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# Polarisation of T-cadherin to the leading edge of migrating vascular cells in vitro: a function in vascular cell motility?

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**Abstract** Both histological and in vitro studies indicate a relationship between T-cadherin levels and acquisition of a modulated, migratory phenotype by vascular cells. This study further examines a role for T-cadherin in relation to cell migration and adhesion. Fluorescence microscopic examination of T-cadherin localisation in confluent cultures of human umbilical vein endothelial cells (HUVEC), human aortic smooth muscle cells and the human carcinoma cell line ECV-304 revealed global distribution over the entire cell body, and with only slight enrichment at cell borders. This contrasts with restricted cell–cell junction localisation of classical cadherin (for example, VE-cadherin in HUVEC). In wounded cultures, T-cadherin polarised to the leading edge of cells migrating into the wound area, again contrasting with classical VE-cadherin, which was undetectable in this region. Confocal microscopy demonstrated that potential signalling functions of T-cadherin at the leading edge are unrelated to physical interactions with caveolin. Adherence of HUVEC onto a monolayer of T-cadherin-transfected L929 cells is significantly reduced compared with adhesion onto control (T-cadherin-negative) L929. Thus T-cadherin is not required for maintenance of intercellular adhesion, but may rather function as a signalling molecule involved in cell–cell recognition and sensing of the environment in processes where cell detachment occurs.

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## Introduction

Cadherins are cell surface glycoproteins that mediate homophilic calcium-dependent intercellular adhesion (Angst et al. 2001). The "classical" cadherins are composed of five extracellular domains, the first extracellular amino terminal domain bearing the binding site for a homotypical cadherin on a neighbouring cell, a transmembrane and a cytosolic domain which is linked to actin cytoskeleton. T-cadherin (T-cad) is an atypical member of the cadherin superfamily: while possessing the general extracellular structure of the classical type I cadherins, it lacks the transmembrane and cytoplasmic domains and is bound to the plasma membrane via a glycosylphosphatidylinositol anchor (Ranscht and Dours-Zimmermann 1991; Vestal and Ranscht 1992). It is not clear whether this truncated cell surface molecule, obviously not directly linked to the cytoskeleton, can mediate mechanical coupling of cells in vivo, although in suspensions of transfected cells T-cad was shown to mediate weak cell aggregation (Vestal and Ranscht 1992; Niermann et al. 2000). The only physiological function of T-cad proven so far is regulation of motor axon projection in the embryonic nervous system via contact inhibition mechanism (Fredette and Ranscht 1994; Fredette et al. 1996). The role for T-cad in the adult has been studied mostly in tumours, where it was usually downregulated as compared to normal tissue, while transfection of cancer cells with T-cad cDNA decreased their proliferative and invasive potential (reviewed in Takeuchi and Ohtsuki 2001). These data imply that T-cad is a signalling and recognition receptor rather than a true adhesion molecule.

T-cadherin is highly expressed in the cardiovascular system and is present on endothelial cells, pericytes and smooth muscle cells from all layers of the vascular wall (Ivanov et al. 2001; Kudrjashova et al. 2002). Histological analysis of T-cad expression in diseased human vessels

demonstrated that its level was markedly increased in smooth muscle cells during atherosclerosis and restenosis after percutaneous balloon angioplasty (Ivanov et al. 2001; Kudrjashova et al. 2002), and on endothelial cells of tumour-penetrating vessels (Wyder et al. 2000). Since these disorders are characterised by enhanced vascular cell motility and growth (Schwartz 1997; Conway et al. 2001), upregulation of T-cad in atherosclerotic lesions, restenotic tissue and angiogenic vessels is contrary to what has been found in most tumour cells where an increase in T-cad is associated with decreased cell growth and invasion (Takeuchi and Ohtsuki 2001). We have proposed that in vascular cells T-cad may contribute to the acquisition of a less adhesive, promigratory cell phenotype (Schwartz 1997; Conway et al. 2001).

In this study we have examined the role for T-cad in relation to cell migration and adhesion in vitro. Since directional cellular locomotion and adhesion–deadhesion processes are thought to involve lateral transport/redistribution of cell surface molecules we performed immunocytochemical analysis of T-cad localisation in quiescent and migrating cultures of human umbilical vein endothelial cells and human aortic smooth muscle cells. T-cad localisation was also studied in ECV-304 cells, a human bladder carcinoma cell line (Dirks et al. 1999) which has many endothelial features (Suda et al. 2001) and is widely used as a model cell line for endothelia. We also addressed whether elevation of surface T-cad expression within a cell monolayer might affect the ability of other cells to adhere to that monolayer.

