In Vitro Cell. Dev. Biol.—Animal 33:763–773, November–December 1997

1071-2690/97 $05.00 + 0.00

IN VITRO ANGIOGENESIS IS MODULATED BY THE MECHANICAL PROPERTIES OF FIBRIN GELS AND IS RELATED TO $\alpha_v\beta_3$ INTEGRIN LOCALIZATION

BRUNO VAILHÉ, XAVIER RONOT, PHILIPPE TRACQUI, YVES USSON, AND LÉONE TRANQUI[1]

Laboratoire de Bioénergétique Fondamentale et Appliquée, Université Joseph Fourier, B.P.53, 38041 Grenoble Cedex 9, France (B. V., L.T.); Laboratoire Dynamique de l'Organisation des Génomes, Institut Albert Bonniot, Université Joseph Fourier, 38706 La Tronche Cedex, France (X. R., Y. U.); Laboratoire TIMC, UMR CNRS 5525, Faculté de Médecine, Institut Albert Bonniot, Domaine de la Merci, 38700 La Tronche, France (P. T.); Laboratoire de Neurobiologie du Développement, E.P.H.E., 34095 Montpellier Cedex 05, France (X. R.)

(Received 18 September 1996; accepted 10 December 1996)

SUMMARY

This study deals with the role of the mechanical properties of matrices in *in vitro* angiogenesis. The ability of rigid fibrinogen matrices with fibrin gels to promote capillarylike structures was compared. The role of the mechanical properties of the fibrin gels was assessed by varying concentration of the fibrin gels. When the concentration of fibrin gels was decreased from 2 mg/ml to 0.5 mg/ml, the capillarylike network increased. On rigid fibrinogen matrices, capillarylike structures were not formed. The extent of the capillarylike network formed on fibrin gels having the lowest concentration depended on the number of cells seeded. The dynamic analysis of capillarylike network formation permitted a direct visualization of a progressive stretching of the 0.5 mg/ml fibrin gels. This stretching was not observed when fibrin concentration increases. This analysis shows that 10 h after seeding, a prearrangement of cells into ringlike structures was observed. These ringlike structures grew in size. Between 16 and 24 h after seeding, the capillarylike structures were formed at the junction of two ringlike structures. Analysis of the $\alpha_v\beta_3$ integrin localization demonstrates that cell adhesion to fibrinogen is mediated through the $\alpha_v\beta_3$ integrin localized into adhesion plaques. Conversely, cell adhesion to fibrin shows a diffuse and dot-contact distribution. We suggest that the balance of the stresses between the tractions exerted by the cells and the resistance of the fibrin gels triggers an angiogenic signal into the intracellular compartment. This signal could be associated with modification in the $\alpha_v\beta_3$ integrin distribution.

Key words: endothelial cell; neovascularization *in vitro*; fibrin; matrix mechanical properties; dynamic analysis; $\alpha_v\beta_3$ integrins.

INTRODUCTION

Angiogenesis is rare in the human adult's physiological processes; but in numerous pathological states, an unregulated neovascularization develops (15,16). During capillary formation, the degradation of the extracellular matrix (ECM) allows the dispersion of the endothelial cells, which also acquire the capacity to migrate toward pathological tissue and then to reassociate to form the lumen of new capillaries (17). *In vivo* as well as *in vitro* experiments emphasize the multifactorial aspect of this process. Angiogenic factors can be divided crudely into soluble and insoluble substances. Soluble factors include mainly cytokines and a large number of peptides (17,34,40). Insoluble factors that promote capillarylike structure (CLS) formation *in vitro* include naturally occurring extracellular matrix such as Matrigel, or purified matrix proteins such as laminin, fibrin, or type I, II, IV, and V collagens (21,29). Among the insoluble proteins having angiogenic properties, fibrin occupies a specific place since it can be considered as a provisional matrix involved in the early phases of CLS formation during wound healing (9,24). Although ECM plays a fundamental role in determining cell morphology, its function is far from clear because both its composition and its mechanical properties, referred to as malleability (27), appear to be crucial. It is well established that the mechanical properties of biogels are directly determined by the monomer concentration into the gel. Such effects include modifications of gel elasticity and viscosity, and thus are related to the resistance of the gel to environmental stresses (22,23). Numerous studies have dealt with the effects of the ECM composition on cellular shape and differentiation, but fewer consider its mechanical aspect. The angiogenic properties of fibrin gels have been described (4,13,18,36,38), but a precise correlation between variations in fibrin concentration and the extension of a capillarylike network has not been established. Indeed, when cells are seeded on a matrix, the biological signal can be initiated by forces resulting from the balance between forces exerted by the cells on the matrix and the mechanical resistance of the matrix (27,28). This variation in tension across the cells may cause changes in expression of genes and consequently induce differentiation (25,30,32,39,43). The forces will be transmitted by the integrins that are attached to the matrices (27,30,41). Endothelial cells are attached to fibrinogen and fibrin through the integrin $\alpha_v\beta_3$ (8,10) and it seems likely that this integrin contributes to the regulation of angiogenesis (6,19). These integrins are physically associated with cytoskeletal proteins and co-localize at sites called focal contacts (7). Much attention has been focused

[1]To whom correspondence should be addressed.

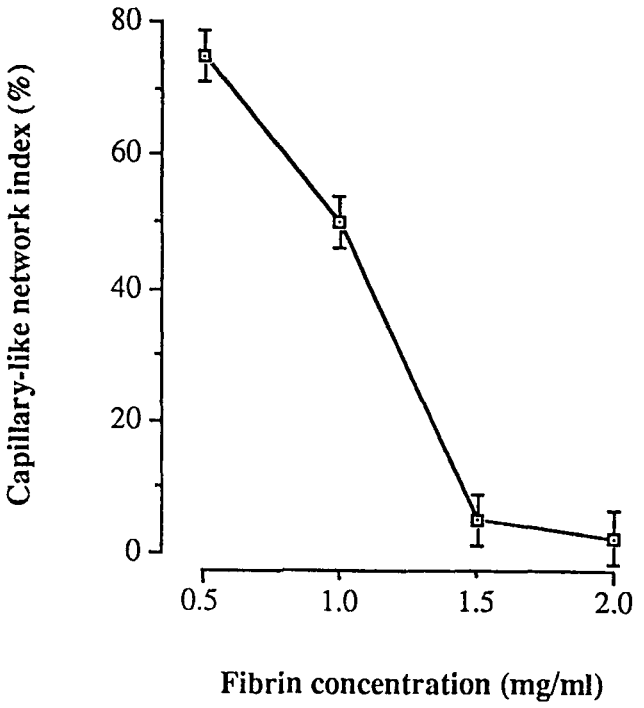

FIG. 1. Capillarylike network index depends on fibrin concentration. 2 × 10^5 cells/ml were plated on fibrin gels. The concentration of fibrin gels decreased from 2 mg/ml to 0.5 mg/ml. The medium contained 2% human serum (HS). Results were observed 24 h after seeding. The capillarylike network index was obtained by the ratio (expressed as a percentage) between the cell-free area delimited by the capillarylike network and the total area of the culture (*see* "Materials and Methods"). A hollow tubular network is formed when the capillarylike network index approached 70%. Results were expressed ± standard deviation.

on focal contacts, however, several other types of substratum contacts have been reported, such as dot or point contacts (37,42). It has been suggested that the presence of dot contacts instead of focal contacts at the periphery of the cells could be related to cell motility (12,44).

The pivotal role of the matrix and its mechanical properties in CLS formation were explored by comparing rigid matrices of fibrinogen to fibrin gels. Moreover, the influence of the mechanical properties of fibrin gels in initiating angiogenesis was assessed by modifying fibrinogen concentration, which leads to modifications of the viscoelastic properties of the gels. A study of the spatiotemporal dynamic of CLS formation was made and the relationships between human umbilical vein endothelial cell (HUVEC) differentiation and modifications in the distribution of the $\alpha_v\beta_3$ integrins were established using immunofluorescence microscopy. Our results reveal that the formation of a large capillarylike network is obtained as the concentration of fibrin gels decreases, indicating that the mechanical properties of the matrices are essential to promote capillarylike structure formation.

