Effects of prolonged exposure to hypoxia on morphological changes of endothelial cells plated on fibrin gel

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Since tissue oxygenation has a profound effect on capillary growth, the effect of pO2 on endothelial cell functions was studied. Under normoxic conditions, EA.hy926 endothelial cells and HUVEC plated onto fibrin gels in low-serum culture medium underwent rapid and profound morphological changes within 12 to 48 hours depending on the cell line used. Their characteristic cobblestone organisation was transformed into a network of cord-like or tube-like structures. We showed that when exposed to low oxygen concentrations for 3 days, HUVEC and EA.hy926 have their ability to rearrange reduced to around 50 %. With EA.hy926 this effect was amplified by 79% after 9 days of hypoxia. The altered behaviour of hypoxia-adapted cells was not caused by a loss in their fibrinolytic activity. In fact, the fibrin degradation rate and the generated fibrin fragments appeared identical in normoxia and hypoxia. Confocal microscopy and gel densitometry showed that in normoxia the remaining undegraded fibrin gel underwent a dynamic remodeling whereas in hypoxia it remained undisturbed. It is likely that hypoxia induces modification in the factors that integrate matrix information and cytoskeletal organisation in order to contract fibrin.

Introduction

The endothelium situated at the interface of blood and the underlying tissues is a dynamic structure that responds to signals coming from either blood or tissue. Oxygen concentration is one of the major factors to which endothelial cells can respond to. Endothelial cell sensitivity and responses to hypoxia play an important role in the pathogenesis of numerous diseases. In fact, early studies have demonstrated that in some regions of solid tumors, where low oxygen tension (hypoxia) and necrosis are common features, endothelial cell recruitment from the surrounding stroma allows tumor growth beyond several millimeters in diameter (Folkman, 1995; Rockwell and Knisely, 1997). Similarly, retinal hypoxia leads to vascular expansion (Pe’er et al., 1995). In myocardial ischemia, hypoxia induces the development of collateral cardiac vessels (Ware and Simons, 1997) however the compensatory response to hypoxic stress is often insufficient. However, the basic mechanisms by which hypoxia changes endothelial cell function are poorly understood. Studies performed in vitro have shown that the lack of oxygen itself is sensed and transduced as a nuclear signal. Indeed a specific nuclear factor, hypoxia-induced factor 1 (HIF-1), is induced in hypoxia (Semenza, 1998). HIF-1-binding sites have now been defined in the control sequences of a wide variety of genes which regulate the expression of metabolic and angiogenic factors (Franklin-Bunn and Poyton, 1996). One of these factors, vascular endothelial growth factor (VEGF), produced by a variety of cells (Plouët et al., 1989; Leung et al., 1989) (for review see (Ferrara and Davis-Smyth, 1997)), is regulated by oxygen tension and is known to be linked to vascular expansion in poorly perfused tissues (Ladoux and Frelin, 1993; Shweiki et al., 1992; Shweiki et al., 1992; Aiello et al., 1994). Angiogenesis is a multistep process that requires partial degradation of the surrounding matrix and invasion of the tissue by proliferating endothelial cells before formation of new vessels (Pepper et al., 1996; Madri et al., 1996). The effect

Abbreviations: BSA Bovine serum albumin. – DME Dulbecco’s modified Eagle’s medium. – DPBS Dulbecco’s phosphate-buffered saline. – ELISA Enzyme-linked immunoassay. – FBS Foetal bovine serum. – FDP Fibrin degrady product. – HIF-1 Hypoxia-induced factor 1. – HUVEC Human umbilical vascular endothelial cells. – MMPs Matrix metalloproteinases. – uPAR Urokinase plasminogen activator receptor. – VEGF Vascular endothelial growth factor.

