Pericytes influence endothelial cell growth characteristics: Role of plasminogen activator inhibitor type 1 (PAI-1)

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Abstract

Objective: Pericytes, located in close proximity to the underlying endothelium, form an integral component of the microvasculature. These cells are intimately involved in angiogenesis, which is of fundamental importance in many physiological and pathological processes. We evaluated the influence of pericyte-conditioned medium (PCM) on endothelial cell growth characteristics and modulation of endothelial gene expression.

Methods: Migration and tubule formation assays were performed in vitro to determine the effect of PCM on endothelial growth characteristics. cDNA microarray analysis was used to identify alterations in gene expression following exposure of human microvascular endothelial cells (HMEC-1) to PCM. Overexpression of PAI-1 using recombinant protein or transient transfection, and inhibition using an inhibitory antibody against PAI-1, were used to determine whether up- or down-regulation of this gene was responsible for the changes in endothelial cell characteristics observed in response to PCM exposure.

Results: We have shown that PCM exerts a dramatic inhibitory influence on endothelial cell migration in vitro. In addition, endothelial cells cultured on Matrigel™ and exposed to PCM were found to generate significantly fewer angiogenic branches. Microarray analysis of endothelial cells exposed to PCM identified PAI-1 as the gene showing the greatest level of differential expression (3.4-fold induction). Studies using an inhibitory antibody to PAI-1 suggest that induction of this protein by PCM is pivotal to the observed inhibitory influence on the migratory and angiogenic potential of HMEC-1. We further investigated this by overexpressing PAI-1, which was shown to have a potent inhibitory influence on EC migration and angiogenic branching, although the concentration of PAI-1 was clearly important.

Conclusion: Collectively, these findings suggest that PCM contains a bioactive element(s) that controls both endothelial cell migration and tubule formation in vitro and that these responses may be partially controlled by increased endothelial cell expression of PAI-1.

Keywords: Pericytes; Endothelial cells; Angiogenesis; Microvasculature; Plasminogen activator inhibitor 1

1. Introduction

Pericytes are intimately involved in angiogenesis, which is of fundamental importance in many physiological and pathological processes, including atherosclerosis, diabetic retinopathy, rheumatoid arthritis, tumour development and wound healing [1]. Evidence suggests that pericytes play a pivotal role in the maintenance of microvascular integrity via the control of blood flow and regulation of endothelial cell proliferation [2,3]. Pericytes also have a role in the
maintenance of microvascular integrity by the expression of an ecto-ATPase that facilitates platelet aggregation [4]. There is considerable evidence that pericytes are involved in the control of angiogenesis. After destabilization of the vessel wall, endothelial cells proliferate, migrate and form a tube, which is stabilized by pericytes and smooth muscle cells [5]. Ultrastructural analysis of a diverse range of mammalian vascular beds has shown pericytes to be universally distributed, however, levels of abundance appear to be variable [6]. Interestingly, tissues with the greatest number of associated pericytes such as the brain and retina [7] were found to have the lowest microvascular endothelial cell turnover rate [8]. Studies on diabetic retinopathy also support the suggestion that pericytes inhibit endothelial cell growth; prior to the rapid neovascularisation characteristic of this disease, there occurs a selective loss of pericytes associated with the retinal capillaries [7].

In a seminal experiment utilising a co-culture system to examine pericyte–endothelial cell interaction, it was shown that pericytes suppress endothelial cell proliferation [2]. Pericyte modulation of endothelial cell growth was dependent upon contact or proximity and the inhibitory mechanism was mainly due to the activation of latent transforming growth factor beta (TGF-β1). TGF-β1 is a cytokine secreted by both endothelial cells and pericytes and it is known to have a potent influence on cell growth, motility and differentiation [9,10]. There is clearly a high degree of contact between the two cells types, often resulting in an intercellular space of less than 20 nm. In addition, there are numerous mechanisms by which ‘cross-talk’ is facilitated between pericytes and endothelial cells, for example, peg and socket junctions, cell surface adhesion molecules and gap junctions [11]. The areas of pericyte–endothelial cell contact create a possible receptor system in which the cells respond to soluble mediators secreted in the other cell type [12].