MATERIALS AND METHODS

Protein purification. Human fibronectin was purified by affinity chromatography according to the method of Engvall and Ruoslahti (14). Human fibrinogen was purified according to the method of Keckwick et al. (33); its purity, determined by gel electrophoresis, was greater than 99.9%. We checked the absence of plasmin activity as follows: after coagulation of the fibrinogen solution, the fibrin clot was incubated 3 d at 37° C in phosphate-buffered saline (PBS) containing 1 m*M* Ca 2^{++}, 1 m*M* Mg 2^{++} without degradation.

Culture of HUVECs. HUVECs were isolated according to the method of Jaffé et al. (31). Cells were seeded on fibronectin and grown in 199 medium (Sigma Chemical Co., St. Louis, MO) supplemented with 20% human serum (HS), glutamine, and antibiotics, until confluence. Cells were used within three *in vitro* passages, seeded either on fibrinogen or fibrin, and grown in 199 medium (Sigma) with variable percentages of human serum (HS), glutamine, and antibiotics. Cell counts were made with a Neubauer hemacytometer. Before use, HS was heat deactivated at 56° C for 30 min.

Fibrin gels. Variations of fibrin monomer concentration into fibrin gels was obtained by mixing various fibrinogen concentrations (0.5 mg/ml to 2 mg/ml) with a constant quantity of thrombin (0.2 units/ml; Sigma) in Hank's balanced salt solution (HBSS). Fibrin gels were 1 mm thick.

Fibrinogen coat. Tissue culture wells were coated overnight with a solution of human fibrinogen at 1 mg/ml in PBS. The fibrinogen concentration on the plastic was determined using the radiolabeled protein as a tracer and was 12 $\mu g/cm^2$ (45). The adsorption of fibrinogen on plastic dishes is not linearly correlated to the concentration of the fibrinogen solution.

Microscopy. Phase-contrast images of living cells were recorded using an inverted microscope (Diaphot, Nikon Inc.) equipped with a Phl plan 10 and a PhL plan 4, NA 0.5 objective lenses.

Indirect immunofluorescence labeling: $\alpha_v\beta_3$ integrin was detected on cells grown during 3 h on coated glass coverslips. Monoclonal antibodies against $\alpha_v\beta_3$ (clone LM609) were obtained from Chemicon International Inc. (Temecula, CA) and the modified method of Granger and Lazarides (20) was used. Briefly, cells were washed three times with PBS at 37° C and then immersed in freshly prepared 3% paraformaldehyde in PBS at 37° C for 15 min. Permeability to antibodies was achieved by incubation in 1% Triton in PBS, for 30 min. After a 5-min wash in PBS, the fixed cells were incubated with the monoclonal antibody for 30 min at 37° C followed by a 30-min incubation with a solution of rhodamine conjugated F(ab')2 solution at 37° C. Finally, the cells were washed for 15 min in PBS and water, and mounted in a mixture of mowiol and glycerol to which 1,4-diazobicyclo-(2,2,2)-octane (DABCO) has been added. The labeling obtained was specific, as no labeling was obtained with nonimmune IgG or by using the $F(ab')_2$ directly. Observations were carried out with a Nikon microphot microscope equipped for epifluorescence.

Confocal microscopy was carried out with an LSM 410 Zeiss confocal imaging system equipped with a Plan apo ×63 oil immersion, NA 1.40 objective lens. Reorganized capillarylike tubes were fixed with freshly prepared 3% paraformaldehyde in PBS at 37° C for 15 min, washed with PBS, and stained with phloxine 2 min (0.2%; Sigma). Cells were washed in PBS and mounted in the same manner. A helium neon laser (λ = 633 nm) was used to excite phloxine and the emission signal above 650 nm was collected. The micrographs represent serial optical sections.

Nomarski Differential Interference Contrast (DIC) microscope images were recorded in scanning modes and their contrast was improved using a similar approach as AVEC enhancement techniques. Concisely, the signal-to-noise ratio was improved when 16 frames were accumulated to form an image (1).

Cell proliferation assays. HUVECs were labeled overnight with [^{3}H] thymidine (0.6 μCi/ml) and washed five times with HBSS containing 1 m*M* $Ca^{2+}Mg^{2+}$. A 5% trichloracetic acid (TCA) solution was then added to lyse cells and precipitate the DNA. Five minutes later supernatants were removed and 1 ml of KOH was added to the tissue culture dishes for 20 min. The contents of the dishes were transferred to scintillation vials, 10 ml of scintillant were added (Amersham Aqueous Scintillant, Buckinghamshire, England) and radioactivity measured in a β counter.

Inhibition of cell attachment. Microtiter 24-well polystyrene plates were coated overnight with either fibrinogen (1 mg/ml) or fibrin. Fibrin-coats on microtiter wells were obtained by adding thrombin (0.2 U/ml) to fibrinogen (0.5 mg/ml) in HBSS. Wells were washed three times with HBSS before use. HUVECs labeled overnight in the presence of [^{3}H] thymidine (0.6 μCi/ml) were washed, dissociated by trypsinization, and 5 × 10^3 cells/ml were resuspended in 199 medium containing 1% albumin. Suspended cells were incubated with purified anti-$\alpha_v\beta_3$ antibody (Monoclonal, Clone LM609, Chemicon) for 15 min at 37° C, then allowed to attach at 37° C for 3 h to tissue culture wells coated with fibrinogen or fibrin. Cell adhesion was monitored by rinsing the cultures with HBSS, lysing the attached cells in KOH, and counting the lysate as described. Assays were performed in duplicate and repeated in two separate experiments.

