Atypical GPI-Anchored T-Cadherin Stimulates Angiogenesis
In Vitro and In Vivo

Maria Philippova, Andrea Banfi, Danila Ivanov, Roberto Gianni-Barrera, Roy Allenspach, Paul Erne, Thérèse Resink

Objective—T-cadherin (T-cad) is an atypical GPI-anchored member of the cadherin superfamily. In vascular tissue, T-cad expression is increased during atherosclerosis, restenosis, and tumor neoangiogenesis. In vitro, overexpression and/or homophilic ligation of T-cad on endothelial cells (ECs) facilitates migration, proliferation, and survival. This study investigated T-cad effects on angiogenesis.

Methods and Results—In vitro, T-cad homophilic ligation induced arrangement of ECs into a capillary-like network in a 2-dimensional model of EC differentiation and stimulated in-gel endothelial sprout outgrowth in an EC spheroid model and a modified Nicosia tissue assay. Sprouting from spheroids composed of adenoviral-infected T-cad overexpressing ECs or T-cad siRNA transfected ECs were significantly increased or reduced, respectively. In vivo, T-cad potentiated VEGF effects on neoangiogenesis in a model of myoblast-mediated gene transfer to mouse skeletal muscle; vessel caliber after co-delivery of T-cad and VEGF was significantly greater than after delivery of VEGF alone.

Conclusions—We unequivocally identify T-cad as a novel modulator of angiogenesis and suggest that this molecule can be exploited as a target for modulation of therapeutic angiogenesis, as well as for prevention of pathological conditions associated with abnormal neoangiogenesis. (Arterioscler Thromb Vasc Biol. 2006;26:2222-2230.)

Key Words: angiogenesis ■ cadherin ■ endothelial cell differentiation ■ VEGF

Angiogenesis, a structural and morphogenetic process by which new blood vessels are generated by sprouting from preexisting vessels, plays an important part in embryogenesis and in the adult for physiological repair and restoration of blood supply to damaged tissues. In the cardiovascular system, pathological neoangiogenesis induced in response to inflammation and tissue ischemia during atherogenesis can predispose the atherosclerotic plaque to intramural hemorrhage and rupture causing thrombosis and subsequent facilitation of coronary artery stenoses, occlusion, and myocardial ischemia.1 Deregulated angiogenesis can promote and aggravate pathological conditions such as tumor formation, diabetic retinopathy, and psoriasis.2 However, therapeutic neoangiogenesis is a promising strategy to treat myocardial and peripheral ischemia. Many experimental and preclinical trials reported that vascular endothelial growth factor (VEGF), the major endothelial-specific regulator of angiogenesis, significantly improves tissue perfusion and function when delivered as recombinant protein or by gene transfer.3 Some concern, however, has been raised regarding adequacy in structure, function and stability of newly generated blood vessels as well as unwanted side effects such as formation of hemangiomas.4–6 Consequently, there is compelling need for alternative strategies that would allow to finely regulate therapeutic angiogenesis rates and avoid unwanted side effects without impairing beneficial effects of growth factors on tissue perfusion.

Cadherins are transmembrane receptors mediating homophilic calcium-dependent intercellular adhesion.7 T-cadherin (T-cad), an atypical member of the cadherin superfamily, shares the general molecular organization of cadherin extracellular domains, but lacks transmembrane and cytoplasmic domains and is attached to the plasma membrane via glycosylphosphatidylinositol (GPI) anchor.8 A role for T-cad in the regulation of vascular cell function has been recognized only recently. T-cad is widely expressed in the cardiovascular system9 and its expression in vascular cells is markedly increased during atherosclerosis, restenosis after balloon angioplasty, and tumor neoangiogenesis.9–11 In contrast to classical cadherins, T-cad is absent from adherens junctions, is located within lipid rafts of the plasma membrane, and is redistributed to the leading edge of migrating cells.12,13 These characteristics invoke a function for T-cad as a signaling receptor involved in interpretation of extracellular cues rather than as a true adhesion molecule. In vitro, homophilic ligation of T-cad receptors on the endothelial cell...
(EC) surface with soluble recombinant T-cad protein or with agonistic antibody induces the motile phenotype via activation of Rho and Rac pathways and facilitates cell migration, whereas T-cad overexpression increases cell cycle progression, proliferation, and survival via activation of PI3-kinase/Akt pathway. Because EC phenotypic modulation, growth and migration are critical steps in the angiogenic process, we hypothesized a role for T-cad in neovascularization and examined its effects on EC angiogenic properties in vitro and in vivo.

Materials and Methods
The materials and methods used in this study are fully described in the online data supplement section (http://atvb.ahajournals.org).