## Materials and methods

### Cell culture

Human umbilical vein endothelial cells (HUVEC) and endothelial cell growth medium containing endothelial cell growth supplement were purchased from PromoCell (Heidelberg, Germany). HUVEC at between passage 2 and 6 were used in the described experiments. Human bladder carcinoma cell line ECV-304 was a kind gift of Dr. V. Stepanova, Cardiology Research Center, Moscow. ECV-304 were cultured in Dulbecco's modified essential medium (DMEM) supplemented with 10% fetal calf serum (FCS), 10 mM TES/HEPES (pH 7.3), 100 U/ml penicillin/streptomycin and 20 mM Glutamax (Invitrogen, Basel, Switzerland). Human aortic smooth muscle cells (hSMC) were isolated and phenotypically characterised in primary cultures as described previously (Scott-Burden et al. 1989) and cultured in DMEM supplemented as described above for ECV-304 with addition of smooth muscle growth supplement (Cascade Biologics, Portland, USA). For the experiments described herein hSMC were used at between passage 6 and 13. Transformed mouse fibroblast cell line L929 was cultured in DMEM supplemented as described for ECV-340.

### Immunocytochemistry and microscopy

Cells were plated in 24-well plates onto round 12-mm glass coverslips precoated with 0.1% gelatine at a density of $1.5\times10^5$ cells/well. Confluent, quiescent monolayers were either fixed with 4% paraformaldehyde immediately or wounded with a scraper made of a bent needle (5 mm wide) and fixed after 18–24 h incubation for analysis of migrating cells. After preincubation in blocking solution (10% FCS in PBS) samples were processed for single or double immunostaining. Polyclonal antibody against recombinant amino terminal domain of human T-cad was raised in our laboratory (Ivanov et al. 2001) and purified on affinity column with immobilised recombinant domain coupled to CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech, Uppsala, Sweden). The following commercially available antibodies were used: monoclonal anti-VE-cadherin, monoclonal anti-caveolin (both 1:100; BD Transduction Laboratories, Heidelberg, Germany) and secondary anti-species Cy-2- and Cy3-labelled IgG (both 1:500; Jackson Immunoresearch Laboratories, West Grove, USA). For controls, samples were incubated with non-immune mouse or rabbit IgG as a substitute for primary antibodies. Nuclei were counterstained with Hoechst (1:3,000 in PBS; Molecular Probes, Leiden, Netherlands), and slides were mounted upside-down in FluorSave reagent (Calbiochem, Darmstadt, Germany). Single immunostained samples were studied under a Zeiss Axiophot fluorescent microscope (Zeiss, Feldbach, Switzerland), and photos were taken using a digital camera and AnalySIS software (Soft Imaging System, Munich, Germany). Double immunostained samples were examined using a laser scanning confocal microscope LSM 510 (Zeiss). Images were processed and analysed for colocalisation on an O2 Workstation (Silicon Graphics Computer Systems, Mountain View, CA, USA) using Imaris 3.0 and Colocalization Bitplanes software (Bitplane, Zurich, Switzerland). Micrographs present typical images.

### Transfection of L929 cells with human T-cadherin

Full-size human T-cad cDNA was generated by RT-PCR using mRNA from cultured human aortic smooth muscle cells as a template. cDNA was cloned into pcDNA 3.1 vector (Invitrogen, San Diego, USA) and used for transfection of L929 cells with Lipofectin reagent (Invitrogen). Luciferase cDNA fragment in antisense orientation was cloned into pcDNA 3.1 and used for transfection as control vector. L929 clones carrying inserts were selected by incubation in DMEM/10% FCS containing 200 $\mu$g/ml Geniticin (Invitrogen) and tested for T-cad expression by immunoblotting and surface antibody binding with anti-T-cad antibodies generated in our laboratory (see under Immunocytochemistry and microscopy). The method of immunoblotting was fully described previously (Kuzmenko et al. 1998). For surface binding transfected L929 clones were seeded into 96-well plates and grown to confluent monolayer. Then cells were washed with cold PBS and incubated for 2 h at 4°C with different concentrations of anti-T-cad IgG or non-immune rabbit IgG diluted in PBS containing 3% BSA and 10 mM HEPES pH 7.5. After washing, cells were incubated with secondary horseradish peroxidase-conjugated goat anti-rabbit IgG (1:2,500; Southern Biotechnology, Birmingham, USA) for 1 h. Colour reaction was developed using Fast o-phenylenediamine dihydrochloride tablet kit (Sigma-Aldrich Chemie, Steinheim, Germany), and $OD_{460}$ was measured in a SpectraMax 190 ELISA-reader (Bucher Biotec, Basel, Switzerland). T3 clone with highest level of T-cad expression and two control clones E9 and E15 were selected for further experiments.