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of hypoxia on matrix remodeling is unclear. The few studies performed in vivo in rats and humans remain equivocal (Risberg and Stenberg, 1985; Stegnar et al., 1987; Mangum et al., 1987; Gando et al., 1997). Several investigators using endothelial cells of different origin, have shown that hypoxia modulates this step (Gartner et al., 1993; Wotja et al., 1988; Shatos et al., 1990). Moreover, Graham et al. (1998) suggested that an increased expression of the urokinase plasminogen activator receptor (uPAR) can facilitate cell migration and tissue invasion. At present, the effect of hypoxia on the plasminogen-plasmin system is poorly documented.

In attempt to clarify and identify the factors involved in neovascularisation, many models have been used. In vitro model systems used to study angiogenesis are often based on culture of endothelial cells of different origins onto matrix proteins (Dvorak et al., 1987; Vernon and Sage, 1995). In our lab, we have described the formation of tubes by HUVEC seeded on fibrin gel (Vailhé et al., 1996). Because the EA.hy926 endothelial cells are a clonal, continuous cell line, they possess a wide range of endothelial properties and offer many advantages over primary endothelial cell cultures (Edgell et al., 1983, Emeis and Edgell, 1988). Earlier investigations have used EA.hy926 cells, which when seeded on Matrigel™ or in collagen lattice, undergo morphological changes similar to their endothelial parent (Bauer et al., 1992; Jones et al., 1998; Schönherr et al., 1999).

Herein, we have analysed the adaptation of two endothelial cell lines HUVEC and EA.hy926 to prolonged hypoxia, while they were grown under routine culture conditions. Subsequently, we compared their morphological rearrangement into cord- and tube-like structures on fibrin gel after pre-exposure to hypoxia.

We show that endothelial cells adapt to hypoxia as they grow at the same proliferative rate. However, a prolonged exposure to low oxygen tension leads to an inhibition of endothelial cell reorganisation in our model.

Materials and methods

Reagents

Dulbecco’s modified Eagle’s medium (DMEM), 199 medium, Dulbecco’s phosphate-buffered saline (DPBS), o2-antitpsin, thrombin, plasmin, paraformaldehyde, phloxine B, RNase A, propidium iodide were obtained from Sigma Chemical Co. (St Louis, MO, USA); antibodies from Biological Industries (ATGC, Noisy Le Grand, France) and foetal bovine serum (FBS) from Gibco-BRL. Trypsin was purchased from Boehringer Mannheim (Germany); antibiotics to human fibrinogen and its degradation products (catalog # A0080) were supplied by DAKO A/S (Glostrup, Denmark); goat anti-rabbit IgG linked to horseradish peroxidase (catalog # NA934), TMB peroxidase substrate kit and kaleidoscope prestained molecular mass standards from BIO-RAD Laboratories (Richmond, CA, USA). Chemiluminescence ECL kit and Hyperfilm ECL were purchased from Amersham Life Science (Buckinghamshire, England), and nitrocellulose membranes from Schleicher and Schuell. Quantikine™ Human VEGF immunoassay (catalog # DVE00) and human recombinant VEGF165 were obtained from R&D Systems (Minneapolis, USA).

Cells

The EA.hy926 endothelial cell line was kindly provided by Dr. Cora Jean Edgell, University of North Carolina School of Medicine, Chapel Hill, North Carolina. EA.hy 926 were established by fusing human umbilical vein endothelial cell (HUVEC) with human carcinoma cell line A549. EA.hy926 cells and A549 cell lines were routinely grown in DMEM medium supplemented with 10% heat-inactivated FBS and antibiotics (penicillin 100 U/ml and streptomycin 100 µg/ml) in an atmosphere of 5% CO2/95% air at 37 °C in a humidified incubator (Jouan IG 150, St Herblain, France). HUVECs were isolated according to the method of Jaffé et al. (1973) and cultured on fibronectin matrix in 199 medium supplemented with 20% human serum (HS), antibiotics and 2% glutamine. Each cell batch was harvested and cultured from four umbilical veins.

Hypoxia was induced by culturing cells in an humidified incubator (Heraeus, serie 6000, Hanau, Germany) at 37 °C in a constant 3% O2/5% CO2 atmosphere with oxygen deficit induced by nitrogen replacement. The oxygen level was measured with a CLARK electrode. SD cultures were made from confluent cultures by trypsinization in DPBS containing 0.25% trypsin/0.01% EDTA.