Results
T-cad Ligation Induces Rearrangement of ECs Into Network Structures
Our previous studies demonstrated that T-cad can exert two types of effects on EC. Upregulation of T-cad expression level on the cell surface stimulates proliferation and survival. Ligation of T-cad receptors with recombinant T-cad protein or agonistic antibody included into the matrix imitates homophilic T-cad–mediated intercellular interactions and promotes cell detachment and cell motility. To analyze the effect of T-cad ligation on EC phenotype in the 2-dimensional monolayer system human umbilical vein endothelial cells (HUVEC) or human microvascular endothelial cell line (HMEC-1) cells were plated onto substrata containing recombinant T-cad protein which acts as an external ligand for T-cad molecules expressed on the surface of spreading cells. Cell morphology was examined following fixation of cultures (Figure 1A and 1B) and in living cells (Figure 1C). EC on control substratum displayed the typical cobblestone morphology of endothelial monolayers (Figure 1A, left panels), whereas ECs on T-cad-containing substratum formed a network-like pattern of interconnecting tubular structures (Figure 1A, middle and right panels). Total length of capillary-like tubes on T-cad substratum was ~8-fold higher than on control substratum for HUVECs and ~20-fold higher than on control substratum for HMEC-1.
higher for HMEC-1 (Figure 1B). Similar data were obtained when T-cad ligation was induced by substratum inclusion of agonistic antibody against the first extracellular domain of T-cad. Transfection of HUVECs with T-cad siRNA significantly decreased their tube-formation response to T-cad substratum; decreased T-cad protein level in HUVEC 72 hours after transfection with specific siRNA was confirmed by immunoblotting (Figure 1B). Time-lapse videomicroscopy showed that cells on T-cad substratum failed to spread and aggregated in small clusters from which elongated cells with highly motile lamellipodia protruded toward each other forming a network pattern within 5 hours (Figure 1C). Cells on control substratum spread fully within 1.5 to 2 hours after seeding (not shown). For videos showing EC behavior on control (video1-control.avi) and T-cad–containing substrata (video2-Tcad.avi) (please see http://atvb.ahajournals.org).

T-cad Ligation on ECs Stimulates Angiogenesis in the Spheroid In Vitro Model

The 2-dimensional models for cord-forming have been successfully used for studying effects of cell matrix interactions on EC morphology, but they do not reflect sprouting angiogenesis. Therefore, we implemented the EC-spheroid assay, which has proven to be useful for analysis of active outgrowth of newly formed capillary-like sprouts within 3-dimensional matrices. The effect of homophilic T-cad ligation on sprouting was examined by embedding spheroids into gels prepared with inclusion of T-cad protein. Identical results were obtained with collagen and fibrin matrices (Figure 2 presents data only for fibrin). We observed both VEGF-induced and a background “spontaneous” sprouting probably caused by autocrine cell activity and/or the presence of low concentrations of growth factors (eg, basic fibroblast growth factor) in the medium overlay. Inclusion of T-cad in gels significantly increased sprouting both in the absence and presence of VEGF (see photomicrographs in Figure 2A and graphs in Figure 2B to 2D). T-cad effect was apparently independent of exogenous VEGF since relative increases in sprout length in T-cad-containing gels (~1.5- to 2-fold versus control) were similar without or with VEGF in medium overlay (Figure 2C and 2D). Use of the Hoechst stain to quantitate the total length of endothelial structures composed of ≥2 aligned cells revealed a ~2.5-fold increase (P<0.01) in chain-like organization of sprouting EC within T-cad–containing gels (~1.5- to 2-fold versus control) were similar without or with VEGF in medium overlay (Figure 2C and 2D). This effect of T-cad on EC alignment within outgrowing sprouts resembles its ligation-dependent stimulation of EC reorganization into chains in monolayer cultures.

Figure 2. T-cad ligation stimulates angiogenesis in the in vitro EC-spheroid model. HUVEC spheroids were embedded into fibrin gels without (control) or with inclusion of recombinant T-cad protein (80 μg/mL for A, C, and D; indicated concentrations for B) or BSA as negative control and incubated in the presence or absence of 50 ng/mL VEGF. A, EC spheroid-containing gels were stained with TRITC-phalloidin for visualization of cellular structures (red) and with Hoechst for visualization of nuclei (blue). Bar=100 μm. B to D, Morphometric analysis shows that sprout lengths were significantly greater for spheroids embedded within T-cad-containing gels (**P<0.01). Stimulation of angiogenesis in the presence of VEGF was dependent on T-cad concentration (B). Data for both the total length of all sprouts and the total length of sprouts composed of 2 and more cells are given as absolute units (B, C) and as % of the respective control (D).
control (parental, empty vector, or LacZ-infected) HUVECs were embedded into collagen and fibrin gels not containing T-cad protein as an external ligand. T-cad protein expression in infected HUVEC was monitored by Western-blotting (Figure 3C). Morphometric analysis (data expressed relative to sprouting in parental HUVECs) demonstrated that T-cad overexpression produced 2.5-fold (P<0.01) increase in the total length and number of sprouts (Figure 3A and 3B). Sprouting behaviors of empty vector, LacZ, or parental HUVEC spheroids were comparable. As in the preceding experimental series on spheroids embedded within a T-cad containing gel, inclusion of VEGF in the medium overlay increased sprouting for all spheroids and the relative stimulatory effect of T-cad upregulation on sprouting was similar without or with inclusion of VEGF (Figure 3A). To confirm the importance of T-cad expression level for angiogenesis we studied the effect of T-cad silencing by siRNA transfection on HUVEC sprouting rates. Transfection with T-cad siRNA significantly (P<0.01) decreased sprout outgrowth into fibrin gels in the presence of VEGF (Figure 3D). Sprouting assay from empty vector-infected and TC⁺-spheroids was performed in the absence or presence of 10 μmol/L VEGF receptor inhibitor (E). a indicates significant difference (P<0.05) between empty-vector infected and TC⁺-spheroids; ***significant difference (P<0.001) between control and VEGFR inhibitor-treated spheroids.

