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Origin of Periendothelial Cells in Microvessels Derived From Human Microvascular Endothelial Cells

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Abstract

In microvessels, periendothelial cells expressing alpha smooth muscle actin (α SMA) interact with the endothelial cells and are essential for vessel maturation and stabilization. In adult tissues, the cellular origin of the periendothelial cells is still not clear, in particular in humans. To determine the origin of human periendothelial cells, we used a recently-developed 3D co-culture system that mimics human skin connective tissue. This system is composed of normal human dermal fibroblasts (NHDF), human dermal microvascular endothelial cells (HMEC-1), and a collagen matrix. In this system, “microvessels” composed of an endothelial lumen with associated with periendothelial cells develop. Using this co-culture system, we (i) labelled fibroblasts with the vital dye CFDA-SE, cultured them with unlabeled endothelial cells, and observed that only endothelium-associated CFDA-SE-labelled cells express α SMA; (ii) infected endothelial cells with a retrovirus stably expressing eGFP, cultured them with unlabeled fibroblasts, and observed that cells expressing α SMA did not co-express eGFP, but were associated with the eGFP-expressing endothelial cells of the microvessels. Together, these results indicate that periendothelial cells arise by differentiation from fibroblasts and that they require interaction with endothelial cells to do so.

Keywords

angiogenesis; pericyte; 3D co-culture; stem cell markers; tissue engineering

1. Introduction

Periendothelial cells, also called pericytes, are found surrounding capillaries, precapillary arterioles, postcapillary venules, and collecting venules, where they form a discontinuous layer and are in close contact with the endothelial cells (Allt & Lawrenson, 2001; Armulik, Abramsson, & Betsholtz, 2005; Sims, 1986). Periendothelial cells are essential for vessel maturation and stabilization by depositing extracellular matrix and inhibiting endothelial cell proliferation and migration. Due to their expression of contractile proteins, such as the smooth muscle cell marker α -smooth muscle actin (α SMA), they regulate the blood flow and pressure to provide homeostatic control (Allt & Lawrenson, 2001; Armulik et al., 2005; Carmeliet, 2000).

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The origin of the periendothelial cells in microvessels is not clear, especially in human adult tissues, although it is believed that they are of mesenchymal origin (Armulik et al., 2005). In adult tissues, it has been reported that periendothelial cells present in capillaries and in tumor vessels were derived from the bone marrow (Rajantie et al., 2004). However, recently it has been suggested that cells in the peripheral tissues also may serve as precursors of periendothelial cells (Chambers, Leoni, Kaminski, Laurent, & Heller, 2003; Feugate, Li, Wong, & Martins-Green, 2002; Frid, Kale, & Stenmark, 2002; Ishisaki, Hayashi, Li, & Imamura, 2003; Jones & Jacobson, 2000; Paranya et al., 2001; Tomasek, Gabbiani, Hinz, Chaponnier, & Brown, 2002). Examples are: (i) adult endothelial cells can co-express endothelial and periendothelial cell markers *in vitro* in response to TGF- β treatment or when deprived of FGF-1 (Frid et al., 2002; Ishisaki et al., 2003; Paranya et al., 2001); (ii) in culture, primary adult fibroblasts are able to express periendothelial cell contractile proteins in response to both chicken chemotactic and angiogenic factor (cCAF), the chicken homolog of human Interleukin-8 (IL-8), and TGF- β (R. C. Chambers et al., 2003; Feugate et al., 2002); (iii) *in vivo*, during wound healing, TGF- β and cCAF/cIL-8 stimulate the differentiation of fibroblasts into myofibroblasts, which express contractile proteins similar to those expressed by periendothelial cells (Feugate et al., 2002; Tomasek et al., 2002).

We have previously established a “skin” organ culture composed of microvascular endothelial cells, primary normal human dermal fibroblasts, and normal human epidermal keratinocytes (Martins-Green, Li, & Yao, 2005). In this organ culture system, after 10–12 days of co-culture, microvessels form and have associated with them α SMA-producing cells, whether or not keratinocytes are present. This observation suggests that the endothelial cells, fibroblasts, or both are differentiating into periendothelial cells in our co-culture system. This system closely mimics many aspects of human skin, hence our findings also imply that these cells may differentiate into periendothelial cells *in vivo*. Using specific labeling of human endothelial cells and fibroblasts, we show here that fibroblasts, but not endothelial cells, differentiate into periendothelial cells in this 3D co-culture system.

