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In vitro tubulogenesis of endothelial cells by relaxation of the coupling extracellular matrix-cytoskeleton

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Abstract

Objective: This investigation aimed at determining the importance of the rigidity of the adhesive support and the participation of the cytoskeleton in tubulogenesis of endothelial cells in vitro.

Methods: The morphotype, biosynthetic phenotype and cytoskeleton organization of human umbilical vein endothelial cells (HUVEC) were analyzed on supports of variable mechanical resistance.

Results: Western blot analysis revealed a strong reduction of the expression of actin and focal-adhesion plaque (FAP) proteins in HUVEC organized in tube-like structures (TLS) on soft matrigel or on matrigel co-polymerized with heat-denatured collagen as compared to HUVEC remaining in a monolayer pattern on rigid matrigel-coat or on matrigel co-polymerized with type I collagen. Human skin fibroblasts morphotype was not altered in these culture conditions and the pattern of FAP proteins and actin was not modulated. By using polyacrylamide gels polymerized with various concentrations of bis-acrylamide to modulate the mechanical resistance of the support and cross-linked to a constant amount of gelatin to provide an equal density of attachment sites, it was shown that the less rigid the support, the more endothelial cells switched to a tube-like pattern. Collagen type I-induced tubulogenesis was accompanied by a profound and reversible remodeling of the actin-FAP complex suggesting a weakening of the bridging between extracellular matrix (ECM) and the cytoskeleton. Human skin fibroblasts and smooth muscle cells, used as control cells, adhered strongly to the collagen, did not form TLS and their network of actin stress fibers was not remodeled. The inhibition of collagen type I-induced tubulogenesis by agents altering the actin cytoskeleton-FAP complex including calpain type I inhibitor, orthovanadate, KT5720 and jasplakinolide, further supports the determinant role of mechanical coupling between the cells and the matrix in tubulogenesis.

Conclusions: A reduced tension between the endothelial cells and the extracellular matrix, originating in the support or within the cells is sufficient to trigger an intracellular signaling cascade leading to tubulogenesis, an event mimicking one of the last steps of angiogenesis.

Keywords : Angiogenesis ; Extracellular matrix

1. Introduction

Angiogenesis is a tightly regulated physiological process required in development, growth, uterine physiology, embryonic implantation and healing. Persistent angiogenesis participates in various pathological conditions such as diabetic retinopathy, rheumatoid arthritis and psoriasis, and plays a determinant role in malignant progression and metastatic dissemination. It is also involved in the development of pathologies of the large blood vessels and mainly of the aneurysm of the abdominal aorta [1,2]. The induction of neovascularization and collateral vessels growth by angiogenic factors is a promising therapy for the ischemic myocardium and heart failure [3,4]. The formation of new blood vessels is a multistep process in which activated endothelial cells of existing vessels degrade the underlying basement membrane, migrate and proliferate in the perivascular stroma to form capillary sprouts. These sprouting endothelial cells stop proliferating, align, form tubes with patent lumen and deposit a basement membrane to finally yield operational new blood vessels. Several in vitro models have been designed to investigate the mechanisms underlying angiogenesis using various types of extracellular matrix (ECM) supports and/or growth factors or chemicals interfering with the cellular signaling pathways [5-9]. Most of the reports investigate the early events of angiogenesis [10]. In the models of tubulogenesis in vitro the alignment of the endothelial cells and generation of a patent lumen were induced by interactions between the endothelial cells

and a variety of substrates such as collagen type I, fibronectin, fibrin, collagen type IV laminin or matrigel. This suggests that several subtypes of integrins recognizing these ECM proteins are able to perform a similar function and that another type of signal arising from the support is required for the induction of the tube-like morphotype.

It has been further reported that tube formation is promoted when endothelial cell adhesiveness is reduced by blocking integrins with antibodies [8] or by reducing ligand concentration below a threshold level [11]. Similarly, the tubulogenesis induced by matrigel, that polymerizes as a soft gel, is inhibited by increasing its rigidity by co-polymerization with collagen type I [12]. These observations could be explained by the modification of mechanical signaling arising in the extracellular attachment sites connected to the cytoskeleton through focal adhesion plaques (FAP). We have previously reported that collagen-induced EC differentiation is accompanied by large modifications in expression and structural organization of various FAP-associated proteins as well as actin, strongly supporting the hypothesis that decreasing the mechanochemical coupling between the ECM and the cytoskeleton induces differentiation [13]. However, it is still unclear whether this release of mechanical coupling is sufficient to promote in vitro tube formation.

The aim of the present study was to analyze the involvement of tension forces exerted by the cytoskeleton of EC through FAP on the extracellular attachment sites on the in vitro tubulogenesis. The present results indicate that a decrease of these forces, resulting either from a reduction of the mechanical resistance of the support or from an intracellular mechanism induces tubulogenesis of the endothelial cells in vitro.

2. Methods

2.1. Cell cultures

Human umbilical vein endothelial cells (HUVEC) were isolated according to Jaffe et al. [14] as previously described [13]. The procedure received the agreement of the institutional ethics committee. The cells were cultured on gelatin-coated tissue culture dishes in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 5% heat-decomplemented pooled human serum, 5% decomplemented fetal calf serum (FCS) (Gibco), 20 μg/ml endothelial cell growth factor (ECGF) (Boehringer, Mannheim, Germany), 50 μg/ml heparin, 10 mM HEPES, penicillin (100 U/ml) and streptomycin (100 μg/ml) and used at passage 1 or 2. The endothelial origin of the cells was assessed by their typical cobblestone-like morphology and by positive staining for FVIII-related antigen (FVIII-r.a.). ECGF and heparin supplementation used to initiate the culture was omitted to perform the experiments. Jasplakinolide (J-7473, Molecular Probes, Leiden, The Netherlands), calpain inhibitor I (208719, Calbiochem, San Diego, CA), KT-5720 (420330, Calbiochem) and orthovanadate (159664, ICN, Costa Mesa, CA) were added to the culture medium after attachment and spreading of the cells.

Fibroblasts were isolated by expiant outgrowth from normal human dermis, amplified in DMEM supplemented with 10% FCS (Gibco) and used between passage 6 and 13. Vascular smooth muscle cells were isolated and amplified as previously described [15].

2.2. Cell supports

A collagen gel of 0.4-mm thickness was made by polymerizing a solution of purified bovine skin type I collagen (1 mg/ml) in RPMI 1640 medium [4] for 10 min at 37°C. A solution of gelatin was obtained by heat denaturation (60°C for 10 min) of a bovine type I collagen solution (1 mg/ml). A 0.4-mm thick gel of matrigel was obtained by polymerizing for 15 min at 37°C a matrigel solution at 11 mg/ml, alone or supplemented by native or heat-denatured collagen solution at 1 mg/ml. In some experiments, dishes were coated with matrigel (2 μg/ cm^2).