Recombinant T-Cad Stimulates Angiogenesis in the Nicosia Heart Model

Of the currently available in vitro angiogenesis models, the Nicosia tissue assay is considered to more closely approxi-
mate the in vivo situation; it includes not only ECs but also surrounding nonendothelial cells in their microvascular environment, and the EC growing into fibrin gels from heart tissue fragments have not been preselected by passaging and thus are not in the proliferative state at the time of explantation, although tissue injury and in vitro conditions induce a certain level of spontaneous sprouting.

Morphometric quantification of total gel area invaded by endothelial sprouts and the total sprout length within fibrin gels ± recombinant T-cad demonstrated the stimulatory effect of T-cad on both spontaneous and growth factor (VEGF or bFGF)-induced sprouting (*P<0.01 versus controls) (Figure 4A). Representative photomicrographs are presented (Figure 4B and 4C). Note the presence of prominent cords and the fine interconnecting mesh of tubular structures within T-cad–containing gels (Figure 4C).

**Figure 4.** Angiogenesis in Nicosia model: influence of recombinant T-cad. Pieces of mouse hearts were embedded in fibrin gels without (control) or with inclusion of BSA or T-cad protein and incubated in the presence or absence of VEGF or bFGF (10 ng/mL). T-cad increases the area invaded by ECs and sprout length (**P<0.01).** (A). Representative images of gels (± bFGF only) stained with AlexaFluor 488-labeled isolectin IB4 are shown: low-magnification pictures, bar=500 μm, invasion distances indicated with arrows (B) and high-magnification pictures illustrating formation of a capillary-like net, bar=200 μm (C).

**T-Cad Modulates Dose-Dependent VEGF-Induced Angiogenesis in Mouse Skeletal Muscle**

To demonstrate relevant in vivo angiogenic functions for T-cad we investigated effects of recombinant T-cad protein on VEGF-induced angiogenesis in skeletal muscle. In this model gene delivery is achieved by implantation of retrovirally transduced mouse myoblasts into the posterior auricular muscle of mice where the myoblasts differentiate, fuse with preexisting host myofibers and secrete the proteins of interest into the surrounding tissue. The following previously characterized19 myoblast clones were used: clones VZ6 and VZ3 homogenously expressing distinct VEGF_164 levels (~5 ng/10^6 and ~70 ng/10^6 cells/d in culture, respectively) together with LacZ marker gene, and control myoblasts (Z) expressing LacZ only. For the purposes of estimating the ability of secreted T-cad protein
to induce and/or modulate VEGF-stimulated angiogenesis, the myoblasts were overinfected with a retroviral vector encoding secreted form of recombinant T-cad linked to a truncated form of CD8a (VZ6/T, VZ3/T, Z/T) or with control vector expressing CD8a only (VZ6/C, VZ3/C, Z/C). Therefore, in each clonal population, every cell expressed the same amount of VEGF, ensuring its homogeneous distribution in the myofiber microenvironment. This is crucial because it was previously shown that the phenotype of induced vessels depends strictly on VEGF dose. Moreover, different levels of expression do not average in vivo and remain highly localized in the microenvironment around each fiber. Therefore in order to draw quantitative conclusions it is necessary to base comparisons on a given homogenous expression level of VEGF. The presence of secreted T-cad protein in the culture medium of T-cad/CD8a-transduced myoblasts was confirmed by Western blotting (data not shown). Representative images of the vessels formed at the sites of clone implantation are shown in Figure 5A.

Within the areas around control Z cells, only straight capillaries running parallel to the myofibers were observed. Implantation of VEGF-expressing myoblasts clones stimulated formation of new capillaries which were smaller (VZ6/C) or larger (VZ3/C) than pre-existing vessels depending on VEGF level (Figure 5B), as previously reported. The absence of blue LacZ staining in the case of clones VZ3/C and VZ3/T indicates that implanted VZ3 myoblasts did not remain in the tissue for the entire 4 weeks; nevertheless, the morphological characteristics of the vessels attest to secretion of sufficient VEGF and the angiogenic response is as expected for VZ3 clone. Clones VZ6/T and VZ3/T co-expressing VEGF and T-cad induced formation of capillaries with larger diameters than myoblasts expressing only VEGF (**P<0.01).

Figure 5. T-cad modulates VEGF-induced angiogenesis in mouse skeletal muscle. Mouse myoblast clones expressing LacZ only or LacZ and 2 distinct VEGF levels were overinfected with a control retroviral vector (Z/C, VZ6/C, VZ3/C, respectively) or a vector expressing recombinant T-cad (Z/T, VZ6/T, VZ3/T) and implanted into the posterior auricular muscle of C.B.17/SCID mice. Angiogenic effects were evaluated after 4 weeks. All examined vessels exhibited normal morphology, no aberrant structures or hemangiomas were observed (A, Bar=50 μm). Data for vessel diameter measurements are presented as scatter diagram (B) and vessel diameter distribution (C). Myoblasts co-expressing VEGF and T-cad induced formation of capillaries with larger diameters than myoblasts expressing only VEGF (**P<0.01).
Discussion