Research conducted by our own group [3] suggests that pericytes may exert their influence on microvascular endothelial cells via the secretion of a soluble mediator in a non-contact-dependent manner. It was demonstrated that endothelial cells, when exposed to pericyte-conditioned medium (PCM), exhibited increased mRNA expression of the potent vasoconstrictor endothelin-1. This study was the first to focus on pericyte modulation of endothelial cell gene expression in order to elucidate the mechanism by which pericytes exert their influence on the endothelium. Other researchers have suggested that endothelial cell–pericyte interactions during co-culture lead to alteration in the expression of pericyte adhesion molecules including E-selectin, which, significantly, is not mediated by TGF-β1 [1]. This suggests that endothelial cells and pericytes must communicate via a number of means including the synthesis and release of soluble mediators and production of extracellular matrix molecules.

The ubiquitous distribution of pericytes in normal tissue suggests that they are of significant importance in the formation and regulation of capillary vessels. Identification of vasoactive or growth related genes regulated in endothelial cells by pericytes could help to elucidate the molecular mechanisms involved. In this study we aimed to evaluate the influence of pericyte conditioned medium (PCM) on endothelial cell gene expression and growth characteristics in vitro.

2. Materials and methods

2.1. Cell culture and preparation of conditioned medium

All experiments were routinely carried out at 37 °C in a humidified atmosphere of 5% CO₂/95% air. The immortalised human microvascular endothelial cell line (HMEC-1) was the kind gift of the Department of Ophthalmology, Queen’s University Belfast. The cells were routinely maintained in MCDB-131 medium (Gibco BRL, Paisley, UK) supplemented with 10% fetal calf serum (FCS) (PAA Laboratories, Somerset, UK) epidermal growth factor (EGF, 10 ng/ml) (Roche, East Sussex, UK) and L-glutamine (10 mM) (Gibco BRL, Paisley, UK). Cells were seeded at a density of 1.4 × 10⁵ cells in a T-175 tissue culture flask, grown as monolayers and passed every 3 to 4 days to maintain exponential growth.

Primary cultures of pericytes were established according to the method developed by Gitlin and D’Amore [13]. Cell cultures were maintained in DMEM (Gibco BRL, Paisley, UK) supplemented with 20% FCS, penicillin (20 IU/ml), streptomycin (20 μg/ml) and fungizone (Gibco BRL, Paisley, UK) (2 ml). Cells were grown as monolayers and passed every 5 days to maintain exponential growth. Only cells between passages 2–6 were used in experiments. Purity of the pericyte culture isolated from bovine retina was verified by immunocytochemical staining using an antibody to factor VIII antigen to exclude contamination by endothelial cells.

Serum-free conditioned medium was collected from early passage (2–6) pericytes (PCM) and HMEC-1 cells (endothelial cell conditioned medium; ECCM). Cells were grown until 90% confluent, the monolayer washed twice with PBS and the medium replaced with serum-free MCDB-131. Cells were maintained for 24 h before harvesting the conditioned medium. It was centrifuged at 2000 rpm to remove cellular debris and stored at −70 °C.