### ELIRA adhesion assay

T-cadherin-deficient and T-cad-expressing L929 cell clones were seeded into 96-well plates and grown to confluent monolayer, mildly fixed for 30 s with 0.1% paraformaldehyde and carefully washed several times with PBS to remove all traces of paraformaldehyde. HUVEC were seeded (5,000 cells per well) on top of the L929 monolayers in normal endothelial growth medium (see under Cell culture). Adherent HUVEC were quantitated after 16 h by ELIRA for von Willebrand factor: cells were washed, fixed with 4% paraformaldehyde, preblocked with PBS containing 3% BSA for 30 min, incubated with anti-von Willebrand factor IgG (1:400; Dako, Denmark) or non-immune rabbit IgG for 1 h and, after washing, with secondary horseradish peroxidase-conjugated goat

anti-rabbit IgG (1:2,500; Southern Biotechnology) for 1 h. Colour reaction was developed using Fast o-phenylenediamine dihydrochloride tablet kit, and $OD_{460}$ was measured in a SpectraMax 190 ELISA-reader. L929 monolayers without HUVEC served as background controls.

## Results

### T-cadherin in vascular cells does not localise to intercellular junctions in monolayer cultures and concentrates at the leading edge in migrating cells

Immunofluorescence staining for T-cad in confluent monolayers of cultured hSMC, HUVEC and ECV-304 revealed a global and punctuate localisation over the entire cell body (Fig. 1A–D). Occasionally a mild enrichment of T-cad at cell–cell borders was seen (see within Fig. 1A or D). However, the generally diffuse distribution of T-cad was quite distinct from that of VE-cadherin (in HUVEC) which was restricted at cell–cell borders in the pattern typical of classical cadherins (Fig. 1E, F).

The distribution of T-cad in migrating cells was examined after wounding of confluent cell monolayers and images are presented in Fig. 2. Cells migrating into the wound area displayed typical "migratory" appearances, namely elongated and bearing pseudopods at the leading edge with a rear-end protrusion in the case of hSMC (Fig. 2A–C), and rather more ovally polarised with a broad leading edge in the case of endothelial cells (Fig. 2D–I). Immunofluorescent localisation of T-cad revealed a distinct enrichment at the leading edge of migrating hSMC (Fig. 2A–C), ECV-304 (Fig. 2D–F) and HUVEC (Fig. 2G–I). VE-cadherin was never enriched at the leading lamella in migrating HUVEC, and in fact was barely detectable in single migrating cells (see, for example, Fig. 2J). Images (Fig. 2K, L) of cells just starting to protrude into the wound area from the rim of the monolayer demonstrate the presence VE-cadherin only at intercellular contacts and not at the leading edge. Black-and-white contrast versions (Fig. 2M–O) of the original fluorescence images (Fig. 2J–L) of VE-cadherin in migrating HUVEC are presented to enable clear visualisation of the cell body.

### T-cadherin does not colocalise with caveolin at the leading edge in migrating cells

Glycosylphosphatidylinositol (GPI)-proteins are known to be associated with caveolae domains of the plasma membrane (Harder and Simons 1997). Our previous studies demonstrated that extracts of Triton-insoluble plasmalemmal domains from human aortic media and aortic SMC were enriched in T-cad together with other GPI-proteins and caveolin (Philippova et al. 1998). Since caveolin has been shown to be distributed to the leading edge of migrating SMC (Okada et al. 1995), the polarised