Our previous studies demonstrated that T-cad can exert two types of effects on EC: T-cad overexpression stimulates cell cycle progression, proliferation, and survival, whereas homophilic ligation of T-cad receptors on the EC surface with recombinant extracellular domain of T-cad molecule included into the matrix causes cell detachment and promotes motility.\(^\text{14–17}\) The main findings of this study are: (1) a further consequence of T-cad ligation-dependent modulation of cell adhesion properties in a 2-dimensional model of EC differentiation is induction of tubular structures and arrangement of EC into multicellular interconnecting chains which form a capillary-like/networked pattern closely resembling the initial response of EC to a pro-angiogenic environment; (2) T-cad stimulates in-gel outgrowth of endothelial sprouts in 3-dimensional in vitro EC-spheroid and heart tissue models of angiogenesis; and (3) T-cad facilitates VEGF-induced angiogenesis in mouse skeletal muscle in vivo.

It is recognized that EC of all origins share a common ability to organize in vitro into tubular networks that are very similar to vascular beds formed by vasculogenesis or angiogenesis in vivo.\(^\text{20}\) Mechanical forces applied to cells from the matrix, dynamic control of cell matrix adhesion, and resulting alterations in cell shape are of crucial importance for determination of EC function.\(^\text{21}\) The relationship between EC adhesivity and their ability to migrate or differentiate is not direct and unequivocal. Promotion of integrin-mediated attachment by ephrinB1/EphB4 tyrosine phosphorylation is necessary for EC migration and neovascularization,\(^\text{22}\) and integrin blockade with anti-adhesive molecules decorin\(^\text{23}\) or rhodostomin\(^\text{24}\) elicits anti-angiogenic effects. However, dynamic inhibition of adhesion can be equally important for EC conversion toward differentiated angiogenic phenotype. Negative regulation of integrin function by class 3 semaphorins, ephrinA1/EphA2 and blocking anti-αβ\(_2\) and αβ\(_3\) integrin antibodies promotes formation of cord structures on collagen I or fibrin in vitro\(^\text{25}\) and stimulates neovascularization in vivo.\(^\text{26,27}\) By analogy we suppose that induction of cord-like structures in response to T-cad ligation in monolayer EC might be caused by T-cad–dependent inhibition of adhesion to the matrix,\(^\text{14}\) resulting in an “angiogenic switch” in the cell differentiation program and a facilitation of cell motility.

T-cad ability to influence neovascularization is supported by the 3-dimensional in vitro angiogenesis experiments. In both EC-spheroid and Nicosia tissue assays inclusion of T-cad ligand (recombinant T-cad protein) into gels promotes assembly of multicellular EC sprouts and increases total length and number of capillary-like structures invading the gels. Angiogenic behavior of ECs was also affected by modulation of cellular T-cad expression levels per se without inclusion of T-cad ligands in the gel. Adenovirus-mediated T-cad overexpression enhanced angiogenic rates in fibrin and collagen gels, whereas specific siRNA-driven downregulation of T-cad consistently reduced angiogenic sprouting, possibly caused by effects of T-cad expression levels per se on cell cycle progression and proliferation rates.\(^\text{16}\) Furthermore, in multicellular in vitro systems and in vivo it is likely that T-cad ligation and overexpression represent 2 sides of the same process. Upregulation of surface T-cad levels would increase the chance of homophilic intercellular T-cad ligation on neighboring cells during vascular remodeling. One also cannot exclude the possibility of T-cad overexpression causing lateral cis contacts between T-cad molecules and a subsequent generation of intracellular signals caused by increased clustering and T-cad interactions with its putative membrane signaling partners. This hypothesis is supported by our observation that both overexpression and ligation of T-cad can activate the same signaling pathway and induce Akt phosphorylation (supplemental Figure I, available online at http://atvb.ahajournals.org). However, overexpression does not induce cell detachment and phenotypic modulation, and ligation results in phenotypic switch only when homophilic T-cad ligands are included in the matrix and not simply added to culture medium. Thus the functional consequences of T-cad activation might depend on the cell state (spreading cells versus already attached monolayer) and T-cad interactions with molecules participating in cell adhesion to the matrix.

In vivo effects of T-cad on neovascularization were studied using myoblast-mediated gene transfer to mouse skeletal muscle, a model that has specific advantages over other in vivo methods. Precise control of angiogenic stimulation in the target tissue is achieved by implantation of myoblast clones retrovirally transduced to homogenously express specific VEGF levels. Use of a single stimulatory growth factor at precise doses allows to standardize experimental conditions and to study even subtle effects of angiogenic regulators, these being more difficult in hind limb ischemia, Matrigel plaque, or tumor angiogenesis models where new vessel formation is induced by complex and multifactorial stimuli such as hypoxia or tumor environment. Our data demonstrate that implantation of myoblast clones co-expressing VEGF and secreted T-cad protein induces formation of capillaries of larger diameters compared with respective clones expressing the same dose of VEGF alone, unequivocally confirming the proangiogenic properties of T-cad. Importantly, whereas significantly increasing the caliber of the vessels at the sites of myoblast implantation, T-cad did not induce formation of any aberrant structures such as hemangiomas, often observed in response to delivery of higher VEGF concentrations.\(^\text{5,19}\) Small increases in vessel size bring about large improvements in blood flow which is proportional to the fourth power of vessel radius, according to Poiseuille’s law. Therefore, these results suggest an interesting application for T-cad co-delivery to enhance the efficacy of VEGF-induced therapeutic angiogenesis at low and safe levels.