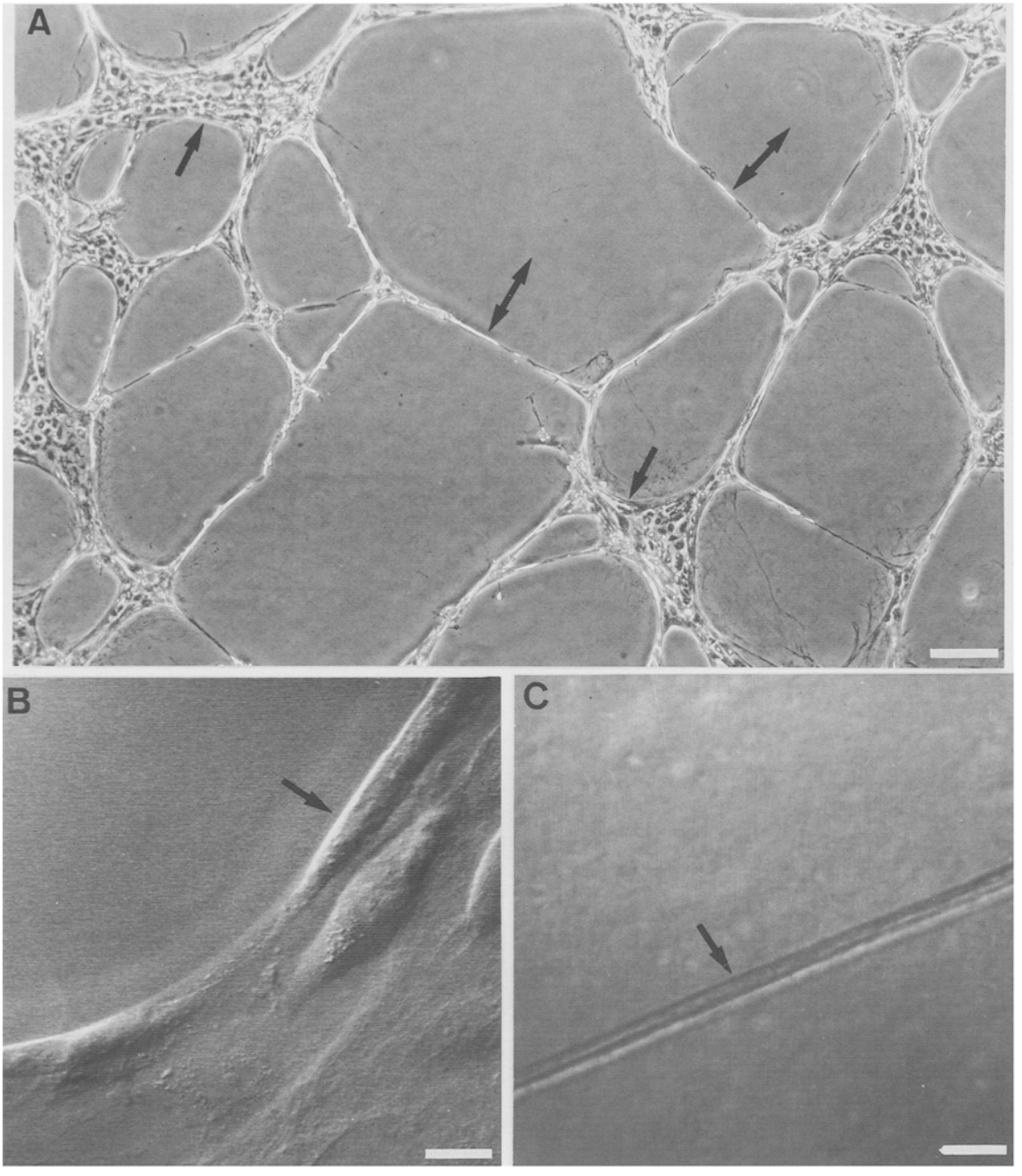

FIG. 2. *In vitro* angiogenesis of fibrin gels. *A*: capillarylike network obtained *in vitro* with cells cultured during 24 h on fibrin gels, 0.5 mg/ml concentration, 1 mm thick. *Arrows* indicate elongated cells and *double arrows* hollow tubular structures. *B*: enlargement part of a ringlike structure bordered with elongated and/or twisted cells (*arrow*). *C*: enlargement of a hollow tubular structure. *A* was photographed with a phase contrast microscope (Diaphot, Nikon Inc.). *B* and *C* were photographed with a Zeiss DIC microscope as described in "Materials and Methods." [*Bars* represent 250 μm (*A*), 15 μm (*B*), 5 μm (*C*)]

Morphometric analysis. Cells were allowed to adhere to the different matrices for 3 h, then were rinsed three times with HBSS, immersed in 3% paraformaldehyde in PBS at 37° C for 10 min, and stained with Trypan blue to improve cell visualization. The slides were analyzed by means of a SAMBA 2005 image cytometer (Alcatel TITN, Grenoble, France) connected to a Zeiss microscope (objective ×40, 1.3 NA,). Four different regions selected systematically from the top to the bottom of each slide were analyzed. The average number of cells measured per slide was 300. The projection area and a form factor were determined for each cell. The form factor is equal to the ratio of the squared perimeter to the projection area weighted by 4 π. These variables indicate how far a cell shape diverges from a perfect circle. The form factor is equal to one for a perfect circle and increases as the cell shape becomes elliptical or as the cell contour becomes more complicated.

Quantitative in vitro capillarylike network index. A quantitative estimation of the extent of the capillarylike network was given by the ratio (expressed as a percentage) between the cell-free area delimited by the capillarylike network and the total area of the culture. This ratio is called capillarylike network index. When the capillarylike network index approached 70%,

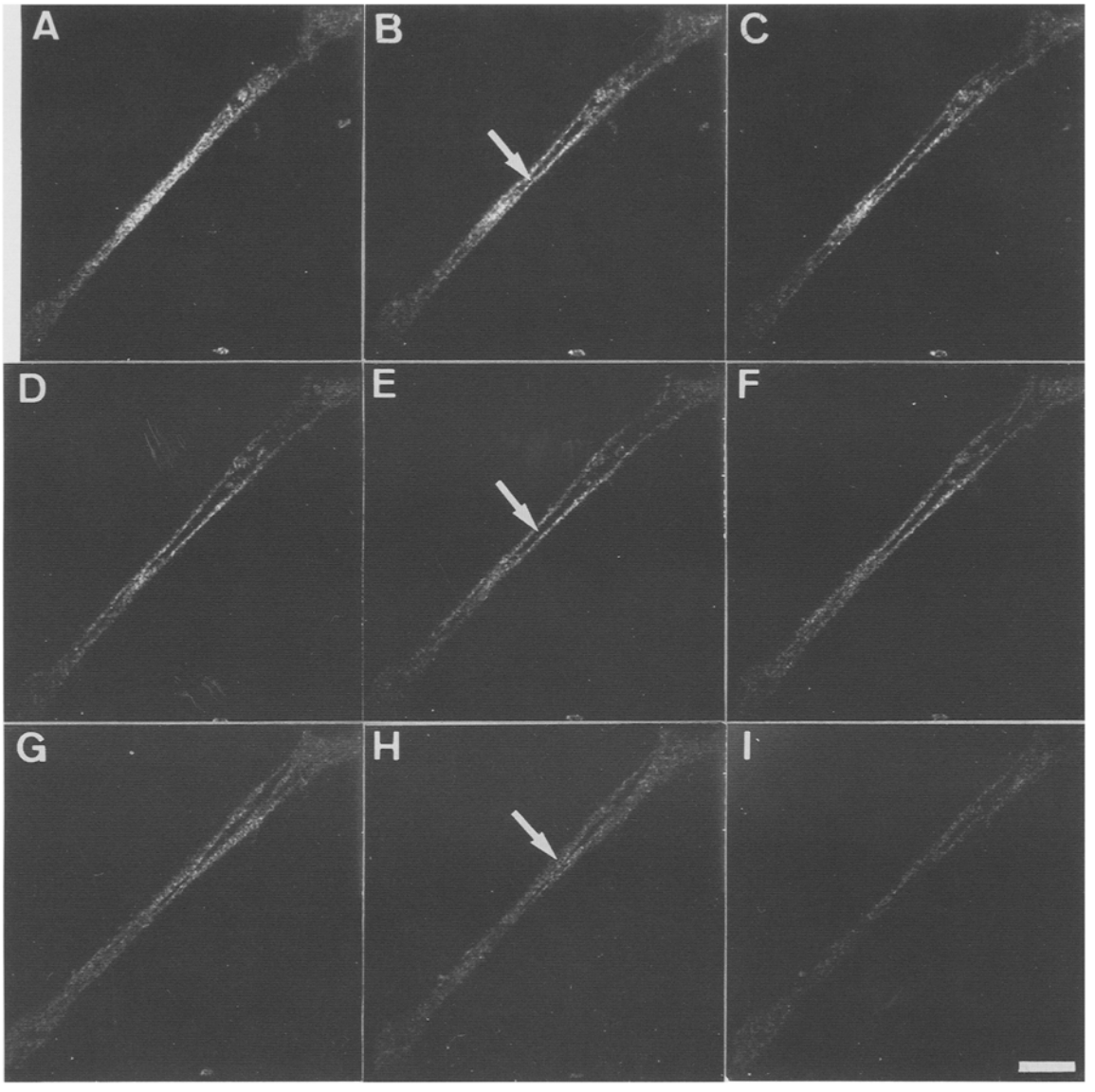

FIG. 3. Series of transversal confocal micrographies of a hollow tubular structure. Phloxyne staining was used to localize the whole cell body (*see* "Materials and Methods"). Micrographies represent optical sections at different levels of the cell. Fig. *A,B,C,D,E,F,G,H,* and *I* are optical sections located at 0; 2; 2.5; 4.5; 5; 5.5; 7.5; 8.75; 10.5 μm from the top to the bottom of the cell, respectively. Dark areas represent the inner parts of the tube unlabeled by phloxyne. *Bar* = 50 μm.

HUVECs layered on fibrin form a hollow tubular network. Conversely, low values of the index indicated that cells delimit cell-free areas without the formation of a hollow capillarylike network. This index evaluation was performed as follows: HUVECs were seeded and grown on fibrin for 24 h, then rinsed three times with HBSS, fixed with 3% paraformaldehyde in PBS at 37° C for 10 min, and stained with Trypan blue. Area measurement was performed using a SAMBA 2005 image cytometer (Alcatel TITN) connected to a SV11 Zeiss Stereomicroscope.