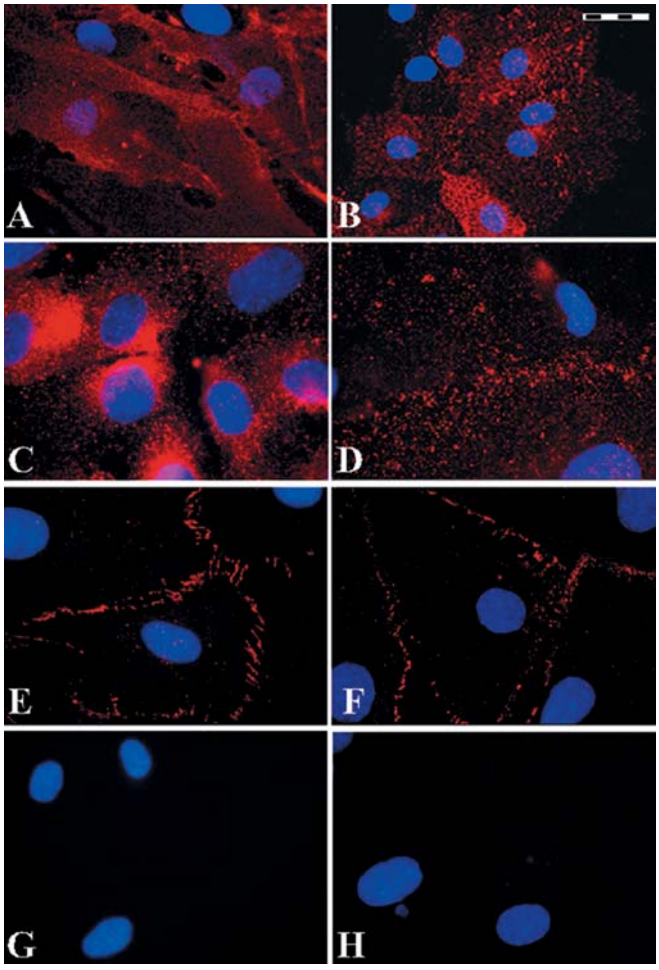



**Fig. 1A–H** Cadherin localisation in cell monolayers. Confluent monolayers of human aortic smooth muscle cells (hSMC; **A**), ECV-304 (**B**) and human umbilical vein endothelial cells (HUVEC; **C–F**) were stained with antibodies against T-cadherin (T-cad; **A–D**) or VE-cadherin (**E**, **F**). Nuclei were counterstained with Hoechst. **G** Non-immune control for anti-T-cad antibody. **H** Non-immune control for anti-VE-cadherin antibody. *Bar length* 20 $\mu$m

distribution of T-cad observed in migrating cells might be related to its interaction with caveolin. To address this issue we performed double immunostaining for caveolin and T-cad in migrating SMC and HUVEC and analysed the samples by laser scanning confocal microscopy. In total, 20 different images for each cell type were analysed, of which 3 are presented in Fig. 3 as being typically representative of cells either migrating already some distance away from the wound edge (Fig. 3 *panel sets I and III*) or cells in the process of migrating out of the wound edge (Fig. 3 *panel set II*). Each image was scanned and "dissected" into stacks of 20×0.5 $\mu$m horizontal slices. Figure 3A–D shows the slice focused for maximal T-cad staining at the leading edge, while Fig. 3E–H shows another slice focused for maximal caveolin staining. The double-channel (Fig. 3A, E) and single-channel (Fig. 3B, C, F, G) images demonstrate that while T-cad (red staining) can be detected at the leading