2. Materials and Methods

2.1. 3D human co-culture system

HMEC-1 (human microvascular endothelial cells; CDC, Atlanta, GA) and primary normal human dermal fibroblasts (NHDF; Cambrex, Walkersville, MD) were used to generate a 3D co-culture system that closely mimics human connective tissue (Martins-Green et al., 2005). The cell culture media used to culture the endothelial cells and fibroblasts were EGM-2MV and FGM2, respectively (Cambrex, Walkersville, MD). To generate the co-culture system, an interstitial collagen matrix, Vitrogen collagen (Cohesion, Palo Alto CA), was combined at 4°C with 10X 199 (GIBCO, Carlsbad, CA), and 0.33N NaOH at 2:1:17 ratio. The fibroblasts were evenly resuspended within this matrix at a concentration of 1×10^5 cells/300 μ l matrix, and were then placed in 24-well plates at 37°C for 30 minutes to solidify. After the collagen layer gelified, a suspension of 1×10^5 HMEC-1 cells/300 μ l EGM-2MV was added to cover the collagen gel, and these endothelial cells were allowed to attach for 4 hrs. After cell attachment, the media was removed and a new fibroblast-containing collagen suspension, prepared using the method described above, was applied on top of the endothelial cell layer. After the upper collagen gel gelified, 1 ml of EGM-2MV was added on top of the culture system. This co-culture system was incubated at 37°C for 10–12 days, and the media was changed every 24 hours (Martins-Green et al., 2005).

2.2. Diff Quick staining

After 10–12 days of culture, the 3D cultures were fixed in 4% paraformaldehyde for 2 hrs, then washed with PBS and quenched with 0.1M glycine in PBS for 30 mins. After quenching,

cultures were incubated in 15% sucrose at 4°C overnight, then incubated in 30% sucrose at 4°C overnight, and subsequently embedded in Tissue-Tek O.C.T (Optimal Cutting Temperature) compound (Sakura Finetek USA Inc, Torrance, CA). Sections 6µm thick were obtained using a cryostat microtome (International Medical Equipment Inc.) and placed on gelatin-coated slides. The slides were stained using the Diff-Quick kit (Lab Aids); slides were initially placed in solution 1 (composed of an anionic/acidic dye (eosin) that stains positively-charged proteins red) for 20 mins and rinsed with ddH₂O. The slides were then placed in solution 2, composed of a cationic dye (methylene blue and derivatives that stain nuclei and negatively charged molecules blue) for 20 mins, rinsed with ddH₂O, and then mounted using krystalon (Fisher Scientific).

2.3. Immunostaining

After 10–12 days, 3D cultures were fixed in 4% paraformaldehyde for 2 hrs, embedded and sectioned as described above, and sections were placed on gelatin-coated slides. For immunostaining, sections were rinsed with PBS, fixed in 4% paraformaldehyde for 15 mins, washed with PBS, incubated in 0.1M glycine for 10 mins, and blocked with 10% preimmune serum of the species of the secondary antibody. Slides were subsequently incubated with mouse anti-human PECAM-1 (CD31) at a dilution of 1:500 (R&D Systems, Minneapolis, MN) or rabbit anti-human VE-Cadherin at a dilution 1:200 (Sigma- Aldrich, St. Louis, MO), or mouse anti-human collagen type IV (Sigma-Aldrich) at a dilution of 1:200, or mouse anti-human fibronectin (EP5) (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:200, or mouse anti-human β-catenin (BD Biosciences, San Diego, CA) at a dilution of 1:50 at room temperature 1 hr, followed by washing and incubation with goat anti-mouse Cy3 (Jackson Immuno Research, West Grove, PA at a dilution of 1:1000), or goat anti-mouse FITC (DAKO, Denmark) at a dilution of 1:50, or donkey anti-rabbit Alex 594 (Invitrogen, Carlsbad, CA) at a dilution of 1:500, or goat anti-rabbit FITC (Zymed, S. San Francisco, CA) at a dilution of 1:50 at room temperature for 1 hour. For αSMA immunostaining, cultures were blocked with 3% BSA in 0.1% Triton X 100 in PBS. The sections were incubated with monoclonal anti-α smooth muscle actin-Cy3 antibody (Sigma-Aldrich, St. Louis, MO) at a dilution of 1:200 at room temperature for 1 hr. Sections were then washed with 0.1% BSA in PBS and mounted using Vectashield (Vector Laboratories Inc., Burlingame, CA. For immunostaining with stem cell markers, the fibroblasts were cultured to confluency in the 35mm culture plates or in the Lab-Tek II Chamber slides (Nalge Nunc International, Naperville, IL), and fixed as described above. The cells were then incubated with rabbit anti-human Nanog (Chemicon, Temecula, CA) at a dilution of 1:250, or mouse anti-OCT4 (BD Biosciences) at a dilution of 1:250, or mouse anti-human VCAM-1 (CD106) (Chemicon) at a dilution of 1:50 at room temperature for 1 hr, followed by washing and incubation with goat anti-mouse FITC (DAKO, Denmark) at a dilution of 1:200, or goat anti-rabbit FITC (Zymed) at a dilution of 1:50 at room temperature for 1 hr. Fluorescent images were taken a Nikon MICROPHOT-FXA or a Leica SP2 scanning confocal microscope. 3-D image reconstruction of confocal images was performed using the Imaris software (Bitplane AG, Switzerland).