2.2. In vitro migration assay

The in vitro migration assay used in these studies is a modified version of the method described by Ashton et al. [14]. Briefly, HMEC-1 were plated (2 × 10⁵ cells/500 μl growth medium) on to uncoated glass microscope slides (BDH Laboratory supplies, Dorset, UK), placed in sterile square tissue culture dishes and allowed to adhere for 1 h. HMEC-1 growth medium (15 ml) was added and the cells
were left overnight to form a monolayer. Cell monolayers were aseptically wounded using the pointed edge of a 200 μm pipette tip; all slides were wounded at the same time and returned to the incubator. Post-wounding, slides were bathed in PCM, ECCM, serum-free MCDB-131 or normal growth medium. One slide was removed every hour until wound closure was observed. The cells were then fixed in 4% paraformaldehyde in PBS for 1 h at room temperature. The slides were washed in PBS, allowed to air dry and protected by a coverslip using Vectashield mounting medium (Vector Laboratories, Peterborough, UK). The extent of ‘wound’ closure was assessed microscopically and quantified using a calibrated eyepiece graticule (1 mm/100 μm graduation) at 20× magnification (Olympus BX 50); the observer was blinded to the treatment. The wound size was determined by measuring the distance from one wound edge to the opposing edge; the edge was identified by focusing on cells which had migrated farthest while still in contact with other endothelial cells. For each slide, 20 measurements were taken in 1 mm² fields of view at random intervals across the length of wound. Two slides were processed in each treatment group and the entire experiment repeated in triplicate. Data were expressed as a percentage of the initial wound size at time zero \( T_0 \). A paired students t test was used to compare the results.

2.3. In vitro tubule formation assay

The in vitro tubule formation assay used in these studies is a modified version of the method described by Ashton et al. [14]. In brief, assays were conducted using BD BioCoat™ Matrigel™ Matrix Thin Layer 24-well Multiwell Plates (BD Discovery Labware, Oxford, UK). The Matrigel™ was rehydrated with 500 μl MCDB-131 serum-free medium and incubated at 37 °C for 30 min. Excess medium was removed and HMEC-1 were seeded at a density of 1 × 10⁴ and the plates incubated at 37 °C under 5% CO₂/95% air for 1 h. Once the cells had adhered, excess medium was removed and replaced with PCM or PCM containing varying concentrations of inhibitory antibody to PAI-1 (1, 3, 6, 12, 25 and 50 ng ml⁻¹; American Diagnostica Inc, Axis-Shield, Cambridgeshire, UK), ECCM, MCDB-131 (– serum) or MCDB-131 (+ serum). An IgG antibody 7.5 ng ml⁻¹ (Sigma, UK) was used as a control for the PAI-1 inhibitory antibody. Plates were incubated for a further 17 h, treatment medium was then gently removed and the cells were fixed as before. Endothelial cells are known to form polygonal structures to form an endothelial cell sprout, a 5* score was given. Five fields of view were recorded for each well, with each treatment group comprising 4 wells. An independent investigator assessed each well blindly. Each data point was expressed as an average of 3 readings per well in 3 independent experiments. An unpaired students t test was used to compare the results for PCM, ECCM and serum-free controls. Data were expressed as the mean number of cell-to-cell tube connections.

2.4. cDNA microarray analysis

In order to evaluate differential gene expression induced in HMEC-1 exposed to PCM, a microarray screen was carried out. HMEC-1 were grown to approximately 90% confluence, the monolayer washed twice with PBS and exposed to PCM for a period of 6 h, cells treated with ECCM were used as a control. Total cellular RNA was isolated using the guanidium acid phenol method [16] and the mRNA then isolated using Oligotex Kit (Qiagen, West Sussex, UK). Approximately 800 ng of Poly A+ mRNA (50 ng/µl) from cells exposed to PCM/ECCM was dispatched to Incyte Genomics, Inc (Delaware, USA), where the cDNA microarray analysis was conducted. Incyte’s Lifearray chips enable the screening of 10,000, 100% verifiable, human genes and provides a highly accurate and quantitative measurement of the relative gene expression level in the ECCM and PCM samples.