2.3. Culture on acrylamide gels of different rigidity

Gelatin-coated polyacrylamide gels of variable flexibility were prepared as described by Pelham and Wang [16]. Briefly, glass coverslips (diameter, 22 mm) were flamed in a Bunsen burner, covered with 200 μl of 0.1 N NaOH and air dried. Then 200 μl of 3-aminopropyltrimethoxysilane was spread on the treated face of the coverslips. After 5 min, the coverslips were washed with distilled water and immersed in 0.5% glutaraldehyde (Merck, Darmstadt, Germany) in PBS for 30 min. They were then extensively washed with distilled water and air dried, placed in wells of 12-well plate with the activated face up, and immersed in 300 μl of a mixture containing 10% acrylamide (Pierce) and a concentration of bis-acrylamide (Pierce) ranging from 0.06 to 0.25%. After polymerization, the gels were rinsed three times with 200 mM HEPES (H-7523, Sigma), pH 8.5. The buffer was then drained off the surface of the gels and 300 μl of 2 mM sulfosuccinimidyl 6 (4'-azido-2'-nitrophenyl-amino) hexanoate (Sulfo-SANPAH, Pierce) in 200 mM HEPES, pH 8.5, deposited on the surface of the gel and exposed

to UV for 5 min. The sulfo-SANPAH solution was removed and the gels further exposed to UV for 5 min. After photoactivation, the polyacrylamide gels were washed three times in 200 mM HEPES, pH 8.5. A 0.2-mg/ml solution of heat-denatured (60°C, 10 min) bovine skin type I collagen was layered on the gels and allowed to cross-link to the activated acrylamide overnight at 4°C. Finally, the gels were equilibrated with several rinses in RPMI 1640 medium. In some experiments, heat-denatured [^{3}H]collagen prepared as previously described [17] was cross-linked to polyacrylamide gels to determine the amount of the protein bound on the surface of the various polyacrylamide gels. The efficiency of the photochemical cross-linking of the gelatin was not significantly altered by varying the bis-acrylamide concentration. First passage HUVEC were seeded on these supports at a density of $4X10^4$ cells/cm^2.

2.4. Cytoskeleton labeling

After fixation for 10 min in 3% formaldehyde in phosphate-buffered saline (PBS), pH 7.4, and washing in PBS, cells in monolayer or in tube-like structures (TLS) were permeabilized for 3 min with 0.2% Triton X-100 in PBS and incubated for 30 min with FITC-Phalloidin (Sigma P-5282, Deisenhofen, Germany) to label actin filaments. In some experiments, the FITC-Phalloidin solution was supplemented with 50 µg/ml of bis-benzimide H 33258 (382061, Calbiochem) to label the nuclei. Labeling of focal adhesion-associated vinculin was performed, after extraction of the cytosolic vinculin with Nonidet P-40 [13], using an anti-vinculin mAb (Sigma V-9131, 1:200) and Oregon Green-conjugated goat anti-mouse IgG (Molecular Probes) diluted at 1:20. Samples were examined with a Leitz epi-fluorescence microscope.

2.5. Western blot analysis

Cells cultured on a coat of matrigel or on matrigel co-polymerized with gelatin or native type I collagen were collected after digestion with 0.03% trypsin (Gibco BRL) and/or 0.1% bacterial collagenase (C-9891, Sigma) in RPMI 1640 medium, washed and suspended in PBS. An aliquot of the cell suspensions was used to measure the DNA content. The cell suspensions were lysed in RIPA buffer (sodium dodecyl sulphate (SDS) 1%, sodium deoxycholate 1%, Triton X-100 0.1%, ethylenediaminotetraacetic acid 10 mM in PBS). An amount of lysate equivalent to 0.1 µg DNA was electrophoresed under reducing conditions on a 6.25 or a 12.5% SDS-polyacrylamide gel according to the technique of Laemmli [18] and Western blotting performed as described by Towbin et al. [19] using monoclonal antibodies directed to actin (Sigma A-1804), α-actinin (Sigma A-5044), talin (Sigma T-3287), and vinculin (Sigma V-9131). The secondary antibody was a peroxydase-conjugated goat anti-mouse IgG (Dako P447, Copenhagen, Denmark). The anti-integrin α_2 subunit rabbit polyclonal antibody (AB1936) was from Chemicon (Temecula, USA), the anti-FVIII-r.a. rabbit polyclonal antibody (A082) from Dako and a rabbit polyclonal directed against the p42 MAPKinase (E1B4) was a kind gift of J. Pouysségur (CNRS, Unité Mixte de Recherche 6543, Nice, France). The secondary antibody was a peroxidase-conjugated goat anti-rabbit IgG from Sigma (A-6154). Peroxidase was revealed by using the Enhanced ChemoLuminescence assay (Amersham Pharmacia Biotech, Rainham, UK). The intensity of the signals was recorded by laser scanning densitometry (Ultroscan, LKB, Sweden). No qualitative nor quantitative alteration of the investigated proteins was caused by the enzymatic isolation procedure as previously tested [13].

3. Results

3.1. Tubulogenesis is controlled by the mechanical properties of the support

Seeded on a coat of matrigel, HUVEC proliferated to form a confluent monolayer while seeded on a gel of matrigel they organized in TLS within 24 h. Addition of gelatin to matrigel did not significantly modify the formation of TLS (Fig. 1A, a). In contrast, when the matrigel was supplemented and polymerized with type I collagen to form a mechanically more resistant gel, TLS formation did not occur even after 3 days of observation and HUVEC proliferated to form a monolayer (Fig. 1A, b). To complement these observations previously reported by Vernon et al. [12], the concentration of actin and focal adhesion-associated proteins was investigated by Western blot analysis in HUVEC on TLS-permissive and non-permissive supports (Fig. 1B). The FVIII-r.a. and the p42 MAPKinase (not illustrated) were used to control the cellular proteins loading. In monolayer after 24 h of culture on a matrigel-coat (Fig. 1B, lane 1), the signals for the integrin α_2 subunit, talin, vinculin, α-actinin and actin were clearly visible. A strong reduction of the concentration of these proteins for a similar amount of FVIII-r.a. was observed in HUVEC organized in TLS on matrigel mixed with heat-denatured collagen (Fig. 1B, lane 2). By contrast, when the same matrigel was co-polymerized with native type I collagen on which HUVEC multiply to form a monolayer, such a reduction was not observed (Fig. 1B, lane 3). These results show that alteration of the rigidity of the support but not the chemical signals issued from the matrix modulates the EC morphotype and the expression of the FAP-associated proteins.