2.4. Labeling of cells with CFDA-SE vital dye

CFDA-SE (Molecular Probes, Eugene, OR) was reconstituted in DMSO. Endothelial cells or fibroblasts were treated with 10 µM CFDA-SE for 15 mins in 37°C 5% CO₂. After a 15 min incubation, cells were washed with 1X Tris-glucose (27.8mM Tris, 136.8 mM NaCl, 5.15 mM KCl, 0.7 mM Na₂HPO₄, pH 7.4), trypsinized for 3 mins in 37°C, and then used to generate the co-culture system.

2.5. Retrovirus-labelled endothelial cells

The eGFP (enhanced green fluorescent protein) open reading frame was cloned from pEGFP-N1 (Clontech, Mountain View, CA) into the EcoRI/BamHI site in the retroviral vector pIB-2. The pIB-eGFP vector was packaged using a VSV-G-producing 293T packaging cell line to generate the complete retrovirus. Fresh retroviruses were collected, filtered, and used to infect HMEC-1. Infected cells were sorted 3 days later with a FACSaria instrument (BD Biosciences, San Jose, CA), and cells expressing eGFP 1000-fold over the background level were collected and expanded under optimal growth conditions. After expansion, the eGFP-labelled cells were examined with confocal microscopy, and then used to generate the co-culture system.

2.6. Treatment of fibroblasts with EC conditioned media

Endothelial cells were cultured with EGM-2MV medium for 48 hours, the conditioned medium collected and centrifuged to eliminate dead cells. This conditioned medium was then used to treat fibroblasts for 8, 10, and 12 days with change of conditioned medium every 24 hours. Control fibroblasts were treated with EGM-2MV medium alone. The fibroblasts were then immunolabelled with anti- α SMA (see above Immunostaining section), and the cells labelling for α SMA counted/HPF (high power field).

2.7. Statistical Analysis

In table I data are shown as mean $\pm$ standard deviation (SD). Data analysis was performed using the unpaired Student's t-test on raw data using GraphPad Instat software (GraphPad Software Inc.).

3. Results

3.1. Development of microvessels in a 3D tissue culture system that mimics human skin connective tissue

Human skin connective tissue consists primarily of blood vessels, fibroblasts, and extracellular matrix (Sorrell & Caplan, 2004). To mimic this primary human dermal connective tissue structure, human dermal microvascular endothelial cells (HMEC-1) were co-cultured with primary human adult dermal fibroblasts (NHDF) in our 3D culture system, which contains collagen types I and III (Junqueira, Montes, Martins, & Joazeiro, 1983). After preparation, the complete culture system was allowed to develop for 10–12 days. Diff Quick staining of cross sections on day 12 showed formation of a network of tubes, each consisting of a layer of cells with a well-formed lumen (Fig 1A and 1B). This “microvessel” network was embedded in the collagen matrix containing fibroblasts. In addition, several cells were observed adjacent to some microvessels. In order to ascertain that the observed network of tubes was formed by endothelial cells, we immunostained the sections with an antibody against PECAM-1, a specific marker of endothelial cells (Fig. 1C and D). Much like microvessels *in vivo*, the tube-like structures are composed of endothelial cells that are surrounded by a well-defined basal lamina (Fig. 1E and F).