Dynamic analysis of capillarylike structures formation. The evolution of the cultured cells was performed by image cytometry using an Axiovert 135M inverted microscope (Plan Neofluar 1.25 × 0.035 objective lens) equipped with a temperature controlled stage and an incubation chamber (37° C, 5% CO_2, 90% hygrometry; Carl Zeiss, Jena, Germany). To avoid cell photodestruction, the cultures were exposed to the light source only during image acquisition. A SIT camera was used (Silicon Intensified Tube, C2400-08, Hamamatsu, Photonics K.K, Joko-cho City, Japan), allowing low light level detection and short exposure times. Images were acquired every 30 min during a 48-h period using a microscopic imaging work station (Samba 2640 system, Alcatel TITN, Answare, France). The behavior of cell cultures inside the incubation chamber was compared to the behavior of cell cultures in a CO_2 incubator (IG 150, Saint Herblain, France) and no differences were found. Computer-assisted image processing was used to enhance the contrast of the original images.

RESULTS

Role of fibrinogen matrices and fibrin gels in CLS formation. Because fibrinogen and fibrin are closely related proteins, the modification of matrix mechanical properties can be evaluated by comparing a rigid fibrinogen matrix made of fibrinogen molecules attached to plastic dishes and fibrin gels. Modifications in the mechanical

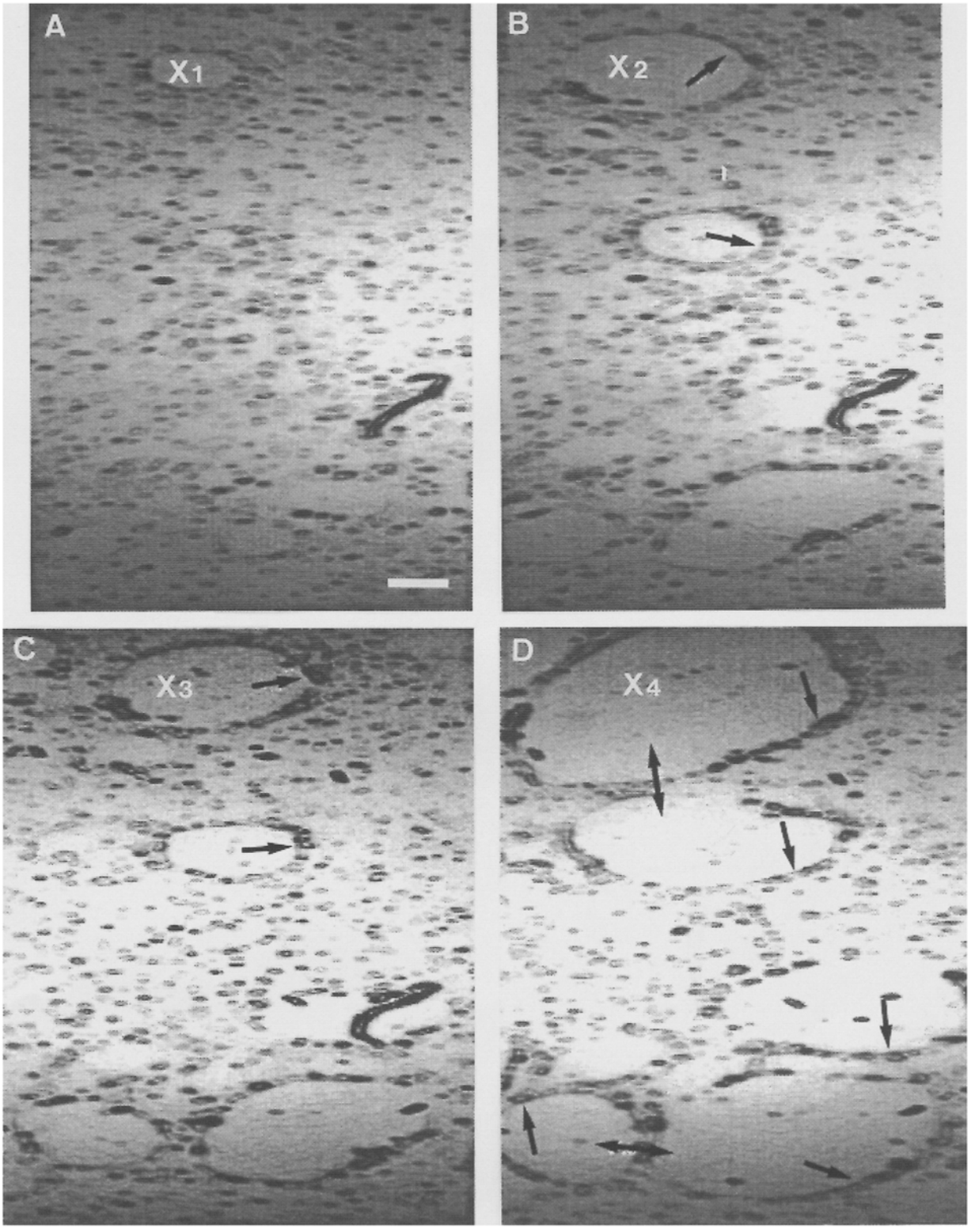

FIG. 4. Representative views of the dynamics of capillarylike structures (CLS) formation. The experiments were performed as described in "Materials and Methods." *A,B,C,* and *D* represent the same field photographed respectively 9, 18, 19, and 24 h after cell seeding. Image processing by enhancing the contrast discriminated between cells located at different distances from the objective lens. Thus, the grey and dark points represent the cells attached to the fibrin matrix. Cells are reorganized into ringlike structures (*arrows*). In the same area, the number of rings increased progressively from one in Fig. 4 *A* to five in *D*. The ringlike structure X1 grew in size over time and became successively X2, X3, and X4. As the ringlike structures enlarged, they came into contact and at the junction of two ringlike structures a capillary was formed (*double arrows*). *Bars* = 250 μm.

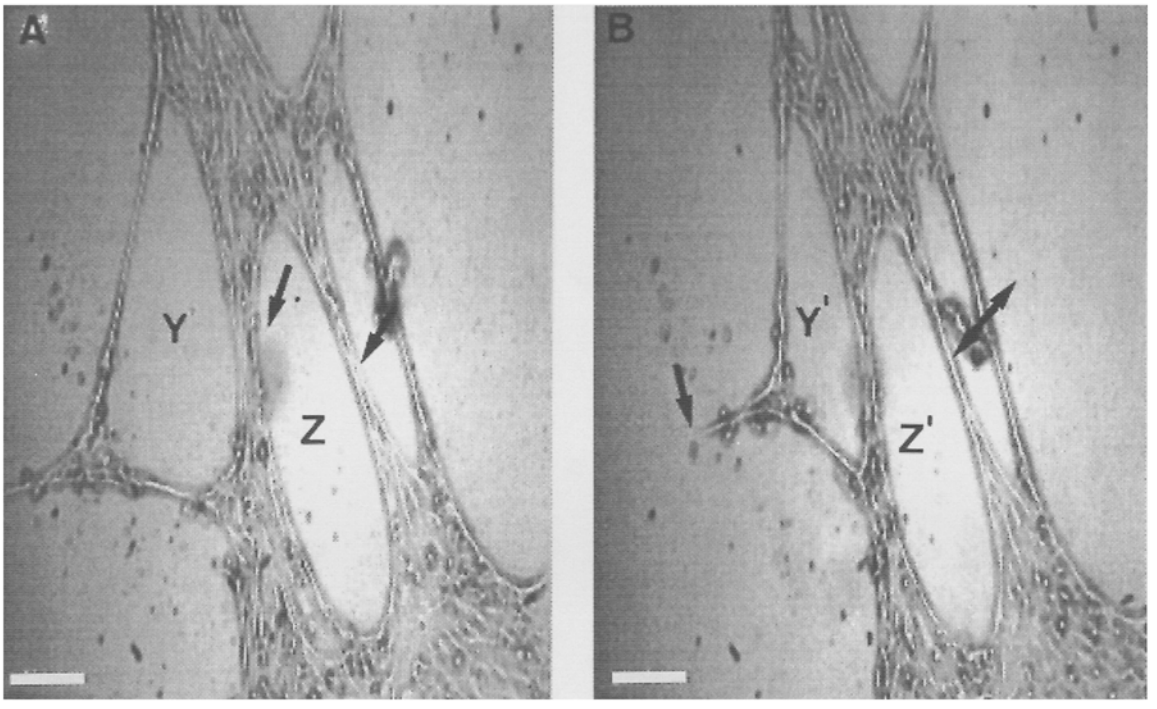

FIG. 5. Images of a ringlike structure photographed with a phase contrast microscope, 22 h (*A*) and 24 h (*B*) after seeding. The experiments were performed as described in "Materials and Methods." At this stage of the experiment, there was no enlargement of the ringlike structures. The shapes of the ringlike structure Y,Z (*A*) are modified into the shapes Y',Z' (*B*). They became more elongated. It is obvious that this deformation was induced by cell forces pulling out the fibrin gel along the main axis of the ringlike structures Y' and Z'. The *arrows* in *A* show the border of ringlike structures. The *arrow* in *B* points to localized disruption of fibrin gels. The *double arrow* in *B* localizes a capillarylike structure (CLS) formed at the junction of two ringlike structures. *Bars* = 200 μm.