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The phenotypical changes observed in HUVEC cultured on matrigel or on matrigel mixed with heat-denatured collagen did not occur in fibroblasts (Fig. 2) or smooth muscle cells (SMC, not illustrated). In these culture conditions, fibroblasts formed 'hills and valleys' but never acquired a more organized morphology (Fig. 2A) and the concentration of the focal adhesion-associated proteins did not change (Fig. 2B).

To further investigate the role played by the mechanical resistance of the substrate, gelatin was photochemically cross-linked to acrylamide gels polymerized with decreasing concentration of bis-acrylamide and used as supports of varying mechanical resistance but equal density of recognition sites by integrins as determined by using [^{3}H]gelatin as detailed in Methods. As observed by phase contrast microscopy, HUVEC cultured for 24 h on gels containing 0.25% bis-acrylamide (Fig. 3b) displayed a typical monolayer pattern similar to that of HUVEC cultured on gelatin-coated culture dish (Fig. 3 a). On a less resistant gel containing 0.125% bis-acrylamide, the morphology of the monolayer was already altered as the cells were less spread (Fig. 3c). Finally, on the most flexible gel containing 0.06% bis-acrylamide, HUVEC formed cordlike structures (see arrows) (Fig. 3d). These morphological observations demonstrate that the decrease of the mechanical resistance of the support is sufficient to switch EC from a monolayer to a differentiated TLS morphology.

Fig. 1. *(A) Phase contrast microscopy photographs of HUVEC cultured for 24 h on matrigel containing heat-denatured (a) or polymeric type I collagen (b). (B) Comparative Western blot analysis of lysates from HUVEC cultured on dishes coated with matrigel (2 μg/cm^2) (1), on matrigel mixed with heat-denatured collagen (2), or on matrigel co-polymerized with native collagen (3), using specific antibodies against integrin α_2 subunit (α_2), talin, vinculin, α-actinin, actin and FVIII-r.a. The experiment was performed using three different strains of EC with similar results. Bar=25 μm.*

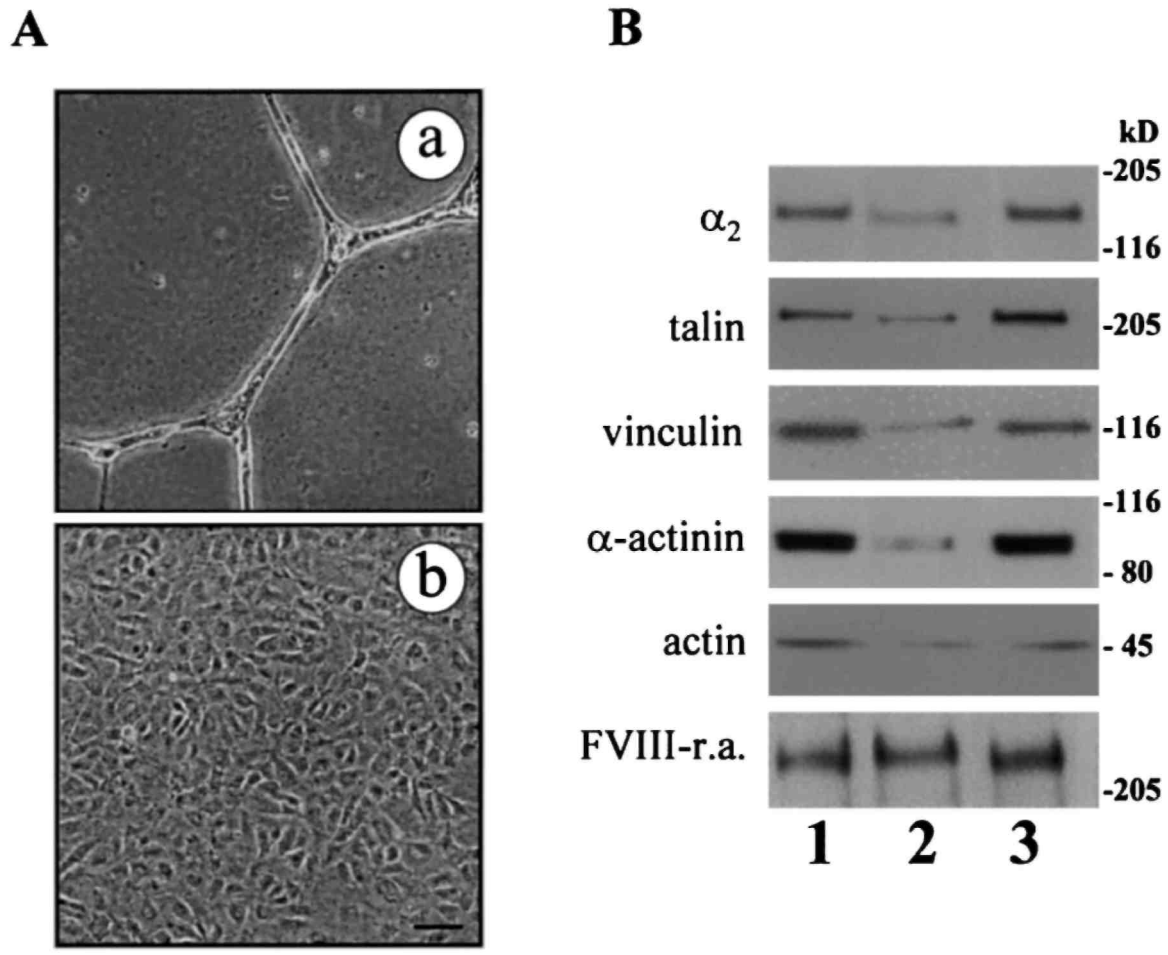

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Fig. 2. *(A) Phase contrast microscopy photographs of fibroblasts and (B) comparative Western blot analysis of lysates from fibroblasts cultured for 24 h on matrigel containing polymeric collagen (a, 1) or heat-denatured collagen (b, 2). The experiment was performed twice with similar results. Bar=25 μm.*