3.2. Periendothelial cells expressing α SMA are associated with the “microvessels” in the 3D co-culture system

The data described above show that the endothelial cells in our system form microvessel-like structures and that other cells are associated with portions of the “microvessel” wall. To determine whether the latter cells could be periendothelial cells, we immunostained sections of the 3D culture system with the periendothelial cell marker α SMA (Fig. 2A and B) (Armulik et al., 2005; Gerhardt & Betsholtz, 2003). In all cases, the cells that stained positively were closely associated with the microvessel walls but some regions of the vessel wall were not

associated with such cells, suggesting a discontinuous periendothelial cell layer surrounding the microvessels similar to what is seen with pericytes *in vivo* (Shepro & Morel, 1993).

To ensure that the presence of cells containing α SMA in our co-culture system was not due to contamination of the cell population with α SMA-positive cells, fibroblasts and endothelial cells were immunostained with α SMA prior to their use in the 3D culture. We found that neither of these cell populations contained α SMA-positive cells before they were used to generate the 3D culture (Fig 2C–F). Furthermore, to look into the possibility that stem cells are present in the fibroblast cultures which could eventually differentiate into α -SMA-producing periendothelial cells we stained cultures of fibroblasts for: (1) Oct4, a transcription factor needed for the replication and maintenance of the stem cell phenotype (Takahashi & Yamanaka, 2006); (2) VECAM, a marker for mesenchymal stem cells (Kolf, Cho, & Tuan, 2007); and (3) Nanog, a transcription factor that is known to be expressed in stem cells but not be needed for maintenance of the stem cell phenotype (Takahashi & Yamanaka, 2006) and that is also expressed in fibroblasts isolated from porcine skin (Carlin, Davis, Weiss, Schultz, & Troyer, 2006). We also used embryonic stem cells as positive control for both Oct4 and Nanog (Fig. 3A and B) and the fibroblast cultures stained only for secondary antibody against anti-nanog as negative control (Fig. 3C). We found that the fibroblasts did not stain for Oct4 (Fig 3D) or VECAM (Fig 3E) but staining for Nanog was found in virtually all fibroblasts (Fig. 3F) as also shown previously for porcine fibroblasts (Carlin et al., 2006). This suggests that Nanog is a transcription factor involved in maintaining the plasticity of fibroblasts.

3.3. Origin of periendothelial cells associated with “human” skin microvessels

In order to determine the origin of the periendothelial cells in our 3D culture system, our first strategy involved labelling one cell type with the vital dye CFDA-SE and then co-culturing these labelled cells with the other cell type, which was left unlabelled. Therefore, one cell type could be distinguished from the other in sections of the 3D culture system, allowing us to determine the cellular origin of the periendothelial cells based upon whether or not they possessed the vital dye. When CFDA-SE labelled cells divide, labelling is distributed equally between daughter cells, decreasing the fluorescence of each cell to half that of the parent cell. In order to know how long this dye can be detected in the endothelial cell and fibroblast populations, we incubated each cell type with 10 μ M CFDA-SE for 15 mins and then replaced the media to remove the excess dye. The labelled cells were then cultured for several days. Although both types of labelled cells retained the green vital dye in 2D cultures for at least 12 days post labelling, the green dye retained by the labelled endothelial cells was lower than that of labelled fibroblasts, (Fig. 4A and B). Therefore, we performed our studies using CFDA-SE-labelled fibroblasts co-cultured with unlabelled endothelial cells. Labelled fibroblasts, like unlabelled fibroblasts, did not express α SMA (Fig. 4C–E). Sections of these co-cultures were immunostained with α SMA (Fig. 5A–F). α SMA was positive only in periendothelial cells associated with the microvessels (Fig. 5B, E) and the stained cells also contained vital dye (Fig. 5C, F), showing that these cells were derived from the fibroblasts. Moreover, the lack of α SMA-staining in fibroblasts distant from the microvessels strongly suggests that direct interaction with endothelial cells is required for fibroblast differentiation into periendothelial cells. To further confirm this possibility we cultured for several days fibroblasts in the presence of endothelial cell conditioned medium which contains soluble factors secreted by these cells. The results showed that the conditioned medium had no effects on increasing the number of α SMA-producing cells (Table I). A few fibroblast-derived cells attached to the microvessels did not express α SMA (double arrowheads in Fig. 5C and F), which could indicate that they did not have sufficient time to fully differentiate.