Measurement of cell growth

EA.hy 926 cells were seeded at a density of 5 × 10³ cells in DMEM supplemented with 10% FBS and placed under hypoxic or normoxic conditions. Every third day, cells reached confluence and were washed with sterile PBS, trypsized and counted in a Neubauer hemocytometer. (Polylabo, Paul Block & Cie, Strasbourg, France). HUVECs were used within three passages after isolation and grown under hypoxia or normoxia. For each passage, cells were seeded at a density of 2.5 × 10³ cells in M199 supplemented with 20% HS. After each passage, cells were reseeded and replaced under the appropriate oxygen concentration.

Cells grown either in normoxia or hypoxia were used for in vitro morphogenetic assays. After removal, culture media were centrifuged (10 minutes, 5000g, 4 °C) and frozen (–80 °C) for subsequent detection of VEGF165 by ELISA.

In vitro morphogenetic assays on fibrin gels

Human fibrinogen was purified according to the method of Keckwitz et al. (1955). The purity and integrity of the protein were assessed by SDS-PAGE.

In order to obtain Petri dishes coated with 1 mm thick fibrin gels, 1 ml of fibrinogen solution was mixed with a constant quantity of thrombin (0.083 UI/ml in Ca²⁺/Mg²⁺-free DPBS for EA.hy926 or HBSS (Hanks’ Balanced salts; for 1 liter: 8.18 g NaCl, 0.3 g KCl, 1.38 g glucose, 3.57 g HEPES, pH 7.5) for HUVEC, in 3.5 cm diameter dishes. Fibrin was allowed to polymerise overnight at 37 °C. For the assays, 2 × 10⁵ EA.hy926 cells or 1.5 × 10⁵ HUVEC resuspended in 1 ml of culture medium containing a low concentration of heat-inactivated (0.5–2%) serum and antibiotics, were seeded on fibrin gels. After cell seeding, the plates were incubated under normoxic or hypoxic conditions. Culture media were withdrawn at the onset of cell rearrangement, centrifuged for 10 min at 5000g 4 °C and frozen (–80 °C) for subsequent determination of fibrin degradation products (FDP).

Microscopy

Phase-contrast photomicrographs of cell rearrangement were prepared after examination on an inverted microscope. Briefly, 12 to 24 hours after cell seeding, plates (cells and fibrin gels) were fixed with 4% paraformaldehyde in DPBS at room temperature during 15 minutes. The fixed plates were washed for 15 minutes in DPBS and photographs were taken (Nikon, USA, Garden City, NY). Cell visualization was performed by staining cultures with propidium iodide. Plates were fixed and washed as described above. Attached cells were permeabilized by incubation in 1% Triton X-1000.1% gelatin in DPBS for 30 minutes, washed 5 minutes in DPBS and then stained with propidium iodide (20 µg/ml) in DPBS containing RNase A (0.5 mg/ml) for 30 minutes at room temperature. After 3 washes with DPBS and water, observations were carried out with an LSM 410 Zeiss confocal imaging system. Confocal microscopy (λ = 543 nm) was associated with light reflected microscopy of fibrin gel. Micrographs represent serial optical sections (from the bottom to the top of the fibrin gel by steps of 5 µm).
Gel densitometry
Cells cultured in normoxia or hypoxia, were fixed with 4% paraformaldehyde in DPBS at room temperature for 15 min and then washed 3 times with DPBS. Cell-to-gel contrast was improved by staining the plates with an aqueous solution of 0.2% phloxine for 2 minutes. The culture dishes were analyzed by means of the SAMBA 2005 image cytometer (Alcatel TNT; Grenoble, France) connected to a Zeiss laser microscope (objective × 20, 1.3 NA). Gel density was measured by the amount of light transmitted through the fibrin gel. Computer-created coloring enhanced gel structure modifications and fibrin distribution over the culture plates. Two representative regions were analyzed per experiment.