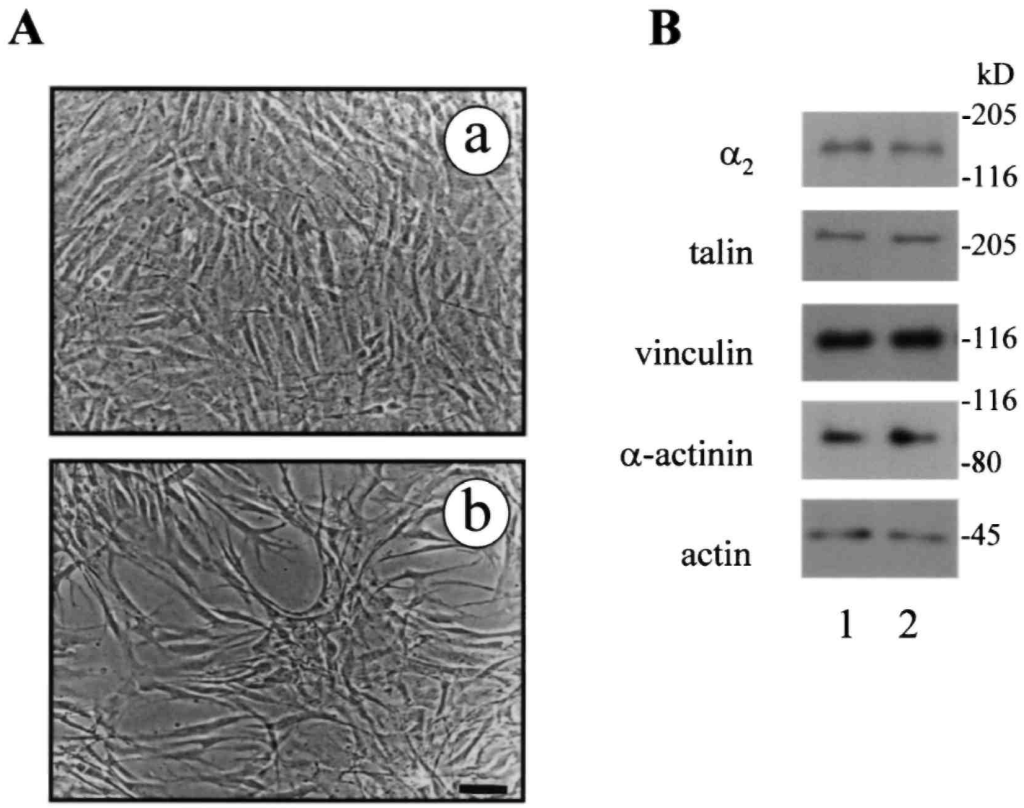

Fig. 3. *Phase contrast microscopy photographs of HUVEC cultured on a gelatin-coat (2 μg/cm^2) (a) or gelatin photochemically cross-linked polyacrylamide gels (b-d). The polyacrylamide gels contain 10% acrylamide polymerized with bis-acrylamide 0.25% (b), 0.125% (c) or 0.06% (d). The arrowheads point to the translucent lining of the cord-like structures suggestive of a tubular organization. Bar=20 μm.*