Mechanisms mediating T-cad–dependent stimulation of angiogenesis have yet to be clarified. T-cad–induced sprouting in vitro is not caused by induction of VEGF synthesis in EC. First, T-cad activation does not influence VEGF gene expression (see http://atvb.ahajournals.org). Second, VEGF receptor inhibition does not eliminate T-cad–induced increase in angiogenic rates. Stimulation of sprouting by T-cad in the spheroid and Nicosia models both in the absence and presence of VEGF further argues for VEGF-independence. However, in vivo T-cad failed to induce significant neovascularization in the absence of VEGF. The discrepancy between in vitro and in vivo findings with respect to VEGF-dependence
could be explained by differences in EC activation states. In culture, cells exist in a state of activation caused by the presence of serum and growth factors in the culture medium, whereas in vivo EC are normally quiescent and in contact with pericytes and have a differentiated phenotype. Further, in vitro T-cad also increased VEGF-induced sprouting, whereas in vivo it did not affect vessel number, but only its diameter. These results are not contradictory if one considers that in vitro sprouting assays are really testing effects on proliferation and migration, but do not reproduce actual angiogenesis in vivo. The pro-migratory and proliferative effects of T-cad on EC in vitro translate into a modulation of VEGF-induced angiogenesis in vivo, affecting vessel diameter, but not length. Therefore, our data suggest that T-cad is not a primary angiogenic stimulus, but rather a modulator of angiogenesis that requires initial destabilization of the vessel by growth factors (eg, VEGF) to exert its facilitatory influence on EC phenotype conversion, proliferation and survival.

Identification of T-cad, a molecule that also participates in guidance of motor axons, as a regulator of angiogenesis further supports the surmise that nerves and blood vessels not only share architectural similarity and follow parallel trajectories during development but also often utilize identical mechanisms that regulate projection of neural and vascular structures and their organization into complex networks. Interestingly, many regulators of neurogenesis and/or neovascularization localize in lipid rafts and caveolae, plasma membrane domains that function as assembling platforms for complexes between activated receptors and their signaling targets on the cell surface. Among them are guidance molecules ephrins, semaphorins, F3/contactin, Rhö family GTPases, regulatory components of PI3-kinase/Akt pathway that also mediate T-cad effects in EC, as well as major caveolae protein caveolin-1 that mediates growth promotory influence of VEGF by eNOS and Flk-1/KDR translocation to the nucleus and the knockout of which critically inhibits angiogenesis in vivo, supporting a central role for these domains in neovascularization. T-cad is also located in caveolae-like domains. Conceivably, lipid rafts may participate in T-cad effects on angiogenesis by permitting interaction of cell surface GPI-anchored T-cad with molecular adaptors that couple it to intracellular signaling systems.

In conclusion, this study reveals T-cad as a novel proangiogenic molecule. We propose a contribution of T-cad to the process of (patho)physiological neovascularization, given the present findings that T-cad increases endothelial sprouting in vitro and facilitates VEGF-induced angiogenesis in vivo, and the previous demonstrations of upregulation of T-cad on EC from newly formed tumor vasculature and very high expression levels of T-cad on vasa vasorum in the adventitial layer of human aorta. Silencing of T-cad in ECs may offer new possibilities for endothelial-targeted treatment of pathological conditions associated with excessive neovascularization. Alternatively, delivery of recombinant T-cad protein or modulation of T-cad expression on ECs using gene transfer might represent potential strategies to improve the outcome of growth factor-dependent angiogenic therapy of tissue ischemia.

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Disclosures
None.

References
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Supplementary information

Supplementary Materials and Methods

Cell culture

Human umbilical vein endothelial cells (HUVEC) were purchased from PromoCell GmbH (Heidelberg, Germany) and cultured in EC growth medium containing low serum (2 %) EC growth supplement (PromoCell). Cells at between passage 2 and 6 were used in the described experiments. Human microvascular EC line HMEC-1 was cultured in the same medium supplemented with 10 % FCS.

Generation of soluble recombinant T-cad protein

Soluble recombinant T-cad protein without GPI-anchor was generated as described. Briefly, cDNA encoding human T-cad without the propeptide and signal for attachment of GPI anchor was obtained by PCR, cloned into pSecTag2C vector carrying 6xHis tag (Invitrogen AG, Basel, Switzerland) and used for transfection of HEK293 cells using Lipofectamin 2000 reagent (Invitrogen AG). 4 stably transfected clones were selected for Zeocine (Invitrogen AG) resistance and cultured in DMEM/10 % FCS. Culture medium from all clones was routinely collected at 3-day intervals and pooled. Recombinant T-cad protein was purified from medium by chromatography on a Ni-NTA agarose column (Qiagen GmbH, Hilden, Germany), dialyzed against PBS, concentrated in Centricon centrifugation units (Millipore Corp., Bedford, USA) and sterilized by passing through 0.22 μm filters.