Quantification of cell reorganisation
A quantitative estimate of the extent of the cell network was given by the ratio (expressed in percentage) between cell-free area delimited by the network and the total surface of cell culture on fibrin gel. This evaluation was done as follows: cells were seeded and grown on fibrin, at the onset of cell reorganisation cells were fixed with paraformaldehyde (4%) at room temperature for 10 minutes and stained to enhance contrast with a 50:50 mixture of Azur II 1%-H2O/methylene blue 1% in borax 1%. Cells were then rinsed with distilled water. For quantification of cell reorganisation, the entire cell cultures in Petri dishes were observed and photographed using a binocular microscope (LEICA CLS 150E) coupled to a digital camera (SONY 3CCD, software Vision Explorer, ETC3000, Graftec). Measurements were performed using Visiolab System (BIOCOM). At least 4 to 8 Petri culture dishes were quantified for each culture condition.

Immunoaassay of fibrin degradation products
The rate of fibrin degradation was assessed by ELISA as previously described (Vailhé et al., 1999). Briefly, after the conditioned culture media were allowed to adsorb onto 96-well Nunc-immuno plates overnight at 4°C, the excess medium was removed from the wells and 100 μl of 2% bovine serum albumin (BSA) in DPBS was added for 35 minutes at 37°C to saturate the wells. Each well was washed at room temperature for 5 min with 200 μl DPBS containing 0.05% Tween 20; then 100 μl of the polyclonal rabbit anti-human fibrinogen antibody, was added to each well. After a two hour incubation at room temperature, the antibody excess was removed and the wells were washed 3 times for 5 min with 200 μl DPBS-Tween 20 and once with DPBS, after which the peroxidase substrate was added. Absorbance was measured at 450 nm 5 minutes later, using an ELISA microplate reader (Labsystem, Multiskan MCC/340, type 347). Results are expressed as the percentage of total fibrin gel degraded (mean ± s.d.).

Effect of prolonged hypoxia on cell proliferative rate
Since hypoxia is known to stimulate angiogenesis in some organs in vivo, we first investigated the effect of hypoxia on cell proliferation in vitro.

Experiments were conducted with EA.hy926 and HUVEC cells. Cells were grown under either standard oxygen tension (20% O2) or low oxygen tension (3% O2) over 9 days and passaged every third day. They were counted after each passage. Figures 1A and 1B show that the ratio between cells counted in hypoxia versus normoxia is approximately 1 suggesting that hypoxia does not affect the rate of proliferation of endothelial cells.

As VEGF has been linked to new capillary formation in vivo under hypoxia, we investigated the effect of hypoxia on the secretion of this factor during cell cultures.

EA.hy926 cells cultured under 3% O2 responded by producing significantly more VEGF165 as early as after 3 days (VEGF accumulation reached 132.8 ± 31.2 pg/ml/10⁶ cells after a 9-day hypoxic exposure versus 17.9 ± 11.1 pg/ml/10⁶ cells in normoxia, n = 3) suggesting a potential role for VEGF as a survival factor under these hypoxic conditions. Nevertheless, VEGF was barely detected in HUVEC culture medium at any time tested during culture in normoxia or hypoxia.

Under hypoxic conditions endothelial cells cultured on fibrin gels failed to undergo their morphologic changes seen in normoxia
EA.hy926 and HUVEC in routine culture conditions adopted the characteristic cobblestone pattern of endothelial cells at confluence. In contrast, HUVEC (Vailhé et al., 1996) and EA.hy926 cells plated on fibrin gels changed their morphology within 24 and 12 ± 4 hours, respectively. Figures 2A and 2B show that EA.hy926 cells (A) and HUVEC (B) adopted the same spatial reorganisation on fibrin. As we have already observed with HUVEC, the reorganisation of EA.hy926 was strongly inhibited by 0.125 μg/ml α2-antiplasmin in the culture medium (data not shown). When the A549 tumor cells, the non endothelial parent of EA.hy926 were cultured onto fibrin they failed to reorganise (data not shown). Thus the behaviour of EA.hy926 was related to their endothelial parent.