properties of fibrin gels were obtained by varying fibrinogen concentration between 0.5 mg/ml and 2 mg/ml. Fibrin gels below 0.5 mg/ml could not be tested, because they were too fragile. Twenty four hours after the cells were plated on 1-mm thick fibrin gels, in a medium containing 2% HS, we observed that CLS formation was augmented as fibrin concentration decreased (Fig. 1). However, when cells were plated on fibrinogen matrices, CLS were never observed regardless of the absorbed fibrinogen concentration (data not shown). The capillarylike structures are shown in Fig. 2 *A*: endothelial cells differentiated in two patterns: (a) elongated and/or twisted cells delineate ringlike structures (*arrows* in Fig. 2 *A*) and (b) typical CLS (*double-arrows* in Fig. 2 *A*). Fig. 2 *B* shows an enlargement of an elongated HUVEC, and Fig. 2 *C* shows an enlargement of the tubular structure of a CLS. Moreover the series of transversal confocal images in Fig. 3 demonstrate the presence of a hollow tubular structure, since no labeling by phloxyne was observed in the inner part of the tubular structures (dark areas indicated by *arrows* in Fig. 3). The average diameter of a CLS is about 15 μm.

Dynamic analysis of angiogenesis in vitro *underlines the role of the mechanical properties of the fibrin gel.* The photographs shown in Figs. 4 and 5 are representative views of dynamic CLS formation in our model. The cells were seeded on 1-mm thick fibrin gels (0.5 mg/ml), at 2×10^5 cells/ml and cultured in 2% HS medium as described above. Every 30 min, a field representing a quarter of the petri dish was recorded with the image analysis work station. HUVECs adhered to fibrin in a random manner within 3 to 4 h after seeding. Between 10 and 30 h, cells reorganized themselves into ringlike structures (*arrows* in Fig. 4), which implied cell translocation since cells proliferated very slowly in 2% HS. Furthermore, the same ring observed over a period of 15 h (X1, X2, X3, X4 in Fig. 4 *A–D*, respectively) expanded dramatically in place with changes in cell morphology. This size increase was partly due to deformation of the fibrin network by cell forces and partly to modification of the endothelial cell phenotype that shifted to an oblong form as shown in Fig. 2 *B*. Stretching of the fibrin lattice was clearly seen at the end of CLS formation (Fig. 5), where structures Y and Z (Fig. 5 *A*) increase in size along their main axis, while their width decreases (Y', Z' in Fig. 5 *B*). Moreover, the stretching of the gel led to its localized disruption (Fig. 5 *B*, *arrow*) and the partial collapse of the Y' ringlike structures that became smaller than the same structure Y photographed 2 h earlier (i.e., before disruption) (Fig. 5 *A* and *B*).

Most of the CLS observed were formed at the junction of two ringlike structures (*double arrows* in Figs. 4 *D* and 5 *B*).

In experiments on gels performed with increased fibrin concentrations from 1 mg/ml to 2 mg/ml, only a few ringlike structures appeared, but they neither increased in size, nor gave rise to CLS during the 24-h observation period. Neither cell stretching nor disruption of the matrix were observed, even after 3 d. These results clearly indicate that an equilibrium between cell tractions and the mechanical resistance of the matrix is required for the formation of CLS. Increasing the fibrin concentration increases the mechanical properties of the fibrin gels.

The formation of a capillarylike network was favored only by a limited range in cell concentration. The above results clearly showed

that the role of cell forces, and hence the number of the cells participating in the process, might be important. Thus, their role was analyzed in two steps.

First, the capillarylike network index was determined when HUVECs were cultured on fibrin gels in medium containing between 2% and 20% HS (Fig. 6 *A*). In this experiment, the fibrin gel concentration varied between 2 mg/ml and 0.5 mg/ml. In parallel, [^{3}H] thymidine incorporation in DNA was determined as a measure of cell multiplication (Fig. 6 *B*). We observed a decrease of cell multiplication with decreasing HS concentration without any significant influence of fibrin gel concentration on cell proliferation. Thus, the results in Fig. 6 *A* show that as the rate of proliferation decreased and fibrin concentration decreased the capillarylike network index augmented.

Second, the influence of the number of cells seeded on a low fibrin gel concentration (0.5 mg/ml) was tested in a medium that reduces minimal cell growth (2% HS). The number of cells seeded varied between 5×10^4 to 5×10^5 cells/ml. Fig. 7 shows that the capillarylike network formation was strongly dependent on cell number: between 1.5×10^5 and 2×10^5 cells/ml, the capillarylike network index was about 75%. Below or above these values, CLS formation was rapidly inhibited.

Before ringlike structure formation, a specific endothelial cell phenotype was observed. In order to detect whether phenotypic modifications of HUVECs precede ring formation, we performed a morphometric analysis 3 h after cell seeding. Two parameters were studied: cell surface and the form factors. The results in Fig. 8 *A* and *B* show that phenotypic changes occurred rapidly; however, they were dependent on the composition of the matrix on which cells were seeded. As the fibrin gel concentration increased, both the form factor and cell surface decreased, which indicated that cell spreading decreased. Variations in fibrin concentration modulated cell shapes, as soon as cells adhered and spread. In contrast, on fibrinogen-coated dishes, the form factors were smaller than on fibrin gels, whatever the fibrinogen concentration (data not shown). In this case, the cell shapes observed were typical of the egglike structures of endothelial cells observed under normal culture conditions, unfavorable to CLS formation.

Analysis of the localization of the $\alpha_v\beta_3$ integrins during the formation of the capillarylike structure in cells seeded onto fibrin. The distribution of the $\alpha_v\beta_3$ integrins was studied after cell seeding onto a rigid matrix of fibrinogen and less rigid fibrin gels (0.5 mg/ml). Higher fibrin concentrations could not be studied as their opacity hampered correct microscopic observation. As fibrin promoted angiogenesis, the observations were made both before cells reorganized into ringlike structures and at the end of the process of cellular elongation and capillarylike structure formation.

