of endothelial cells in vitro through an N-cadherin/FGF-receptor complex.

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