Quantification of cell rearrangement showed that after a 3 to 9 days hypoxic pretreatment, both cell types grown at 3% oxygen tension and then seeded on fibrin gels demonstrated less reorganisation (Fig. 3). After three days of hypoxic conditions.
exposure, endothelial cells responded to hypoxia in a similar way, with rearrangement reduced approximatively by 50% compared to normoxic cells (Fig. 3A, B). However, the response of the two cell types were different after 6 to 9 days of hypoxic preatreatment. After a 9-day exposure to hypoxic conditions, EA.hy926 cell rearrangement decreased to 79% (Fig. 3A), in contrast HUVEC demonstrated only a slight difference between cell reorganisation in normoxia and hypoxia.

Interestingly, we noted that EA.hy926 initially maintained in hypoxic environment for 6 days and then reexposed to normal oxygen tension at the time of seeding on fibrin gels, maintained their hypoxic phenotype (data not shown). However, the effect was completely reversible if cells were reexposed to standard oxygen tension during at least 6 days after a 9-day hypoxic episode. They reorganised into cord-like structures at the same rate as cell which were never subjected to hypoxia. These experiments were not conducted with HUVEC because significant changes in morphology were observed in HUVEC after 3 passages.

Although VEGF was increased during EA.hy926 cellular proliferation under hypoxic conditions, once the cells were plated on fibrin gels VEGF was barely detected in normoxic or hypoxic cell media. These results suggested either that secreted VEGF has no direct effect on cell reorganisation or that the secreted quantities were insufficient to stimulate reorganisation under these conditions.

To test the second possibility, human recombinant VEGF165 was added to culture media at a final concentration of 40 and 80 ng/ml as cells were plated on fibrin gels. Exogenous VEGF had no significant effect on normoxic cells, while it barely stimulated the network formation of hypoxic cells. The optimal effect was obtained with 40 ng/ml, nevertheless, the structures remained much less numerous than that formed by normoxic cells. These results suggested that a defect in VEGF secretion may not be solely implicated in the inhibition of cellular rearrangement after prolonged hypoxia.

Since degradation of fibrin was shown to be crucial for cell rearrangement (Vailhé et al., 1999; Pepper et al., 1996), we next postulated that hypoxia may affect the fibrinolytic activity of endothelial cells.

**Fibrin gel degradation and cell behaviour under normoxia and hypoxia**

Fibrin gel degradation was measured in normoxia and hypoxia for the two cell types when the major effect on cell...
reorganisation was observed (after 9 days for EA.hy926 and 3 days for HUVEC, respectively). Results were expressed as the percentage of total fibrin gel degraded. For EA.hy926 cells, there was no significant difference in the quantity of FDP detected in media from hypoxic and normoxic cultures (6.4 ± 1.8 % and 8.2 ± 2% in normoxia and hypoxia respectively, n = 6). With HUVEC there was also no significant difference in the quantity of FDP detected in media from hypoxic and normoxic cultures (2.8 ± 1.4% and 4.4 ± 3.1% in normoxia and hypoxia respectively, n = 3).

Although the same amount of fibrin was degraded, it was possible that the pattern of the degradation products was different. Because HUVEC and EA.hy926 reorganisation was dependent on the plasminogen/plasmin system and responded to hypoxia in the same way, we analysed culture media from normoxic and hypoxic EA.hy926 seeded on fibrin gel by Western blot.

The products of fibrin digestion by plasmin were also included to determine whether fibrin degradation was plasmin-dependent in both groups of cells. Immunoblot analysis revealed no major differences between either culture media and plasmin-treated fibrin as most abundant fragments were in the range of 45000 to 250000 daltons (arrows, Fig. 4). In addition EA.hy926 cells cultured under hypoxia or normoxia exhibited the same fibrinolytic activity. We conclude that the difference in matrix degradation does not account for the inability of hypoxic cells to form tubes or cord-like structures. As reorganisation of the endothelial cells was also associated with fibrin remodeling, we compared the reorganisation of fibrin gels by cells precultured in normoxia and hypoxia.