Overexpression of T-cad in cultured EC using adenoviral vectors

T-cad was overexpressed in HUVEC using Adeno-X Expression System (Clontech, Palo Alto, USA) as described previously. Viral titer was determined by End-Point dilution assay.
For infections HUVEC in normal growth medium were seeded at a density of 1-2x10^4 cells/cm^2, allowed to adhere and infected overnight with empty, LacZ (Clontech-Becton Dickinson) or T-cad containing adenoviral particles at a final approximate concentration of 100 pfu/cell. The level of T-cad expression in infected HUVEC was examined by immunoblotting and immunocytochemistry with anti-T-cad antibody generated in our lab.\(^4\)

**siRNA transfection**

SiRNA duplexes for silencing of T-cad in EC were pre-designed by Dr.Philippe Riou (Hôpital Universitaire Paul Brousse, Paris, France) and purchased from Microsynth (Balgach, Switzerland). siRNA sequence (5’GGACCAGUCAAUUCUAAAC3’) was proven to be efficient in downregulation of T-cad expression in HUVEC as demonstrated by Western blotting. siRNA (5’CUCUGUUCGUCCAUGCAG3’) found to exhibit no effects on the expression of T-cad at both transcriptional and protein levels was used as negative control. Transfection of HUVEC with iRNA was performed using Hyperfect™ reagent (Qiagen, Hombrechtikon, Switzerland) according to manufacturer's recommendations.

**Analysis of cell morphology**

96-well plates were precoated overnight at 4°C with either recombinant T-cad protein or BSA solution (50µg/ml) with inclusion of 0.1% gelatine in the case of HUVEC. After adsorption dishes were rinsed with sterile phosphate-buffered saline (PBS). HUVEC or HMEC-1 were detached by brief trypsinization (0.1% trypsin/1mM EDTA in PBS), resuspended in their respective growth medium and seeded onto the precoated plates (in triplicate wells for any given experimental condition) at a density of 10^4 cells/well. Cultures were incubated for 6 hrs (unless otherwise specified in Figure legends) at 37°C, washed with PBS, fixed for 10 min with 4% paraformaldehyde in PBS and stained with 0.05% methylene blue. Nuclei were counterstained with Hoechst (Molecular Probes, Leiden, Netherlands). Images of each entire
well-surface were captured using an Olympus IX-50 inverted microscope equipped with a
digital camera (Olympus Optical, Schwerzenbach, Switzerland), and length of tubular
structures per well was determined morphometrically using AnalySIS 3.2 software (Soft
Imaging System GmbH, Münster, Germany). These experiments were performed on 6 separate
occasions.

**Time-lapse videomicroscopy**

HUVEC and HMEC-1 cells were plated onto culture dishes precoated with recombinant T-cad
as described above and filmed at a rate of 1 frame/15 min using an Olympus IX-81 inverted
time-lapse microscope equipped with a digital camera in the humidified chamber with 5% CO₂
at 37°C (Olympus Optical, Schwerzenbach, Switzerland). Images (from triplicate wells for any
given experimental condition) were processed using AnalySIS D software (Soft Imaging
System GmbH, Münster, Germany). The experiment was performed on four separate occasions
with reproducible findings.

**Spheroid assay of angiogenesis in vitro**

Endothelial cell spheroid assay in 3-D fibrin or collagen gels was performed as described⁵ with
modifications. HUVEC were seeded in U-bottomed non-tissue culture-treated 96-well plates
(Falcon) at a density of 500 cells/well in normal growth medium containing 5% FCS and 20%
(w/v) carboxymethylcellulose (Sigma-Aldrich, Buchs, Switzerland) and incubated for 24 hrs.
Under these conditions all cells in the well aggregate and form one multicellular spheroid of
standard size. Then spheroids were collected and pooled by centrifugation (1000 rpm, 10 min).
For fibrin gels, spheroids were resuspended in basal medium (i.e. without serum or growth
factor supplement) containing 20% (w/v) carboxymethylcellulose without or with inclusion of
recombinant T-cad protein (80µg/ml), and mixed 1:1 with sterile-filtered fibrinogen solution
(6mg/ml, Sigma-Aldrich, Buchs, Switzerland) in basal medium; polymerization was induced by
addition of 1U/ml thrombin, and the mixture was pipetted into prewarmed 48-well plates (200µl, 15-20 spheroids/well). For collagen gels, 8 volumes of collagen I stock (BD Biosciences) was mixed with 1 volume of 10xPBS, 0.023 volumes of 1N NaOH and basal medium up to 10 volumes; spheroids were resuspended in basal growth medium without or with inclusion of recombinant T-cad protein (80µg/ml), mixed 1:1 with collagen solution and transferred to prewarmed 48-well plates (200µl, 15-20 spheroids/well). Polymerization was induced by incubation at 37°C for 1 hr. Polymerized spheroid-containing collagen and fibrin gels were overlaid with 500µl of normal growth medium supplemented with 2% FCS without or with inclusion of 50ng/ml VEGF and incubated under normoxia conditions (21% O₂, 5% CO₂, 37°C) for 24 hrs. There were 2 parallel wells for each experimental condition. The experiments were performed on six separate occasions. VEGF receptor tyrosine kinase inhibitor 4-[(4'-chloro-2'-fluoro)phenylamino]-6,7-dimethoxy-quinazoline was from Calbiochem.