Three hours after seeding, the distribution of the $\alpha_v\beta_3$ subunit in HUVECs was quite different depending on whether the cells were allowed to adhere onto fibrinogen or fibrin. On the fibrinogen matrix,

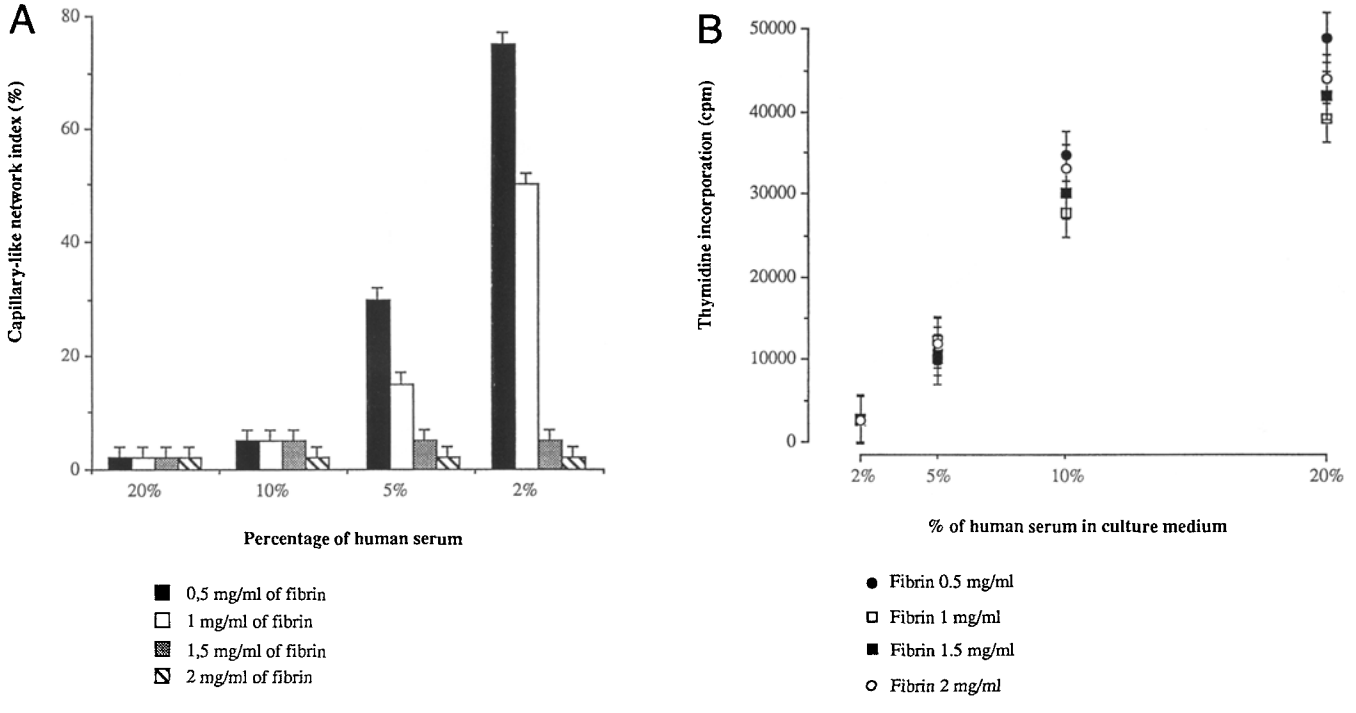

FIG. 6. (*A*) Capillary-network index depends on the concentration of HS in culture medium. 2×10^5 cells/ml were plated on fibrin gels. The concentration of fibrin was decreased from 2 mg/ml to 0.5 mg/ml and HS was decreased from 20% to 2%. The capillarylike network index was calculated as described in the "Materials and Methods" section. When this value approached 70%, hollow tubular structures were formed. The results were analyzed 24 h after seeding. Experiments were done in triplicate, as described in "Materials and Methods," each with a different cell line. (*B*) Cell thymidine incorporation depends on human serum concentration in culture medium. Cells were plated onto fibrin gels at different fibrin concentrations in culture mediums containing different percentages of human serum. Tritiated thymidine (0.6 μCi/ml) was added and the experiment allowed to proceed as described in "Materials and Methods." Experiments were done five times, each with a different cell line. Results were expressed as the mean (± standard deviation) of at least four different experiments performed in duplicate.

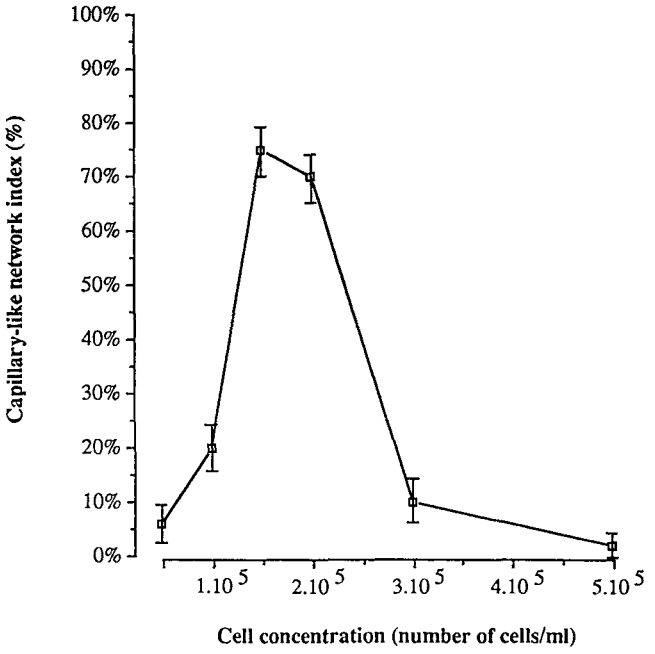

FIG. 7. Capillarylike network index depends on the number of cells seeded. HUVECs were seeded onto fibrin gels at varying cell concentrations, and results were observed 24 h after seeding. The capillarylike network index was calculated as described in the "Materials and Methods" section. Fibrin gels were 1 mm thick, with a fibrin concentration of 0.5 mg/ml. The percentage of HS in the culture medium was 2%. Experiments were done five times, with five different cell lines. Results were expressed as the mean (± standard deviation) of at least four different experiments performed in duplicate.

$\alpha_v\beta_3$ integrins clustered into dense areas called adhesion plaques (*arrow* in Fig. 9 *A*), whereas in cells seeded onto fibrin, a diffuse distribution and dot contacts were observed (*arrows* in Fig. 9 *B*). Most of the labeled integrins observed in these micrographies seem to be located at the cell membrane and linked to fibrin or fibrinogen, since $\alpha_v\beta_3$ antibodies inhibited the adhesion of HUVECs to fibrin or fibrinogen (Fig. 10).

Finally, the labeling of the hollow capillarylike tubes is totally diffuse (*arrows* in Fig. 11 *A* and *B*). This diffuse labeling implies that the integrin $\alpha_v\beta_3$ is not included in adhesion structures. In some places, along the tubular structure (*double arrows* in Fig. 11 *A* and *B*), we observed an area representing the unlabeled empty part of the tube.

DISCUSSION

The present study is the first to demonstrate that the angiogenic properties of fibrin gels vary with the concentration of the fibrin monomers in the fibrin gels. The mechanical stresses resulting from the balance between cell traction and the mechanical resistance of the gel are likely to play a critical role in *in vitro* CLS formation, since this process is inhibited when the fibrin concentration is increased, and hence the matrix resistance increased. Moreover, our dynamic studies showing localized and simultaneous collapse of both CLS and the surrounding matrix provide direct observation of mechanical forces development during CLS formation. Finally, the extracellular matrix receptor $\alpha_v\beta_3$ appears to be involved in CLS formation but may not be required for the maintenance of these structures *in vitro*, because the diffuse labeling we observed indicates