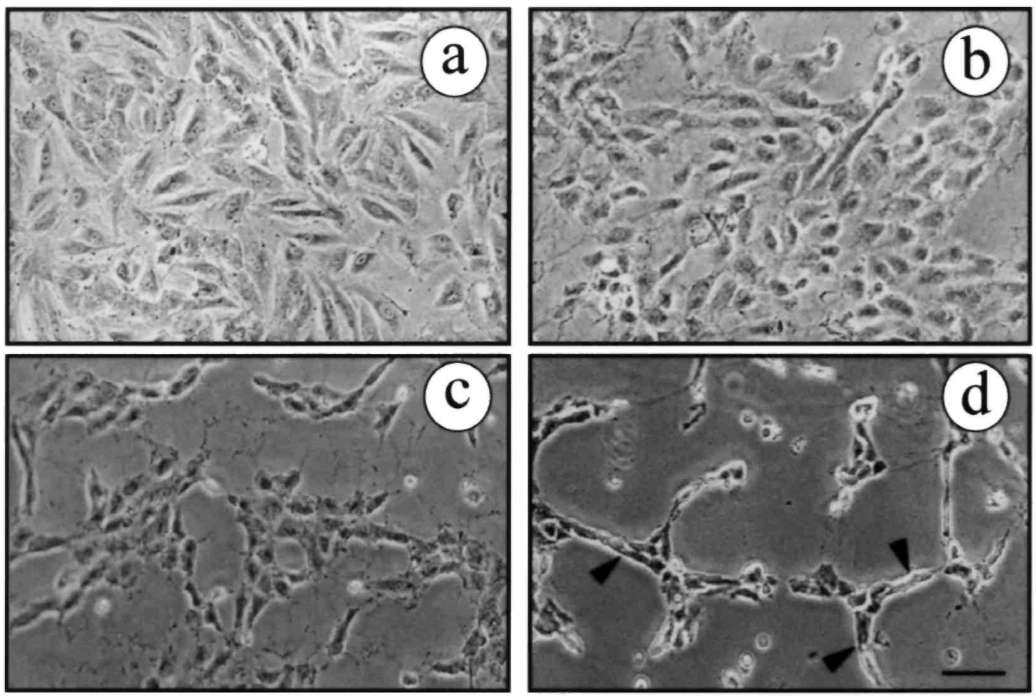

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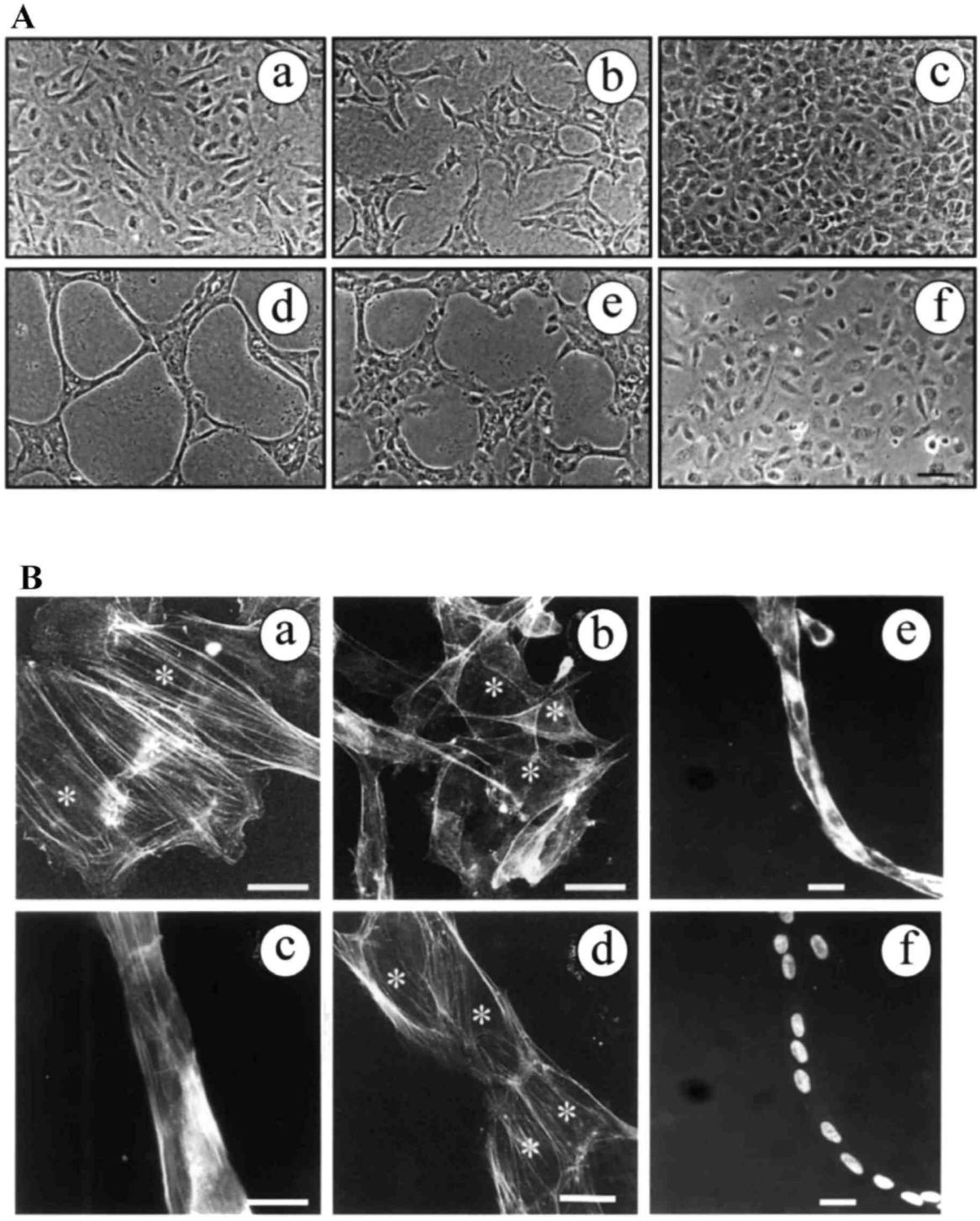
Fig. 4. *(A) Phase contrast microscopy photographs of HUVEC cultured for 5 h (a) or 48 h (c) on a collagen gel; HUVEC cultured for 5 h (b) or 48 h (d) between two collagen gels; HUVEC organized in TLS for 48 h and observed 5 h (e) or 16 h (f) after removal of the overlaying collagen gel. Bar=25 µm. (B) FITC-phalloidin labeled HUVEC cultured for 5 h on a collagen gel (a), for 5 h (b) or 48 h (c) between two collagen gels, or organized in TLS for 48 h and observed 5 h after removal of the overlaying collagen gel (d). Some individual cells are marked (*) in panels a, b, and d. Double labeling of HUVEC cultured for 48 h between two collagen gels with phalloidin-FITC (e) and bis-benzimide (f). Bars=5 µm.*



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3.2. Organization of the cytoskeleton during collagen-induced tubulogenesis and its reversal

HUVEC seeded on a collagen gel attached, spread and proliferated to form at confluence, a monolayer displaying a typical cobblestone pattern (Fig. 4A, a and c). By contrast, subconfluent HUVEC on the same support but covered with an overlaying collagen gel had already changed shape after 5 h of contact (Fig. 4A, b) and progressively formed a network of TLS after 48 h (Fig. 4A, d). A previous study indicated by histological transverse sections that endothelial cells delineated a lumen [13]. By removing the overlaying gel, the TLS morphotype changed after 5 h (Fig. 4A, c) and completely reversed to a monolayer pattern within 16 h (Fig. 4A, f). Staining of actin filaments with FITC-phalloidin revealed prominent stress fibers in HUVEC cultured in monolayer on the collagen gel (Fig. 4B, a). After 5 h of induction, a transient loss of stress fibers was observed (Fig. 4B, b). After 48 h, the TLS were fully organized and thin stress fibers were again present although under a different orientation parallel to the main axis of the TLS (Fig. 4B, c). Upon withdrawal of the collagen gel and reversion to the monolayer, the original pattern of the stress fibers progressively reappeared (Fig. 4B, d). A double labeling with phalloidin-FITC and bis-benzimide of HUVEC in TLS illustrates the alignment of the individual cells in the TLS (Fig. 4B, e and f).

Similar morphological and cytoskeletal reorganization did not occur in fibroblasts (Fig. 5) or SMC (not illustrated). The actin stress fibers seen by FITC-phalloidin labeling in uncovered monolayers of fibroblasts (Fig. 5, a and c) were preserved at least after 5 h of culture between two collagen layers (Fig. 5, b and d). After longer periods of culture, it was impossible to remove the overlaying collagen gel without completely disorganizing the culture as opposed to its easy lifting in HUVEC cultured in the same conditions. This suggested the formation of strong adhesive structures between the fibroblast or SMC and the two collagen gels as opposed to HUVEC which only adhered to the most rigid gel immobilized on the culture dish.

Fig. 5. *Phase contrast microscopy photograph of fibroblasts cultured for 48 h on a collagen gel (a) or between two collagen gels (b). FITC-phalloidin labeled fibroblasts cultured for 5 h on a collagen gel (c) or between two collagen gels (d). Black bar=20 µm, white bar=5 µm.*