3.4. Endothelial cells labelled with eGFP show that they do not give rise to periendothelial cells

The results described above indicate that in our 3D co-culture system periendothelial cells are derived from fibroblasts. However, they cannot distinguish between fibroblast differentiation into periendothelial cells and derivation of the latter by cell fusion of endothelial cells with fibroblasts. To distinguish between these possibilities, we used a retroviral vector encoding eGFP to infect and thus label endothelial cells. A pure population of eGFP-expressing endothelial cells was obtained by FACs sorting. These cells did not express α SMA (Fig 6A–C), and hence were used in the 3D co-culture system with unlabeled fibroblasts. Identification of the periendothelial cells within the sections obtained from these 3D cultures by immunolabeling with α SMA showed that none of the α SMA-positive cells co-expressed eGFP (Fig. 6D and E), thereby excluding the possibility of derivation from fusion events between fibroblasts and endothelial cells. In addition, α SMA-positive cells were again only found in the proximity of the microvessels. Furthermore, sections taken from 3D cultures prepared with non-manipulated endothelial cells and fibroblasts and double immunolabelled for α SMA and β -catenin, a molecule present in the junctions formed between periendothelial cells and endothelial cells (Gerhardt & Betsholtz, 2003), were analysed with the confocal microscope of these junctions. The results showed that the staining for α SMA (green) superimposes with that for β -catenin (red) in specific regions of contact between the endothelial cells and the periendothelial cells giving rise to yellow staining. These findings suggest that both cell types interact/communicate (Fig. 6F). In addition, double immunolabeling for Col IV, a component of the basement membrane, and α SMA shows that the periendothelial cells are surrounded by the microvessel basement membrane (Fig. 6G, H) much like *in vivo* (Hirschi & D'Amore, 1996).

4. Discussion

Using our 3D co-culture system, we show that periendothelial cells appear only in the vicinity of lumen-bearing endothelial microvessels that self-organize during the culturing process. These periendothelial cells cover the vessels discontinuously, as do normal pericytes. By using cells labelled with either a vital dye or eGFP, we show that these periendothelial cells are derived from local connective tissue fibroblasts and not from endothelial cells. It is possible that fibroblasts present within the connective tissue *in vivo* not only maintain ECM and connective tissue homeostasis, but also serve as tissue-resident progenitors of skin-associated lineages. Fibroblasts appear to serve as vascular cell progenitors in the injured rabbit carotid artery (Faggin et al., 1999). Large blood vessels, such as the carotid artery, are composed of three distinct layers of tissue, the intima, media, and adventitia. The intima is composed of endothelial cells and provides the innermost structure of the large blood vessels. The media is composed of periendothelial cells, called vascular smooth muscle cells. The adventitia is predominantly composed of fibroblasts and associated ECM. In the injured rabbit carotid artery, cells from the adventitia proliferate and migrate into the media, extending to the intima. In addition, some of the adventitia cells migrating to the media expressed SM22, a specific marker of vascular smooth muscle cells, suggesting that the adventitial cells may be able to differentiate into vascular smooth muscle cells (Faggin et al., 1999). Since the majority of adventitial cells are fibroblasts, it is possible that fibroblasts may serve as periendothelial cell progenitors *in vivo*, lending support to the findings presented here that in our culture system that mimics human connective tissue, periendothelial cells are derived from fibroblasts.