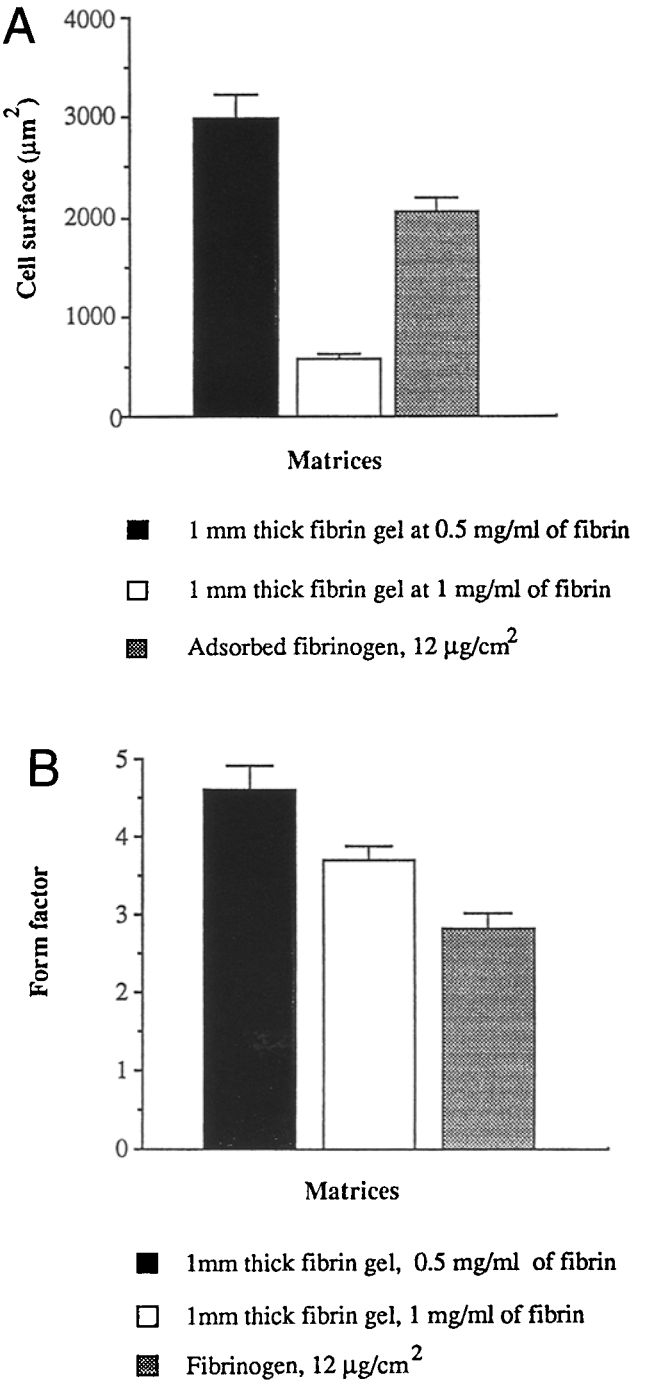

FIG. 8. Morphometric analysis of HUVECs plated on matrices varying in mechanical properties. In *A* and *B*, we show the variation of the cell surface and of the form factor, respectively, when cells were layered during 3 h on fibrinogen coat or fibrin gels at different concentrations. On a fibrin layer, the cells have an elongated shape compared to cells plated on a fibrinogen coat. As the fibrin gel concentration decreases, the surface of the cells increases (*A*), and they became more elongated because the form factor increases (*B*). Results were expressed as the mean (± standard deviation) of at least four different experiments performed in duplicate.

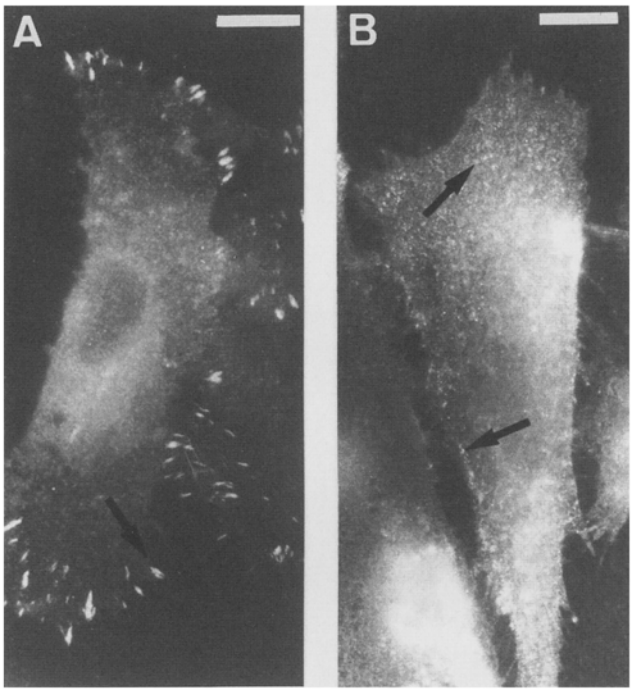

FIG. 9. Immunocytochemical studies of the $\alpha_v\beta_3$ integrins. HUVECs were cultured during 3 h on fibrinogen (*A*) or on fibrin 0.5 mg/ml (*B*). Labeling was obtained using monoclonal antibodies (clone LM609, *see* "Materials and Methods"). (*A*) Human umbilical vein endothelial cells (HUVECs) seeded on fibrinogen-coated coverslips show adhesion plaques (*arrows*). (*B*) HUVECs seeded on fibrin coverslips show dot contacts (*arrows*) at the cell periphery and diffuse labeling. *Bars* = 10 μm.

that the $\alpha_v\beta_3$ integrins are not included into adhesion structures (adhesion plaques or dot-contacts).

The importance of the mechanical properties of the matrix and cell forces during angiogenesis has been recognized (26,29). Most of the studies compared rigid matrices made of molecules attached to plastic dishes to less rigid biological gels. This malleability of the gels was due to their viscoelastic properties, which vary with their protein concentration (2,22). The use as substratum of a complex extracellular matrix for cell attachment does not allow assessment of the respective contributions, if any, of the matrix composition and of its mechanical properties on the angiogenic process. Because we use fibrin gels having increasing fibrin monomer concentration, our study highlights that appropriate mechanical properties of fibrin gels are necessary to induce the reorganization of HUVECs into hollow tubular networks. Indeed, we found that within a time frame of 24 h, an extensive capillarylike network is formed only on fibrin made from fibrinogen solution (0.5 mg/ml) in culture medium containing 2% HS, in the absence of exogenous angiogenic factors. Modification of the mechanical properties of fibrin by increasing the fibrin gel concentration hampered the formation of an extensive capillarylike network when all the other culture conditions remained unchanged. The low HS concentration used (2%) cannot provide the essential angiogenic factors, since we measured a decrease of the capillarylike network index when the concentration of HS was increased (Fig. 6 *A*). In fact, fibrin gels prepared from highly purified fibrinogen can rapidly promote HUVECs rearrangement into a hollow tubular network, as long as the gels are sufficiently viscoelastic. Furthermore, the balance between the cell forces and the mechanical properties of the matrix depends on the number of cells seeded. The latter determines the intensity of the cell forces. Indeed, we have shown in Fig. 7 that, when the number of cells seeded was too low, CLS were not formed, because the cell forces required to reorganize the matrix were too weak. In our study, cell growth needs to be arrested to obtain an extensive capillarylike network (Fig. 6 *A* and *B*). In fact, dividing cells with their disrupted actin filaments may not exert adequate forces and, therefore, cannot counterbalance matrix tension. These results strongly support the hypothesis that HUVECs need to develop synchronous mechanical forces. In previous work, cells seeded on concentrated fibrin gels did not form CLS unless fibrinolysis occurred or other factors were provided (36,40). We believe that proteases, in addition to activating latent angiogenic factors, partially digest the fibrin gel, yielding a less rigid substratum favorable to angiogenesis.

It has been proposed that endothelial cells seeded on an appropriate matrix reorganize to form an interconnected network of capillarylike structures (3,35,47). Direct proof of such an organization is provided by our spatiotemporal study. Twenty-four hours after seeding, a capillarylike network was formed from the rearrangement of cells previously in ringlike structures. These processes imply cell migration, since 3 h after seeding, the cells were still evenly distributed on the substratum and formed ringlike structures only 10 h later. As reported in previous studies, HUVECs that acquire the capacity to build a capillarylike network lose their ability to divide as we show in Fig. 6 *A*. The *in vitro* formation of ringlike structures of increasing

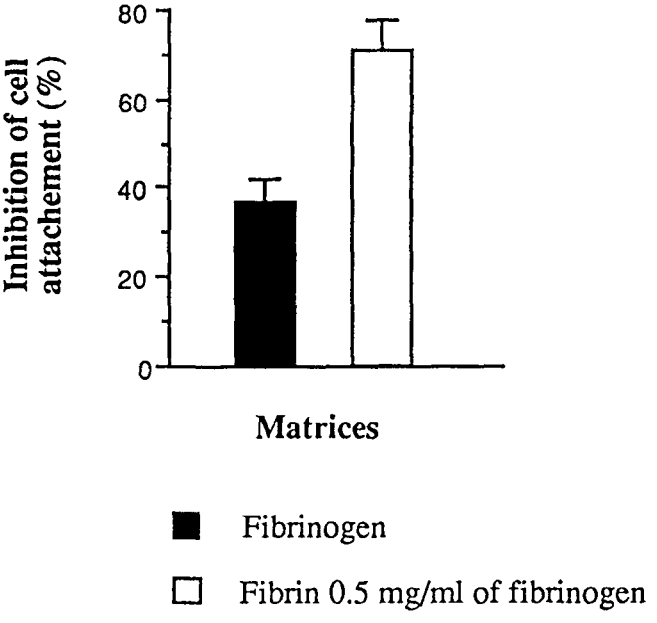

FIG. 10. Inhibition of cell attachment on fibrinogen and fibrin by an antibody against the $\alpha_v\beta_3$ integrins. Cells prelabeled with [^{3}H] thymidine were incubated with 20 μg/ml of anti-$\alpha_v\beta_3$ (clone LM609) in the conditions described in the "Materials and Methods" section. Data are expressed as a percentage of cells attached to the different substrata in the absence of the antibodies ± s.d. Each *bar* represents the mean of two experiments performed in duplicate.