2.5. Northern and Western blot analysis

RNA was isolated (as described above) 0–24 h following exposure to PCM or ECCM. 40 μg of RNA was loaded onto a formaldehyde gel. The RNA samples were then subjected to electrophoresis and transferred overnight via capillary action to Nytran super-charged nylon membrane (S&S, Dassel, Germany) in 20× SSC buffer. The membrane was hybridised for 6 h at 65 °C with labelled probe (25 ng), washed and exposed to X-ray film (n=2). The Northern blots were carried out twice with independently isolated mRNA samples and the blots were then analysed by densitometric analysis and the relative levels (after correction for the 18S loading control) of PAI-1 in PCM treated endothelial cells were compared to those in ECCM controls. For Western blot analysis, cells were lysed in Laemmli buffer (Sigma, UK) at various times up to 24 h after exposure to PCM or ECCM. 40 μg of each sample was electrophoresed at 200 V for 1 h on a 10% Bis–Tris–HCl polyacrylamide gel (Invitrogen, UK). Protein was transferred onto a nitrocellulose membrane (Hybond C, Amersham, UK) for 1 h at 30 V. The membrane was blocked in 1% skimmed milk powder (Sigma, UK) for 1 h at room temperature and then incubated with 1:100 dilution of PAI-1 (Oncogene, Research Products, UK) or actin (Sigma, UK) antibody overnight at 4 °C with gentle shaking. The membrane was then washed 3 times in 25 mM Tris (pH 7.5), 150 mM NaCl and 0.05%
Tween 20 and incubated with the secondary antibody horse-radish peroxidase (HRP) (Santa Cruz Biotechnology, Inc, USA) for 1 h with gentle shaking and washed as described above. The SuperSignal West Pico detection kit (Pierce, UK) was used to assess bound antibody. Densitometric analysis was carried out and the relative levels of PAI-1 in HMEC-1 exposed to PCM or ECCM were compared after correction for loading with actin.

2.6. Generation of a plasminogen activator inhibitor type 1 (PAI-1) cDNA probe

Full-length PAI-1 cDNA was the kind gift of Dr Robert Gerard, University of Texas Southwestern Medical Center. The plasmid DNA was digested with EcoR I and Hind III to release the PAI-1 cDNA insert. Gel purified PAI-1 (25 ng) was labelled with [α-32P] dCTP (Amersham, Buckinghamshire, UK) using Prime-it II Random Primer Labelling Kit (Stratagene, Cambridge, UK), according to manufacturer’s instructions. 18S pTRI linearised vector DNA (Ambion, Oxford, UK) was used to generate an 18S loading control probe.

2.7. Cloning of PAI-1 cDNA into pIRES mammalian expression vector

The pIRES mammalian expression vector was digested with the restriction enzymes EcoR I and Sal I to enable directional cloning of the PAI-1 cDNA insert. PAI-1 had previously been excised from the pACCMV vector using EcoR I and Hind III. Further digestion of the PAI-1 cDNA with Sal I enabled ligation of the insert into the pIRES vector in the correct orientation.

2.8. Influence of PAI-1 on HMEC-1 migration

HMEC-1 were plated (2 × 10⁵ cells/500 μl growth medium) on to uncoated glass microscope slides as described above. After overnight culture, growth medium was removed and the cells washed with serum-free MCDB-131. Cells were then transfected in the presence of Lipofectin (Life technologies, Paisley, UK) with 1.5 μg pIRES-PAI-1, pIRES (empty vector) or exposed to transfection reagent alone. Cells were transfected according to manufacturer’s instructions for 6 h. HMEC-1 growth medium (15 ml) was added to each tissue culture dish and the cells incubated for a further 12 h prior to wounding as described previously. Transfection efficiency was determined by counting the number of cells in five random fields of view (when the slide was viewed at ×200 magnification) and calculating the percentage of cells expressing Green Fluorescent Protein (GFP), which is expressed along with protein to HMEC-1 cells according to the methods previously described. The wound size was evaluated at various times as previously described.

2.9. Statistical analysis

All results were compared using the paired Student’s t test.