It is interesting that some of the fibroblasts located in close proximity to our microvessels expressed α SMA but others did not. This difference may be due to the extent of time of interactions between endothelial cells of the microvessels and fibroblasts. Interaction between endothelial cells and periendothelial cells through cellular junctions may be involved in

regulating the differentiation of periendothelial cells (Luo & Radice, 2005; Tillet et al., 2005). Adhesion molecules such as N-cadherin, and other associated molecules such as β -catenin, are present in adherens junctions and are critical for appropriate recruitment and differentiation of microvascular periendothelial cells, although the mechanisms involved in the process are poorly understood (Paik et al., 2004). Immunocytochemical and biochemical studies showed that N-cadherin is present at the endothelial-pericytic interface (Gerhardt, Liebner, Redies, & Wolburg, 1999; Liebner, Gerhardt, & Wolburg, 2000). β -catenin binds to the tail of cadherins (Geiger, Yehuda-Levenberg, & Bershadsky, 1995; Kemler, 1993) that when activated translocates to the nucleus, modulates gene expression, and plays a crucial role in cell differentiation. It has been shown that siRNA directed against these molecules does not prevent neovascularization of endothelial cells; rather, it profoundly inhibits the acquisition and attachment of α SMA-expressing cells to the vessel wall *in vivo* and *in vitro*. Therefore, in our study, periendothelial cells expressing α SMA may have already established adherens junctions with endothelial cells that then facilitate differentiation, whereas those cells lacking α SMA expression may not yet have established these critical junctions.

One of the possibilities we posed for the origin of these periendothelial cells in our 3D culture system was the presence of stem cells in the fibroblast population introduced into the cultures. A number of markers for embryonic stem cells have been identified such as Oct4 (Rosner et al., 1990; Scholer, Dressler, Balling, Rohdewohld, & Gruss, 1990; Scholer, Ruppert, Suzuki, Chowdhury, & Gruss, 1990), and Nanog (I. Chambers et al., 2003; Mitsui et al., 2003) that are POU (Pit/Oct/Unc) domain-containing proteins that play a central role in the regulation of pluripotency and self-renewal in mouse and human embryonic stem cells. Together with Sox-2, another core transcription factor, they cooperate to regulate the expression of many genes (Boyer et al., 2005). In addition, markers such as VCAM, have been identified for mesenchymal stem cells in the connective tissue. Recently, it has been reported that adult fibroblasts can be made pluripotent stem cells by transfection with only four factors -- Oct 3/4, Sox2, c-Myc, and Klf4 (Takahashi & Yamanaka, 2006). Interestingly, nanog was not needed for this change in potency. In fact, nanog has been found to be present in porcine dermal fibroblasts (Carlin et al., 2006) and here we show that our cultured dermal fibroblasts also express nanog, suggesting that this protein is important in the maintaining of plasticity found in fibroblasts *in vivo*.

In summary, these results show that our co-culture system promotes the formation of microvessels and that fibroblasts associated with the microvessel endothelial cells differentiate into periendothelial cells that have characteristics similar to those of pericytes. Our findings strongly suggest a role for the fibroblast as a periendothelial progenitor cell *in vivo*. In addition, because the microvessels present in our system appear to be mature vessels with associated periendothelial cells, similar to microvascular structure *in vivo*, our system may be useful to further our understanding of the molecular mechanisms of angiogenesis.

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Figure 1. Microvessel formation in the 3D co-culture system

Human dermal microvascular endothelial cells were co-cultured with human dermal fibroblasts in a collagen gel matrix for 10–12 days. **(A)** Diff Quick staining of sections from the 3D culture shows microvessels associated with periendothelial cells. **(B)** Enlargement of the box in **(A)**. **(C)** Endothelial cells immunostained with PECAM-1 (red). Endothelial cells formed microvessels with lumina. **(D)** Enlargement of box depicted in **(C)**. **(E)** Immunolabelling shows that VE-cadherin labelled endothelial cells (red) are surrounded by a well-defined collagen IV-containing basement membrane (green). **(F)** Enlargement of box depicted in **(E)**. Asterisks indicate microvessel lumina. Arrowheads indicate periendothelial cells **(A, B)**. Scale bars, 50 μm **(A, C, E)**, 20 μm **(B, D, F)**.