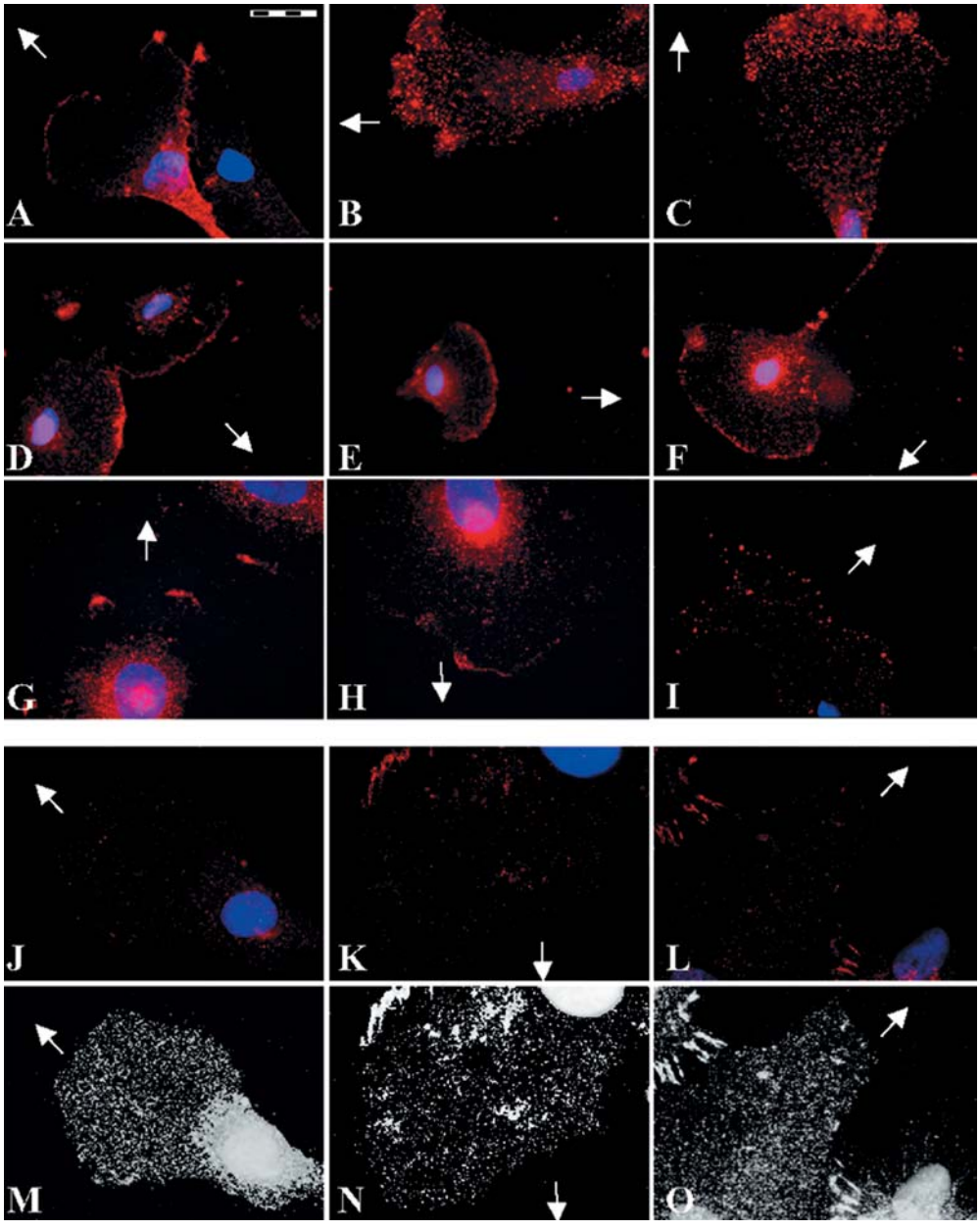
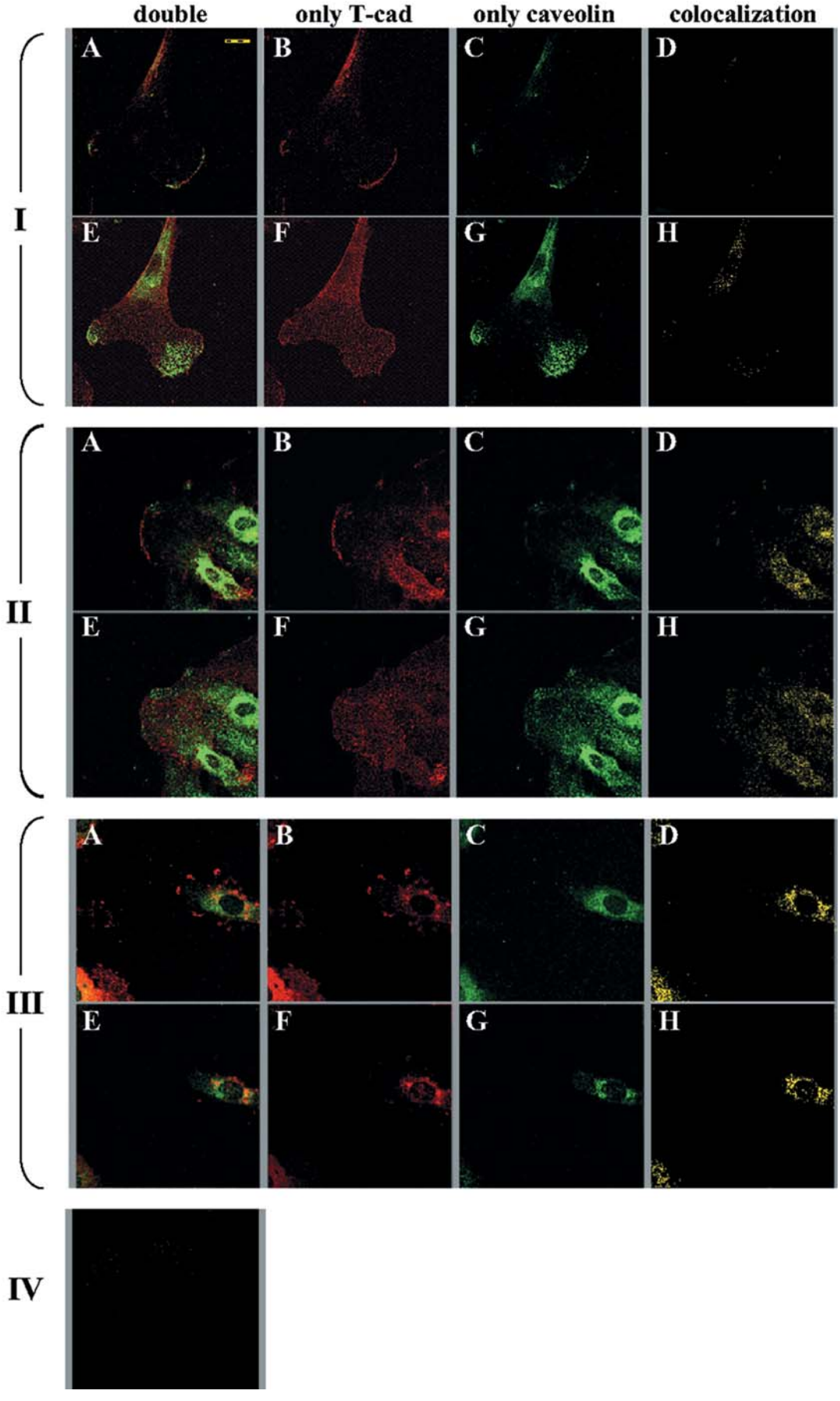