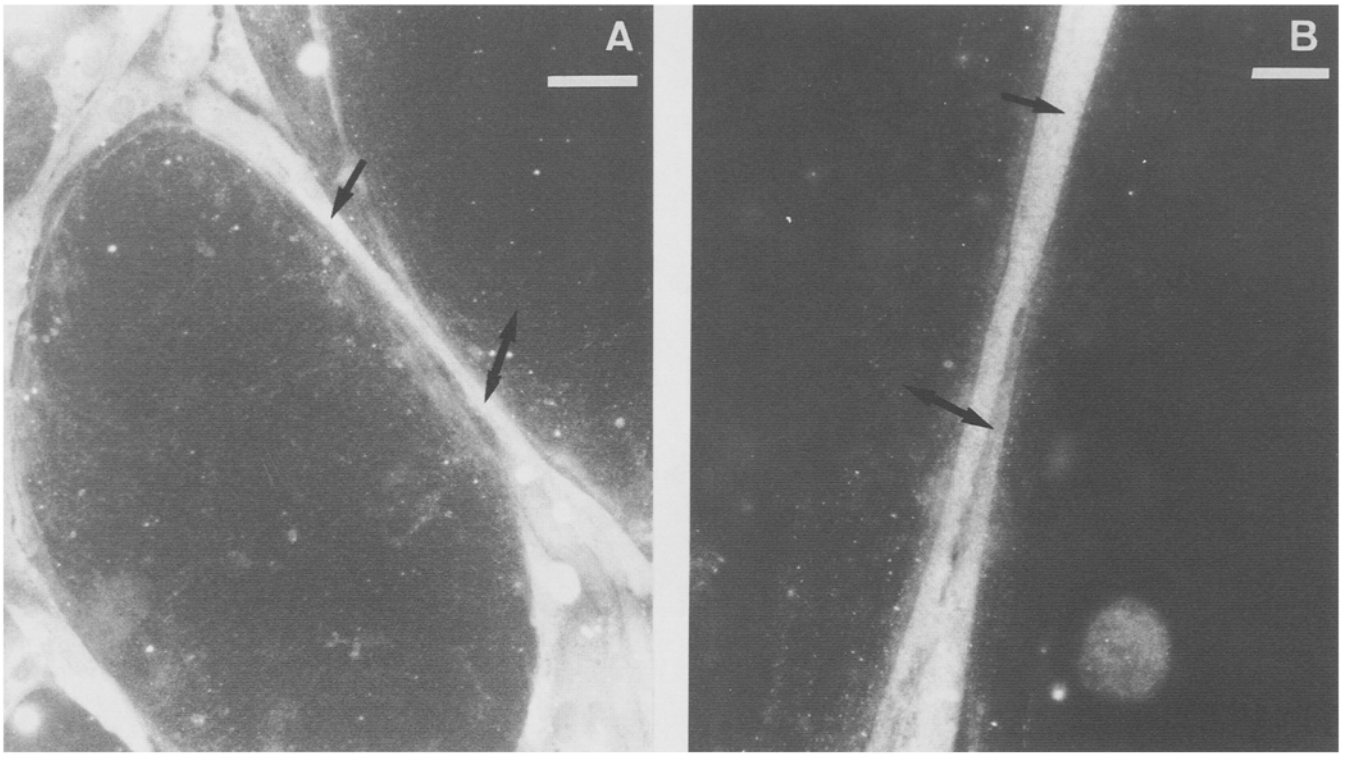

FIG. 11. Immunocytochemical studies of the $\alpha_v\beta_3$ integrins on human umbilical vein endothelial cells (HUVECs) cultured on fibrin when the angiogenesis process has occurred. Labeling was obtained using monoclonal antibodies (clone LM609, *see* "Materials and Methods"). The *arrows* in *A* and *B* show a capillarylike structure. (*A*) HUVECs cultured on fibrin-coated coverslips show a diffuse labeling. The *double arrow* points to the lumenlike structure. (*B*) An enlargement of the capillary-like structure shown in *A* indicates the presence of an empty, nonlabeled zone (*double arrow*). *Bars* = 15 μm (*A*) and 5 μm (*B*).

size does not require cell multiplication, nevertheless, it is stringently controlled by the number of cells. Either too low or too high a cell density strongly hampers cellular rearrangement (Fig. 7). The cell density required to form precapillarylike structures is identical to that found by Bauer et al. (4), although cells were seeded on a more complex Matrigel matrix in the latter study. *In vivo*, apoptosis may regulate the cell number needed to form capillaries. Our study demonstrates for the first time that a fibrin matrix is sufficient to promote angiogenesis *in vitro* in a manner reminiscent of angiogenesis *in vivo*: capillarylike structures originate at the junctions of the elongated cells that delimit the ringlike structures. At this junction, a hollow tubular structure is formed as we demonstrate in Fig. 3. The elongated HUVECs triggered by gel tension, probably represent an intermediate phenotype between undifferentiated HUVECs and CLS. Unlike Vernon et al. (47), we never observed HUVEC clusters.

Previous data (6,8,11) and the results shown in Fig. 10 clearly indicate that the $\alpha_v\beta_3$ integrins are involved in HUVEC adherence to fibrin and fibrinogen. However, the distribution of $\alpha_v\beta_3$ on the surface of HUVECs is highly dependent on the matrix composition (Fig. 9 *A* and *B*) and induced modifications in cell shape (Fig. 8 *A* and *B*). Three hours after plating, cells seeded on fibrinogen exhibit recruitment of $\alpha_v\beta_3$ into areas of focal contact, while on the fibrin gel, the $\alpha_v\beta_3$ integrins accumulate in point contacts at the periphery of the cells. Finally, on hollow capillarylike tubes only a diffuse labeling is observed (Fig. 11 *A* and *B*). The presence of dot contacts may be associated with cell motility via cycling of receptors on and off the cell surface (5,12,46). Our results are in accordance with this suggestion, as this motility is necessary for the organization of the cells into ringlike structures as shown in Fig. 4. Used at the same concentration, antibodies against the $\alpha_v\beta_3$ integrins have a stronger inhibitory effect on cell adhesion on fibrin than on fibrinogen (Fig. 10). This reduced adhesive strength on fibrin may contribute to cell motility. Thus, the angiogenic properties of fibrin gels not only involve the specific mechanical properties of fibrin gels, but also a specific peptide sequence within the fibrin/fibrinogen molecule recognized by the $\alpha_v\beta_3$ receptors.

During the last step, the diffuse labeling we observed on capillarylike structures (Fig. 11 *A* and *B*) suggests that the $\alpha_v\beta_3$ receptors are not included into adhesion structures. This may complement the observations of Gamble et al. (19), who described an increase of capillary tube formation when an anti-$\alpha_v\beta_3$ antibody was added to the cell culture. It is possible that $\alpha_v\beta_3$ may be essential for cell migration; once CLS are formed, a more complex matrix (fibronectin, α-laminin) and other types of integrins may be involved. Based on these results, it seems likely that the $\alpha_v\beta_3$ integrins, besides their role in adhesion, may be involved in regulation of the process of angiogenesis. Therefore, modulation of their localization, associated with the angiogenesis process, may be triggered through a mechanical signal resulting from the balance between cell forces and the mechanical properties of the gels that depend on their protein concentration.

ACKNOWLEDGMENTS

We thank Dr. Alice Barrieux for her comments on the manuscript.

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