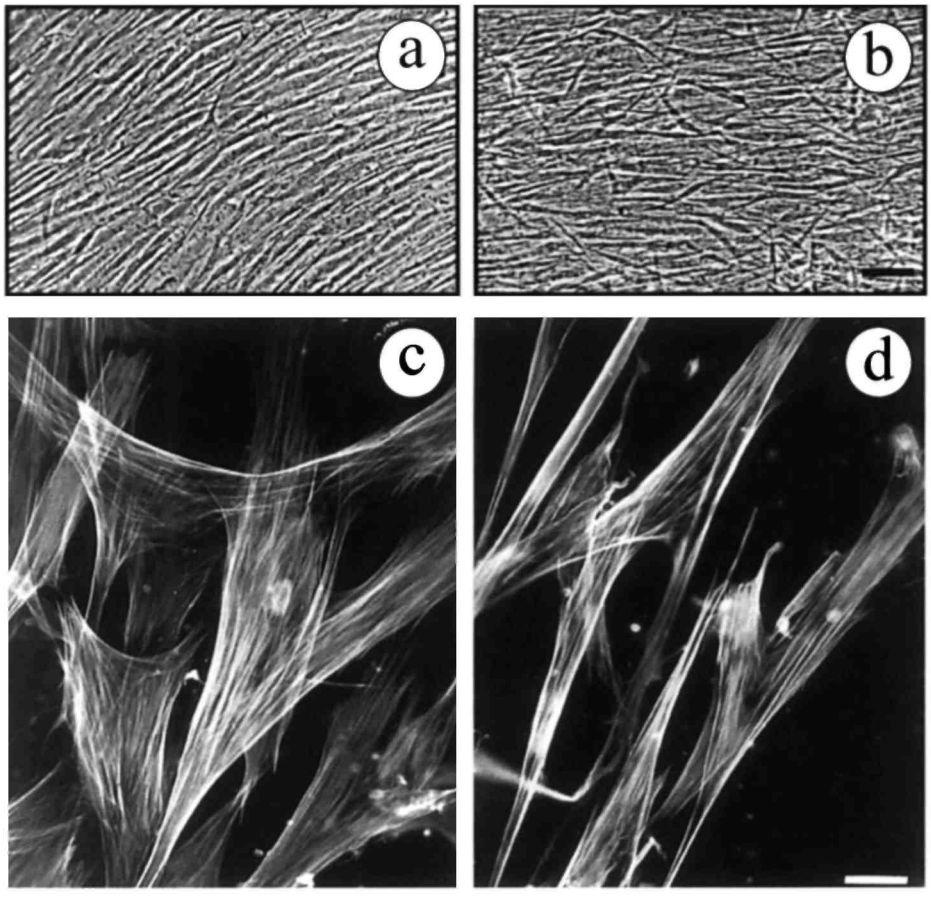
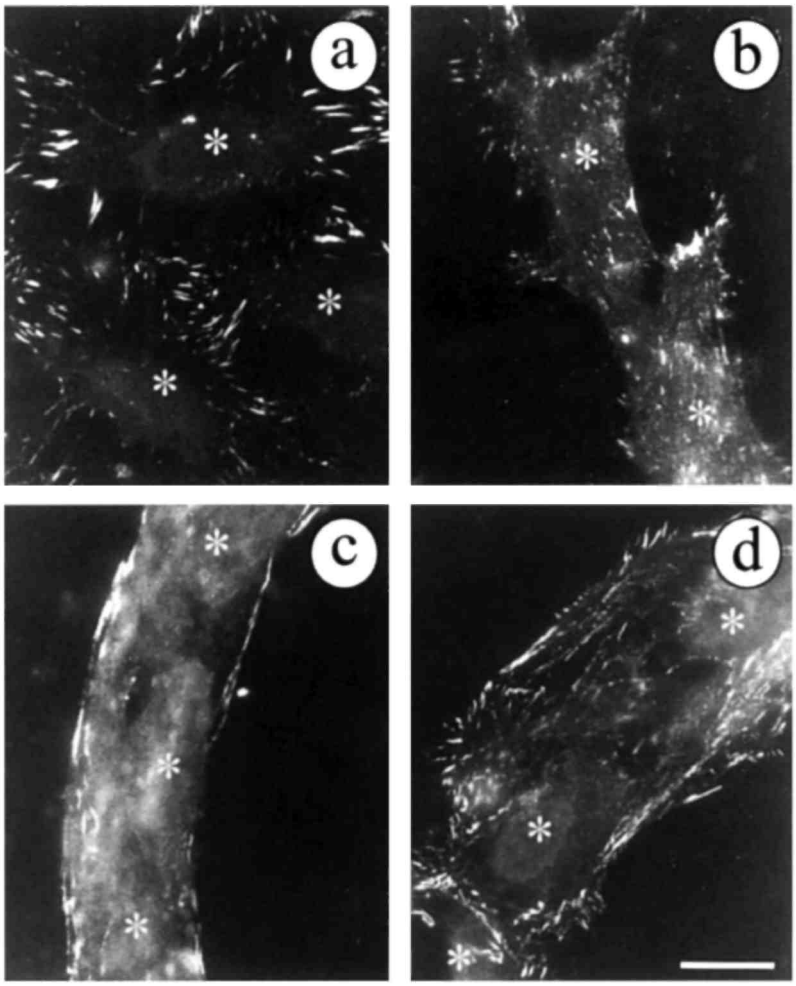


Fig. 6. *HUVEC cultured on a collagen gel for 5 h (a) or between two collagen gels for 5 h (b) or organized in TLS for 48 h (c) and 5 h after removal of the overlaying collagen gel (d) were fixed and permeabilized before labeling with anti-vinculin mAb. Some individual cells are marked (*). Bar=5 μm.*



3.3. Organization of the focal adhesion plaque during collagen-induced tubulogenesis

The morphological organization of the focal adhesion plaque (FAP) in HUVEC was examined by immunostaining of immobilized vinculin after detergent extraction of the cytosolic fraction. In monolayer on a collagen gel, the anti-vinculin mAb revealed a typical arrowhead staining pattern of the FAP mainly located at the periphery of the cells (Fig. 6a). The vinculin labeling in HUVEC cultured between two collagen gels was already modified after 5 h. A significant decrease of the peripheral arrowheads was observed (Fig. 6b) in parallel with the disappearance of actin stress fibers illustrated in Fig. 4B, b. After 48 h, in fully organized TLS, polymeric vinculin appeared as elongated patches parallel to the long axis of the tubes with an orientation similar to the actin filaments (Fig. 6c). Upon removal of the overlaying collagen gel and progressive reversion to a monolayer pattern, the vinculin arrowheads reappeared at the periphery of individualized cells (Fig. 6d). These observations support the existence of a dynamic reorganization of the actin microfilaments and the FAP during TLS formation suggesting that tension forces exerted on the cytoskeleton are reduced by detachment of the extracellular attachment sites and inversely during reversion to a monolayer pattern.