**Modified Nicosia assay of angiogenesis in vitro**

A modification of the Nicosia assay was performed essentially as described before.⁶ Fibrin gels were prepared by dissolving fibrinogen (Sigma-Aldrich, Buchs, Switzerland) in basal DMEM to a final concentration 3 mg/ml. Immediately following induction of polymerization by addition of 0.1 U/ml thrombin, the solution was pipetted into 48-well plates (100 µl/well) and incubated for 40 min at 37°C. 500 µl DMEM was pipetted on top of each gel and left for at least 30 min. 11 week-old mice were sacrificed, hearts were removed, left ventricles were cut into 0.5-1 mm³ pieces, placed on top of fibrin gels (1 piece per well) and overlaid with a second gel layer. After 30 min polymerization 500 µl of DMEM, supplemented with 10% FCS, 300 µg/ml ε-amino caproic acid (Sigma-Aldrich, Buchs, Switzerland) and with or without addition of 1 ng/ml VEGF or bFGF, was pipetted on top of each gel. Gels were incubated for 7-10 days under hypoxic conditions (3% O₂). Growth factors and ε-amino caproic acid were added
freshly every other day. In some wells recombinant T-cad protein or BSA was included (80 µg/ml) in the fibrinogen solution. There were 6 parallel wells for each experimental condition. The experiments were performed on three separate occasions.

**Quantitative analysis of in-gel angiogenesis**

For analysis of outgrowing capillary-like structures in spheroid or Nicosia assays gels were fixed by addition of 500µl 8% paraformaldehyde into growth medium, incubated overnight, washed extensively with PBS and permeabilized with 0.2% Triton X-100. After washing, gels were incubated overnight with either 0.5µg/ml TRITC-conjugated phalloidin (Sigma-Aldrich, Buchs, Switzerland) diluted into FCS for visualization of actin cellular structures, with Hoechst (1:2000, Molecular Probes, Leiden, Netherlands) for staining of nuclei, or with Alexa Fluor 488-labelled Isolectin IB₄ from *Griffonia simplicifolia* (1:100, Molecular probes, Leiden, the Netherlands) diluted into 0.25% BSA in PBS for detection of endothelial cells. For the spheroid assay the length and number of sprouts per spheroid was determined morphometrically using AnalySIS software (Soft Imaging System GmbH, Münster, Germany) and at least 20 spheroids from two parallel wells were analysed for each experimental point. For the Nicosia assay, the morphometric parameters determined for each tissue piece were area invaded by endothelial sprouts and total length of sprouts.

**In vivo angiogenesis in mouse skeletal muscle**

The myoblast-mediated gene transfer model for studying angiogenesis in mouse skeletal mouse was previously described in detail. Experimental animals were treated in accordance with the rules of the Swiss Federal Act on Animal Protection (1998) and the Veterinary Department of the Kanton of Basel Stadt (Switzerland). Primary mouse myoblasts from C57BL/6 mice were retrovirally transduced to express LacZ and murine VEGF₁₆₄ or LacZ only (control, Z). Clones were isolated in which every cell expressed the same amount of VEGF: VZ6 and VZ3
expressing ~ 5 ng and 70 ng/10^6 cells/day in culture, respectively. These populations were overinfected with a retroviral vector expressing secreted form of recombinant T-cad linked to a truncated form of CD8a (VZ6/T, VZ3/T, Z/T) or CD8a only (VZ6/C, VZ3/C, Z/C). CD8a-positive cells were purified using FACS, and expression of secreted T-cad in culture medium from T-cad-transduced populations was proven by Western blotting with anti-T-cad antibodies. Myoblasts (5x10^5 cells/injection) were implanted into the posterior auricular muscle of 6 week-old male C.B.-17/SCID mice (Charles River Laboratories, Germany). This immunocompromised mouse strain was chosen in order to avoid immune response to grafted cells expressing LacZ and human T-cad. After 4 weeks mice were anesthetized, and biotinylated Lycopersicon esculentum lectin (Vector Laboratories, Burlingame, USA) was injected intravenously to label the luminal surface of all vessels, followed by perfusion of fixative solution (1% paraformaldehyde/0.5% glutaraldehyde in PBS). Ears were removed, dissected in the plane of cartilage and subjected to histochemistry procedures that included X-gal staining (1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside in PBS containing 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 1 mM magnesium chloride, 0.02% Nonidet P-40, 0.01% deoxycholate, pH 7.4) to visualize implanted myoblasts, permeabilization with 0.3% Triton X-100 and incubation with avidin-biotin-peroxidase complex (VECTASTAIN ABC System, Vector laboratories), with DAB as the reaction substrate to visualize the vascular network. Tissue samples were whole-mounted onto glass slides with Permount™ mounting medium. Diameter and length of vessels were determined by examination of digital microscopic images of the areas of effect using AnalySIS 3.2 software. 5 ears per group were analysed for clones VZ6/C and VZ6/T, 4 ears for VZ3/C and VZ3/T, 3 ears for Z/C and Z/T. Measurements were performed on at least 8 areas for each experimental condition.