Figure 2. Periendothelial cells expressing α SMA are associated with microvessels in the 3D system
 Double staining of our culture system with the endothelial cell marker VE-Cadherin (green) and the periendothelial cell marker α SMA (red). **(A)** Microvessels observed under epifluorescence microscope. **(B)** Microvessels observed under confocal microscope with three-dimensional reconstruction. Asterisks indicate the lumina of microvessels. Periendothelial cells were closely attached to the microvessels and do not form a continuous cell layer around the microvessels, similar to the characteristics of pericytes *in vivo*. **(C–F)** Cells were treated with antibodies to α SMA (red). Blue staining (DAPI) shows cell nuclei. Neither fibroblasts nor endothelial cells expressed α SMA before co-culture. Arrowheads indicate α SMA-expressing periendothelial cells. Scale bars, 20 μ m

Figure 3. Expression of stem cell markers in cultures fibroblasts

Embryonic stem cells (A, B) and cultured fibroblasts (C–F) were immunolabelled for the presence of the stem cell markers Nanog, Oct4, and VCAM. (A–B) Embryonic stem cells immunolabelled for Nanog (A) and Oct4 (B), served as positive control. (C) Fibroblasts stained only for secondary antibody served as negative control. (D–F) None of the fibroblast stained for Oct4 (D) or VECAM (E), but nearly all fibroblasts stained for Nanog (F). Blue staining (DAPI) shows cell nuclei.

Figure 4. CFDA-SE labelled fibroblasts and endothelial cells in 2D cultures
(A) CFDA-SE labelled endothelial cells at 6, 9, and 12 days post labelling. (B) CFDA-SE labelled fibroblasts at 6, 9, and 12 days post labelling. (C–E) Labelled fibroblasts did not express α SMA. Blue staining (DAPI) shows cell nuclei. Scale bars 20 μ m.

Figure 5. CFDA-SE labelled 3D co-cultures

(A, D) CFDA-SE-labelled fibroblasts retained the green fluorescence of the vital dye after 12 days in the co-culture system; endothelial are not labelled. (B, E) Periendothelial cells were immunostained with α SMA (red). (C, F) Merged images from (A, B) and (D, E), respectively, show that α SMA-labelled periendothelial cells in most cases also show the vital dye, indicating they originated from labelled fibroblasts. Asterisks indicate the lumina of microvessels and the lines delineate the vessels themselves. Arrowheads indicate α SMA-expressing periendothelial cells. Double arrowheads indicate fibroblasts that are adjacent to the microvessels but are not yet expressing α SMA. Scale bars 20 μ m.

Figure 6. eGFP-labelled endothelial cells co-cultured with unlabeled fibroblasts show that periendothelial cells differentiated from fibroblasts
 (A–C) eGFP-labelled endothelial cells did not express αSMA prior to co-culture. (D, E) αSMA-expressing periendothelial cells (red) did not express eGFP (green) whereas endothelial cells of the microvessels are labelled with eGFP. DAPI labelling (blue) indicates cell nuclei. (F) Immunolabelled sections prepared from the 3D cultures using endothelial cells and fibroblast that were not manipulated. Periendothelial cells double immunolabeled for αSMA (green), and junction complexes molecule β-catenin (red), showed superimposed staining, indicating that both cell types are in close contact. (G, H) immunolabeling for Col IV shows that basement membrane (red) also surrounds a peri-endothelial cell stained for αSMA (green),

a feature that is characteristic of peri-endothelial cells *in vivo*. Asterisks indicate the lumen of microvessels, lines delineate the vessels themselves and arrowheads indicate α SMA-expressing periendothelial cells.

Table INumber of fibroblasts/HPF that express α SMA when exposed to endothelial cell conditioned medium

Treatment	Control (n=12)	Conditioned media (8day) (n=12)	Conditioned media (10day) (n=12)	Conditioned media (12day) (n=12)
Cell number (Mean $\pm$ SD)	0.75 $\pm$ 0.87	0.58 $\pm$ 0.67 [*]	0.67 $\pm$ 0.78 ^{**}	0.42 $\pm$ 0.67 ^{***}

HPF = High Power Field

n = number of high power fields counted

^{*} Control vs. Conditioned media (8day): p > 0.05^{**} Control vs. Conditioned media (10day): p > 0.05^{***} Control vs. Conditioned media (12day): p > 0.05

p > 0.05 is considered not significant;

p < 0.05 is considered significant;

p < 0.001 is considered a highly significant.