3.4. Interfering with cytoskeleton or FAP reorganization alters the collagen-induced tubulogenesis

Release of adhesion sites between the cells and their support is likely dependent on a biochemical mechanism acting intracellularly [20]. In migrating cells, it involves the cleavage of components of the focal adhesions that can be inhibited by calpain inhibitor I. This drug was used in our model of tubulogenesis to suppress the release of adhesion sites in HUVEC undergoing TLS. HUVEC cultured between two collagen gels during 48 h in

presence of 20 μg/ml of daily renewed calpain inhibitor I failed to achieve a TLS organization (Fig. 7). As compared to control conditions, the cells were markedly less organized and more dispersed (compare Fig. 7a and 7b) and an appreciable number of them migrated within the collagen gels. Moreover, FITC-phalloidin labeling revealed the persistence of actin stress fibers, crossing the cells, that had disappeared in the control cultures (compare Fig. 7c and 7d). Simultaneous labeling of the nuclei with bis-benzimide also illustrates the defective TLS organization of the cells by the inhibitor. To further investigate the need for cytoskeleton disassembly during tubulogenesis, HUVEC were treated with agents known to stabilize the actin stress fibers, KT5720 and vanadate, inhibitors of, respectively, a cAMP-dependent protein kinase and a protein tyrosine phosphatase [21]. KT5720 (10 μM) (Fig. 8b) and vanadate (40 μM) (Fig. 8c) also interfered with TLS formation. The monolayer was dissociated to some extent but the islands of cells did not further organize to display the final TLS morphotype as seen in the control cells (Fig. 8a). Finally, jasplakinolide (250 ng/ml), a membrane-permeable inhibitor of actin depolymerization [22,23], non-toxic at this concentration in monolayer, also prevented the formation of TLS and the HUVEC remained assembled in small aggregates (Fig. 8d). Altogether, these results support the requirement of a remodeling of the cytoskeleton-ECM coupling as an event required for in vitro tubulogenesis.

Fig. 7. *Phase contrast microscopy photographs (a and b), phalloidin-FITC labeling (c and d) or bis-benzimide labeling (e and f) of HUVEC cultured between two collagen gels during 48 h in absence (a, c and e) or in presence (b, d and f) of 20 μg/ml of calpain type I inhibitor. In b, unfocused HUVEC migrating inside the overlaying collagen gel are marked with arrowheads. Bars = 10 μm.*

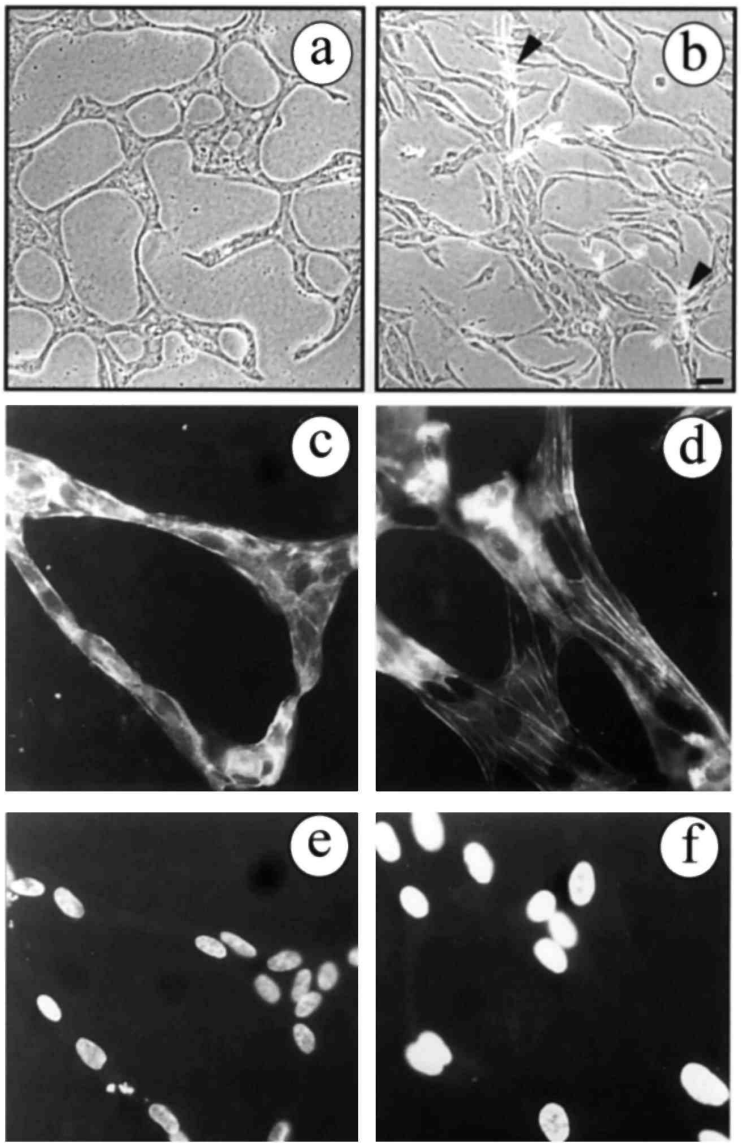
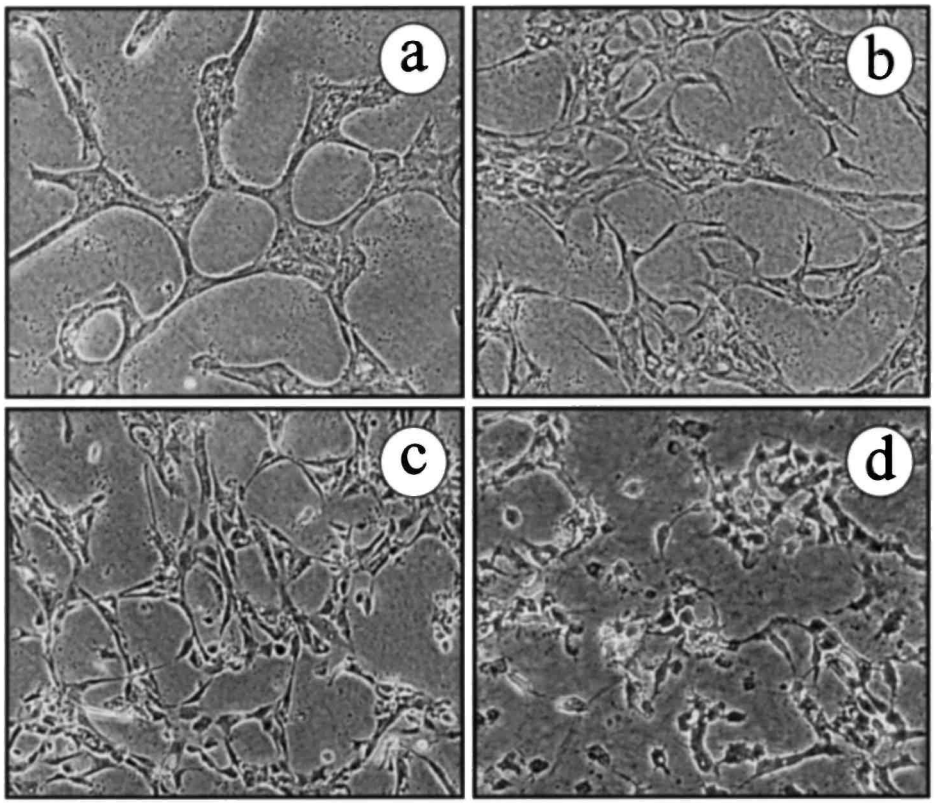