**Fibrin remodeling under normoxia or hypoxia**

The organisation of fibrin has been illustrated with fibrin gels from EA.hy926 cells cultured under normoxia and hypoxia. The density of fibrin remaining in the culture dishes was analysed using computer-enhanced coloring, yellow representing the highest density of fibrin gel and red the lowest. Figure 5A shows that fibrin gels do not polymerise evenly. Plates incubated without cells exhibit large yellow areas alternating with small orange-yellow spots, indicating that fibrin gel presented a slight variation of density. As cells cultured in normoxia reorganised, areas devoid of fibrin (red) alternating with fibrin-rich areas punctated with numerous discrete yellow-orange spots were observed (Fig. 5B). These spots represented fibrin condensed in the neighbourhood of cells. At areas of confluent cells, the matrix appeared intact (bright yellow next to ring-like structures), but where cells formed cord-like structures the fibrin seemed to have been first digested, then partially recondensed on the outside of ring-like cellular structures separating a redish area (arrow, Fig. 5B).

Figure 5C illustrates the fibrin distribution in a representative plate exposed to low pO2 for 9 days. There is partial degradation (arrow, Fig. 5C) however areas devoid of fibrin were never observed.

The position of the cells (red) with respect to the rearranged fibrin (white) at the boundary of cell-free areas (black) is clearly demonstrated in sequential sections performed on normoxic cultures. The cells have aligned along the fibril-like fibrin, leaving large areas free of cells. Reconstruction of a
three-dimensional image by the superimposition of the 6 sequential sections enhances the visualization of the cord-like structure embedded in remodeled fibrin (Fig. 6). This cord-like formation was similar to the tube formation described by Vailhé et al. (1996, 1999). The local rearrangement of the fibrin gel is better seen in an area where cells have detached from their fibrin support (Fig. 7). These results support the hypothesis that hypoxic cells did not effectively remodel fibrin.

Discussion

Angiogenesis represents a physiological adaptation of a tissue to hypoxia or ischemia. Vascular responses to hypoxia requires that cells first survive the hypoxic shock, then adapt to the lack of oxygen before cell proliferation and rearrangement can occur. In vivo, the vascular response to hypoxia is mediated by the endothelial cells as well as underlying tissues. Our interest was to understand the contribution of the endothelium in the vascular response to changing pO₂.

To this end we used in vitro models mimicking some aspects of the angiogenic process. In our models, HUVEC and EA.hy926 plated on fibrin adopted a distinct morphology with the formation of a complex network of cord-like and tube-like structures within 12 to 24 hours (Fig. 2). We have shown that in response to hypoxia this process of tube or cord formation was inhibited by 50% in primary HUVEC and EA.hy926. It is clear that primary HUVEC represent a better indicator of physiologic responses than a cell line did.

Fig. 5. Remodeling of fibrin gel during EA.hy926 cell reorganisation. Computer-generated colors to visualize gel patterning, with fibrin density increasing from red to green. Fibrin gel alone shows a certain amount of heterogeneity (A). Cells grown under normoxic conditions degrade and reorganize the gel matrix (B, black arrow). Cells grown under hypoxic conditions degrade (C, black arrow) but do not reorganize the matrix.
However, their use was restricted, if they were not cultured with growth factors. After several passages they have numerous properties changed, specifically their ability to form tubes as we show in Figure 3B. Thus, in the framework of our study, the response of endothelial cells to three successive hypoxic shocks can be better assayed with the EA.hy926 cell line rather than with HUVEC, insofar as the endothelial properties of EA.hy926 were maintained. Our results give the following...

Fig. 6. Visualization of cells and fibrin lattice by combined confocal and reflexion microscopy. Serial transversal sections of the tube-like structure (A–F) obtained after staining a normoxic culture with propidium iodide were analysed by combined confocal and reflexion microscopy. Micrographs represent optical sections located at −15 (A); −10 (B); 5 (C); 0 (D); 5 (E); 10 (F) from the bottom to the top of the gel. The cells (red) are seen to align along the condensed fibrin lattice (arrow) surrounding areas devoid of cells and fibrin (black). G. Three-dimensional construction obtained by superimposition of the 6 transversal sections, using an optical software. Bar = 100 μm.