**Fig. 2A–O** Cadherin localisation in migrating cells. Wounded cultures of hSMC (**A–C**), ECV-304 (**D–F**) and HUVEC (**G–L**) were stained with antibodies against T-cad (**A–I**) or VE-cadherin (**J–L**). Nuclei were counterstained with Hoechst. *Arrows* show the direction of cell migration. **M–O** Contrasted black-and-white versions of images **J–L**, respectively. *Bar length* 20 $\mu$m

edge of moving cells, it is not always the case for caveolin (green staining). In migrating SMC caveolin is found either at the frontal cell periphery (Fig. 3 *panel set I*) or in the perinuclear area (Fig. 3 *panel set II*), while in most migrating HUVEC it can be detected only around the nucleus and not at the leading edge.

Stringent colocalisation analysis (yellow colour) revealed that caveolin and T-cad were not precisely colocalised at the leading edge. Firstly, maximal edge stainings for either of the proteins were always detected at different depths (i.e. within different optical slices). Secondly, image analysis with colocalisation software consistently revealed a very poor degree of colocalisation at the leading edge. In the colocalisation channel there was an emergence of single occasional pixels that did not form a consistent pattern; this random pixelation is an

**Fig. 3A–H** Localisation of T-cad and caveolin in migrating vascular cells: double immunostaining. Wounded cultures of hSMC (*panel sets I, II*) and HUVEC (*panel set III*) were stained with antibodies against T-cad (red staining) and caveolin (green staining). Samples were analysed using confocal microscopy. Typical images of three different cells are shown in *panel sets I–III*. In each set **A–D** show the optical slice focused on maximal T-cad staining at the leading edge, and **E–H** another slice focused on maximal caveolin staining. **A**, **E** Double-channel fluorescence for T-cad and caveolin. **B**, **G** Single-channel fluorescence for T-cad. **C**, **F** Single-channel fluorescence for caveolin. **D**, **H** Colocalisation pattern alone. *Panel set IV* Double-stained non-immune control. *Bar length* 20 $\mu$m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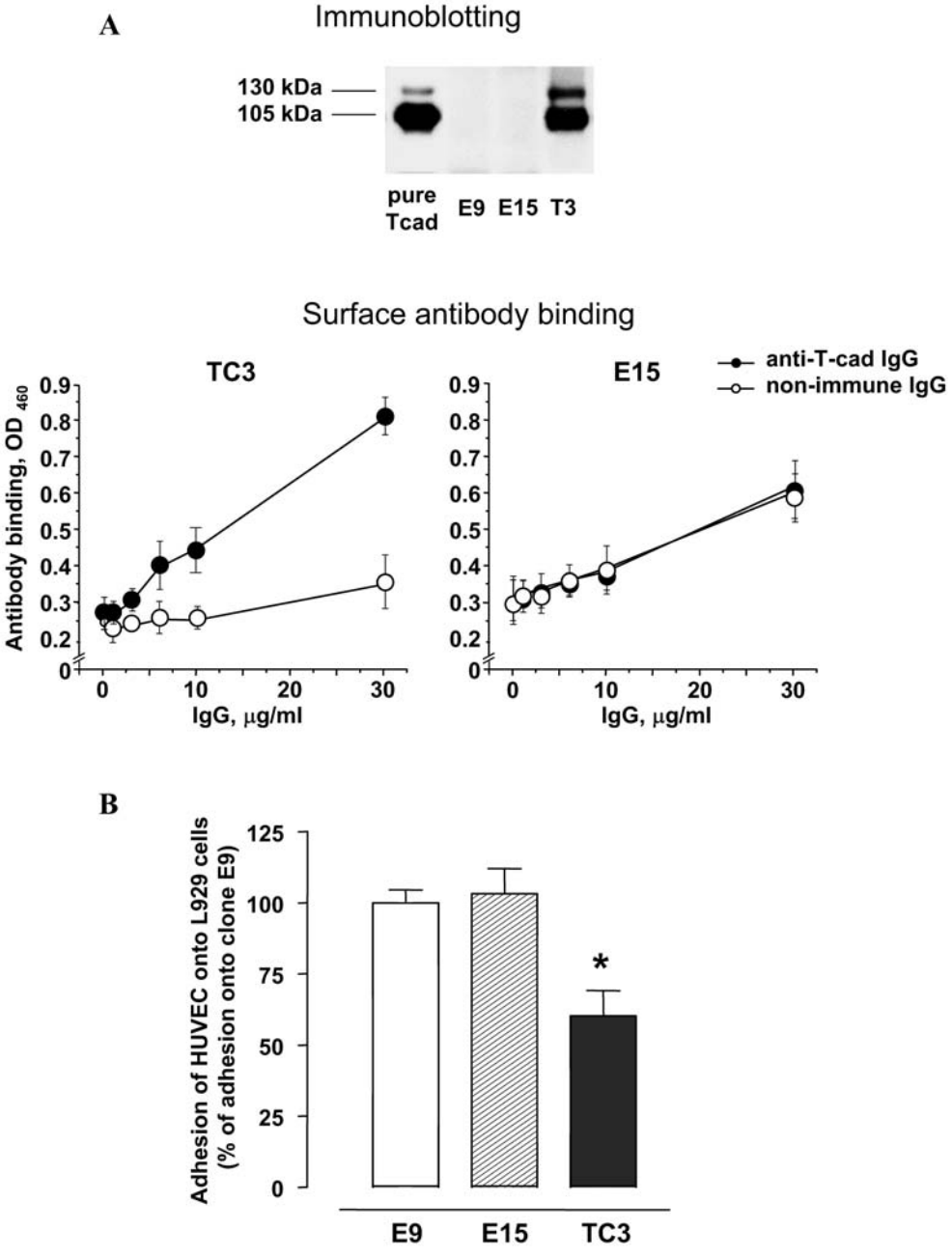


**Fig. 4A, B** Adhesion of HUVEC onto monolayers of L929 cells transfected with T-cad cDNA. T-cad expression in empty vector-transfected (E9 and E15) and human T-cad-transfected (T3) L929 clones was proven by immunoblotting and surface antibody binding (**A**). Partially purified T-cad (*pure Tcad*) from human aorta was used as positive control in immunoblotting. Adhesion of HUVEC onto confluent monolayers of E9, E15 and T3 clones (**B**) was determined. Adhesion is expressed relative to that (arbitrarily taken as 100%) onto E9. Data are given as mean ± SD from three independent experiments, and the *asterisk* indicates adhesion onto T-cad-transfected cells is significantly ($P<0.01$) less than adhesion onto controls

indication of random overlapping of colours but not of true colocalisation (see Fig. 3D, H). Thirdly, even this weak colocalisation pattern never coincided with patterns of maximal edge staining for T-cad or caveolin (compare Fig. 3D, H with single-channel staining for each of the proteins). In contrast, a significant degree of T-cad and caveolin colocalisation was frequently observed in the more "central" part of the cell body, especially in the perinuclear area (see for example, Fig. 3D, H *panel sets II and III*).

Negative adhesive functions for T-cadherin

We also addressed whether elevation of surface T-cad expression within a cell monolayer might affect the ability of other cells to adhere to that monolayer. HUVEC were seeded onto monolayers of L929 cells (which do not express cadherin molecules (Chen and Obrink 1991; Van Itallie and Anderson 1997; American Type Culture Collection) transfected with human T-cad cDNA or control vectors (see Fig. 4A for proof of expression and appropriate surface location). Adhesion of HUVEC to monolayers was assessed by ELIRA targeting endothelial specific von Willebrand factor. We found that adhesion of HUVEC onto T-cad-transfected L929 was reduced to

about 50% of adhesion onto control (empty vector-transfected) L929 (Fig. 4B).