Fig. 8. *Phase contrast microscopy photographs of HUVEC cultured during 48 h between two collagen gels in control conditions (a), with 10 µM KT5720 (b), 40 µM vanadate (c), or 250 nM jasplakinolide (d). Bar=20 µm.*



4. Discussion

Angiogenesis requires a highly coordinated series of events that can be divided into three phases: initiation, proliferation/invasion and maturation [24]. The mechanisms controlling the early steps of angiogenesis have been extensively investigated while fewer studies have focused on the final phase of angiogenesis. The early events of angiogenesis can be studied by using in vitro models reproducing the invasion and sprouting of endothelial cells into three dimensional matrix made of gelled collagen or fibrin as in the monolayer invasion model of Montesano and Orci [6] or the aortic ring model of Nicosia and Ottinetti [25]. The models used in this study reproduce further events of the morphogenetic process, i.e. the intercellular alignment of the cells and generation of a patent lumen, as observed at the end of angiogenesis when endothelial cells shift to a more quiescent phenotype [7]. In the first model described in this study, the organization of HUVEC into TLS was induced by using a soft support, matrigel or matrigel supplemented with gelatin. We investigated the components of focal adhesion plaques (FAP), structures formed by a complex of structural and signaling proteins [26] that assemble at sites of attachment of the cell to the ECM. These structures bridging the ECM to the cytoskeleton through the integrins [27], transfer the mechanochemical information from the ECM to the inside of the cell and, reversely, the mechanical tension issued from the cytoskeleton to the ECM [28]. We showed here that HUVEC organized in TLS on such soft substrates displayed a decreased expression of the α_2 subunit of the integrin $\alpha_2\beta_1$, the main laminin and collagen receptor of HUVEC. Expression of talin and vinculin, two proteins actively involved in the coupling between integrins and the cytoskeleton, and that of actin and α-actinin, two main cytoskeletal components, were also reduced. Similar modifications were reported by us in the overlaid collagen gel-induced tubulogenesis model [13]. By increasing the stiffness of the support upon addition to matrigel of native collagen instead of heat-denatured collagen (gelatin), the remodeling of the FAP and cytoskeleton components did not occur and TLS did not form, although the number of potential attachment sites did not change. These experiments suggest that the mechanical properties of the support are the main determinants in TLS formation confirming the results of Vernon et al. [12] and the data of Vailhé et al. [29] who used fibrin gels at increasing concentrations to enhance the stiffness. Although substrate flexibility was reported to regulate the shape and formation of focal adhesions in epithelial and fibroblastic cell lines [16], fibroblasts and smooth muscle cells did not respond to the stimulus inducing TLS in HUVEC. This observation, in apparent

contradiction to reports of other investigators [12], could be due to the low thickness of the gel of matrigel used here displaying sufficient rigidity to allow a limited spreading of fibroblasts but not that of HUVEC. The role played by the mechanical rigidity of the substrate was more precisely investigated by using gelatin cross-linked to polyacrylamide gels as supports of varying mechanical resistance but similar density of recognition sites by integrins according to the technique of Pelham and Wang [16]. The culture of HUVEC in this model clearly demonstrated that the decrease of substrate rigidity is sufficient to induce the switch from a monolayer to a cord-like pattern resembling the TLS.

Tubulogenesis can be induced by culturing HUVEC between two layers of collagen gel. In this model, HUVEC adher by one face to a rigid support and the contact of the other face with the overlaying soft collagen gel is sufficient to induce the switch from a monolayer to a TLS pattern. The reversion of the process by withdrawing the overlaying gel further points to the significant role of the ECM molecules in the induction and maintenance of TLS. In this model, all the cells are engaged in TLS and the covering gel is easily removed without detaching cells. These features allowed us to investigate the expression and organization of the proteins participating in the ECM-cytoskeleton coupling. Vinculin is a structural protein found both in the FAP and in the intercellular adherens junctions [30] that plays a central role in actin stress fibers formation and in cell shape changes [31]. The early modulation of vinculin and the structural disorganization of the FAP reported here during the monolayer to TLS transition, suggest that the loss of mechanical coupling between the ECM and the cytoskeleton leads to the disappearance of the actin stress fibers. A similar disassembly is observed in floating 3D-collagen gels retracted by fibroblasts [32] and is accompanied by a profound alteration of their biosynthetic phenotype [17]. Actin depolymerisation is required in the initial phase of tubulogenesis as its inhibition by jasplakinolide blocked TLS formation. Interfering with the proteolytic release of cytoskeletal linkages by the type I calpain inhibitor also significantly delayed both the formation of TLS and the disappearance of stress fibers. Moreover, an appreciable number of HUVEC migrated inside the collagen layer, a behavior implicated in the early steps of angiogenesis [7]. Stress fiber disassembly was also impaired by treating HUVEC with two additional drugs, the protein kinase A (PKA) inhibitor, KT5720, and the protein tyrosine phosphatase inhibitor, vanadate. All four drugs interfering with the depolymerization of actin fibers by different pathways inhibited tubulogenesis, further supporting the need for stress fibers disorganization and FAP disassembly in this process. Our observations are in agreement with previous reports demonstrating that calpain activity is involved in the differentiation of osteoblast [33] and adipocytes [34], and PKA activity is associated with terminal differentiation of astrocytes [35] and with blood vessel maturation [36]. The reduced adhesion of endothelial cells to the surrounding matrix required for tube formation is a potential inducer of apoptosis. This process is prevented in functional capillaries by the blood flow-induced shear stress inducing stress fibers and FAP formation [37]. The absence of this compensation might account for the regression of unperfused blood vessel.

In vivo the link between the first and later phases of angiogenesis might be matrix degradation. The activation of endothelial cells by cytokines and other mediators of inflammation alters the proteolytic balance by up-regulating proteolytic enzymes, particularly the MMPs, and down-regulating their physiological inhibitors (TIMPs) [38-41]. This mechanism participating in the invasive stage of the angiogenic cascade by a localized degradation of the perivascular extracellular matrix, provides the sprouting endothelial cell with a less rigid microenvironment favoring the final steps of the angiogenic cascade, tube formation and capillary maturation.

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