**Immunoblotting**
The method of immunoblotting has been described previously. Lysis buffer was PBS containing 1% SDS, 1mM PMSF, 2μg/ml pepstatin, 20μg/ml aprotinin, 30μg/ml bacitracin, 1 mM orthovanadate and 5 mM NaFl. The following primary antibodies were used: polyclonal antibody against the first extracellular domain of T-cad generated in our lab; anti-Akt and anti-phospho-(Ser\textsuperscript{473})Akt (Cell Signaling, New England Biolabs GMBH, Frankfurt, Germany). Scanned images of immunoblots were analyzed using Scion (NIH) Image software. Figures show representative immunoblots.

**Analysis of VEGF gene expression by real-time PCR**

HUVEC were seeded in 6-well plates and either infected with T-cad or LacZ-encoding adenoviral vectors or incubated in the presence of anti-Tcad IgG or non-immune rabbit IgG (100μg/ml). Hypoxia-mimicking agent deferoxamine (100 μM) was used as positive control for VEGF upregulation. mRNA was isolated from cells using Trizol reagent (Invitrogen AG, Basel, Switzerland) and reverse-transcribed using Omniscript RT kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's recommendations. Synthesized cDNA was applied to real-time RT-PCR using 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, USA) using ABsolute SYBR Green ROX Mix (ABgene, Hamburg, Germany) and analysed with 7500 Fast System Software (Applied Biosystems). Real-time cycle conditions were 2 min at 50°C, followed by 10 min at 95°C and then by 40 cycles at 95°C for 15 sec and 60°C for 60 sec. Additional dissociation stage (95°C for 15 sec, 60°C for 60 sec and 95°C for 15 sec) was included in order to confirm the presence of a single PCR product. VEGF expression was normalized to the expression of GAPDH gene. Primer sequences were as follows: 5’-CCCCTGAGGAGTCCAACATC-3’ forward and 5’-GGCCTTGGTGAGGTTGATC-3’ reverse for VEGF; 5’-TCATGACCACAGTCCATGCC-3’ forward and 5’-GCCATCCACAGTCTTCTGGTG-3’ reverse for GAPDH.
Statistical analysis

Unless otherwise stated all results are given as mean ± SD. The number of independent experiments performed are given in corresponding Methods sections. Statistical analyses were performed by One-way ANOVA followed by post hoc Bonferroni’s multiple comparison when appropriate using Prism 3.0 Software. A P value of <0.05 was considered significant.

Supplementary Results

T-cad overexpression and ligation activate Akt pathway in HUVEC

Previously we demonstrated that overexpression of T-cad in EC stimulates cell proliferation and survival via activation of Akt pathway. To investigate whether T-cad ligation leads to stimulation of the same pathway HUVEC were treated with agonistic antibody against the first extracellular domain of T-cad, and levels of phospho-Akt in cell lysates were determined by immunoblotting. As expected, T-cad-overexpressing cells had higher phospho-Akt levels than LacZ cells (Supplementary Fig.I, TC+ vs. LacZ in the presence of non-immune IgG). Anti-T-cad antibody significantly increased Akt phosphorylation in both LacZ and TC+ HUVEC, but to a markedly higher level in TC+ (Supplementary Fig.I). Neither IgG influenced total Akt expression.

T-cad overexpression and ligation do not induce VEGF production in HUVEC

VEGF expression level in HUVEC was measured by RT-PCR. T-cad overexpression did not induce any changes in VEGF message level after 4, 8, 24 and 48 hrs post-infection. VEGF expression was also not modulated by T-cad ligation with agonistic antibody (4, 8, 16 and 24 hrs of incubation). Hypoxia-mimicking agent deferoxamine used as positive control, time-dependently increased VEGF production (data not shown).
Supplementary Figure Legends

Supplementary Figure I. T-cad overexpression and ligation activate Akt pathway in HUVEC.

Total and phospho-Akt levels were measured by immunoblotting in LacZ-infected and T-cad-overexpressing (TC+) cells treated for 6 hrs with agonistic anti-T-cad antibody or non-immune rabbit antibody (n/i) for negative control. The graph shows quantitative data for phospho-Akt level obtained by densitometric analysis of scanned images and expressed as % of total Akt level in the same sample. ** and ***, indicate significant differences (P at least <0.01 and <0.001, respectively) between cells treated with n/i or anti-T-cad antibodies. In both conditions phospho-Akt was significantly higher (P<0.001) in TC+ than LacZ.

Supplementary videofiles. Analysis of T-cad ligation-induced rearrangement of endothelial cells into network structures by time-lapse videomicroscopy.

Time-lapse videomicroscopy showed that capillary network formation by HMEC-1 cells plated onto T-cad substratum followed specific morphological phases (video2-Tcad.avi). During the first hour cells adhered to the culture surface but failed to spread. After 2 hrs small clusters of rounded poorly attached cells began to form. Thereafter elongating cells with highly motile lamellipodia protruded from the clusters, migrated out and aligned into chains with eventual interconnection of clusters, and within 5 hrs a network of cord-like structures formed. However, the established network pattern was unstable; with prolonged incubation, cells continued to retract, elongate and rearrange into new networks (data not shown). Cells on control substratum spread fully, formed a monolayer within 1.5-2 hrs after seeding and retained this morphology over the whole time period analysed (video1-control.avi).
Supplementary Figure I

Supplementary References