Fig. 7. Gel lattice in absence of cells. Confocal microscopy of the fibrin lattice (white) after most of the cells had detached (as described in Materials and methods). Fibrin was distorted and compacted under the traction of cells. Bar = 100 μm.
evidence: they degrade and remodel fibrin to form cord-like structures as HUVEC do when forming tubes (Vailhé et al., 1996). Moreover, their non-endothelial parent, the A459 cells were unable to reorganise on fibrin, as others have observed on different matrices (Bauer et al., 1992), thus, EA.hy926 appear to be a valuable tool to study some of the biochemical and biomolecular events associated with the angiogenic process under hypoxia. In some ways, they permitted a more detailed investigation of the process. For example a 9-day exposure amplified the response to hypoxia, the inhibition of rearrangement increased from 50% to 79% (Fig. 3A). They also provided useful information about the adaptation process, since we observed that the full reorganisation was restored when hypoxic cells were replaced during at least 9 days in normoxia.

Numerous studies suggest that VEGF is required not only for vascular proliferation but also for the maintenance of vessels providing a positive survival signal. Angiogenic activity attributed to VEGF may be due in part to its ability to enhance endothelial cell survival (Gupta et al., 1999; Nör et al., 1999; Gerber et al., 1998; Meeson et al., 1999; Benjamin and Keshet, 1997). We postulated that if VEGF is a survival factor, its secretion should be increased in endothelial cells exposed to hypoxia. In our culture conditions, we observed that hypoxia stimulated the production of VEGF by EA.hy926 cells which also maintained their proliferative rate. Nevertheless, we failed to detect VEGF in culture media of HUVEC although they also maintained their proliferative rate. It is likely that the production of VEGF was too low to be detected. A few laboratories have previously investigated VEGF mRNA expression in HUVEC (Namiki et al., 1995; Minchenko et al., 1994; Liu et al., 1995; Nomura et al., 1995). Some results showed an increase of VEGF expression under hypoxia suggesting an autocrine loop of VEGF (Helmlinger et al., 2000). Whether, in vivo, VEGF is supplied to endothelial cells by an autocrine or paracrine manner is still under investigation.

We postulated that, if VEGF is secreted by the surrounding stroma, the addition of VEGF in culture media will simulate some aspect of the role of VEGF under hypoxia. Thus, we added exogenous VEGF to our angiogenesis assay, but we failed to restore the full capability of endothelial cells to reorganise. Thus with our model we conclude that VEGF may be involved in cell survival but other factors likely act in concert to induce reorganisation.

Beside growth factors, proteases play a crucial role in regulating cellular activity. Plasminogen activators and MMPs (matrix metalloproteinases) act in the local breakdown of the microvascular basement membrane. In vivo, the matrix is complex and, upon stimulation of endothelial cells, it is degraded by MMPs as well as plasmin, giving rise to many degradation products which may stimulate angiogenesis. In our simplified model, fibrin is the only exogenous matrix protein and we have previously shown with HUVEC that fibrin degradation is necessary but not sufficient for the formation of ring-like and cord-like structures (Vailhé et al., 1999). Our assay with EA.hy926 indicates that plasmin was implicated in the fibrin degradation occuring in the early steps of angiogenesis. Obviously, fibrin matrix was degraded in our model since the addition of α2-antiplasmin to the culture medium inhibits cell reorganisation. Local disruption of the fibrin lattice is required so that cells can migrate and engage in morphogenetic events. The gel densitometry and confocal microscopy (Figs. 5 and 6) suggested that fibrin gel remodel-

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In conclusion in our in vitro model hypoxic cells did not rearrange because they failed to induce fibrin contraction. It is likely that prolonged hypoxia induces modification of a wide variety of factors that integrate information and cytoskeletal organisation in order to alter the structure of fibrin which then may facilitate or induce cellular interaction.

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