## Discussion

In this study the cellular localisation of atypical GPI-anchored T-cad in confluent and migrating human endothelial and smooth muscle cells is demonstrated for the first time, and we provide evidence that T-cad functions in cellular deadhesion processes rather than intercellular adhesion.

A first important observation was that expression patterns for T-cad and classical VE-cadherin in confluent monolayers of HUVEC were very different. VE-cadherin, a specific endothelial protein responsible for formation of intercellular contacts and control of vascular permeability (Gory-Faure et al. 1999; Corada et al. 2001), was found, as expected, at cell–cell junctions, while T-cad was distributed globally, with only a slight enrichment at cell borders. Similar global distribution patterns for T-cad were found in confluent monolayers of SMC and also carcinoma cell line ECV-304. These data imply that T-cad is not required for maintenance of intercellular adhesion.

A second striking observation was that T-cad redistributes to the leading edge of migrating endothelial and smooth muscle cells as well as ECV-304, thus invoking a potentially important role for T-cad in directed locomotion of cells. Polarisation of the cell body into the protruding leading lamella and the retracting rear part during directed migration is accompanied by asymmetric distribution of specialised molecules between these compartments. The leading edge contains chemokine receptors and other signalling molecules that sense the environment and induce localised actin polymerisation, which is the basis of lamellopodia motility (Condliffe and Hawkins 2000). Redistribution of T-cad to the leading edge indicates some "recognition" function that might be important for regulation of cell migration. These data imply an interesting analogy with the embryonic nervous system: in extending motor axons T-cad is localised at the growth cones and acts as a repulsive navigation cue inhibiting axon protrusion in the direction where contact with another T-cad molecule on neighbouring cell was established (Fredette and Ranscht 1994; Fredette et al. 1996). A possible function for T-cad as a repulsive cue is supported by our observation that adhesion of HUVEC onto a monolayer of T-cad-transfected L929 cells is significantly less than adhesion onto control (T-cad-negative) L929 cells. It is tempting to speculate that in migrating vascular cells, T-cad, being a signalling and sensing receptor, can also participate in recognition of environmental cues and thereby influence cell behaviour. The ability of atypical GPI-anchored T-cad to mediate contact-dependent navigation of cell movements (Fredette and Ranscht 1994; Fredette et al. 1996) might be important for regulation of endothelial and smooth muscle cell migration, and thereby vascular remodelling. Moreover, since cellular localisation of T-cad in confluent and migrating cultures of ECV-304 carcinoma cells is the same as vascular cells, contact-dependent navigation by T-cad may represent a more general phenomenon in tissue remodelling.

Thirdly, we show that caveolin is not directly involved in T-cad-mediated signalling at the leading edge. GPI-proteins, including T-cad (Philippova et al. 1998), and other lipid-anchored membrane molecules are usually associated with cholesterol- and glycosphingolipid-enriched raft membrane domains (Harder and Simons 1997). Several raft-associated proteins have been shown to accumulate preferentially at the leading lamella (Okada et al. 1995), while raft disruption in cholesterol-depleted cells inhibited cell polarisation and chemotaxis (Manes et al. 1999). Hence, lipid rafts have been proposed to serve as dynamic platforms for the selective delivery of membrane proteins important for cell migration to the leading edge (Gomez-Mouton et al. 2001). We have previously suggested that putative signalling functions of T-cad might be due to its direct physical interaction with caveolar- and raft-associated signalling machinery (Philippova et al. 1998). In examining this possibility we do confirm a previous report of enrichment of caveolin at the leading edge of migrating smooth muscle cells (Okada et al. 1995), but not in endothelial cells. Confocal analysis of migrating smooth muscle and endothelial cells revealed colocalisation in the perinuclear area but insignificant colocalisation of caveolin and T-cad at the leading lamella. These data indicate that caveolin is not involved in T-cad-mediated signalling at the leading edge.

Taken together, our data on T-cad expression patterns in vascular cells suggest that the ability of atypical GPI-anchored T-cad to mediate contact-dependent navigation of cell movements might be important for regulation of endothelial and smooth muscle cell migration. This supports the hypothesis on involvement of this molecule in pathological vascular tissue remodelling associated with abnormal vascular cell motility.

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