3. Results

3.1. The influence of PCM on endothelial cell migration

We have previously shown that pericytes can alter gene expression within endothelial cells via secretion of a soluble mediator in a non-contact-dependent manner [3]. Since pericytes may be important in the formation and regulation of capillary vessels we now wanted to assess the functional significance of potential pericyte-induced changes in endothelial cells, by evaluating the effects of PCM on endothelial cell growth characteristics. Firstly, we evaluated the effects of PCM on endothelial cell migration using a wound scrape assay. Essential controls were used in this assay and included, serum-free MCDB-131 medium that had supported the growth of endothelial cells for 24 h (ECCM) and serum-free MCDB-131. The ECCM controls for any effects caused by medium changes without actually introducing new mediators that are not already present in the MCDB-131 medium, and serum-free MCDB-131 represents the fresh medium used before exposure to endothelial cells.

Representative pictures showing the appearance of wounds before, and after 7 h migration in complete medium versus PCM are shown in Fig. 1a. HMEC-1 exposed to complete medium achieved 50% wound closure by 7 h, with total closure occurring after 16 h (Fig. 1b). The rate of wound closure in HMEC-1 monolayers exposed to ECCM or serum-free MCDB-131 was very similar to that of complete medium (Fig. 1b and Table 1). HMEC-1 monolayers exposed to PCM showed a dramatic inhibition of migration so that complete wound closure required 36 h compared with 16–18 h for controls.

When the end point of the experiment was set at 50% wound closure, which normally occurs after 7 h in complete medium controls, cells exposed to PCM displayed a suppressed migration rate (Fig. 1c), which is significantly less than that observed under serum-free or ECCM conditions (Fig. 1c; p = 0.013). Even larger differences were observed at later time points (Fig. 1b).

3.2. The influence of PCM on tubule formation in vitro

Since we had demonstrated suppressed endothelial cell migration following exposure to PCM, we now wanted to evaluate the effects of PCM in another widely used model
of angiogenesis i.e. tubule formation on Matrigel. Representative pictures showing the effects of PCM compared to complete medium are shown in Fig. 2a. Analysis of the data showed no significant difference in the number of angiogenic branches between samples exposed to ECCM and the serum-free control ($p < 0.2946$) (Fig. 2b). How-
ever, there was a significantly lower incidence of branching in HMEC-1 exposed to PCM ($p<0.044$) compared with the ECCM treated control (Fig. 2b).

3.3. cDNA Microarray

cDNA microarray technology was used to identify genes differentially expressed in HMEC-1 cells exposed to PCM compared to ECCM control conditions at 6 h. Genes regulated by PCM within the differential range +3.4 to −1.5 are listed in Table 2. The most up-regulated of these genes was serine (or cysteine) proteinase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1 AF386492, commonly referred to as PAI-1.

3.4. Confirmation of PAI-1 induction by PCM using Northern and Western blot analysis

The microarray screen generated an overwhelming amount of information so it was decided to focus on the most upregulated gene, PAI-1, especially since PAI-1 had been previously implicated in angiogenic-type responses.

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**Table 1**

<table>
<thead>
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<th>Treatment</th>
<th>Time to 50% closure (h)</th>
<th>Time to complete closure (h)</th>
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<tr>
<td>Complete medium</td>
<td>7</td>
<td>16</td>
</tr>
<tr>
<td>ECCM</td>
<td>8.5</td>
<td>18</td>
</tr>
<tr>
<td>Serum-free medium</td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td>PCM</td>
<td>18.5</td>
<td>36</td>
</tr>
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</table>

**Table 2**

<table>
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<th>Differential expression</th>
<th>Gene name</th>
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<tbody>
<tr>
<td>+3.4</td>
<td>Plasminogen activator inhibitor-1 (PAI-1)</td>
</tr>
<tr>
<td>+2.4</td>
<td>Heat shock protein 70 kDa</td>
</tr>
<tr>
<td>+2.2</td>
<td>Splicing factor proline/glutamine rich</td>
</tr>
<tr>
<td>+2.1</td>
<td>Mitogen activating kinase 8</td>
</tr>
<tr>
<td>−1.9</td>
<td>Phospholipase A2, group B1</td>
</tr>
<tr>
<td>−1.8</td>
<td>Phosphoenolpyruvate carboxykinase</td>
</tr>
<tr>
<td>−1.6</td>
<td>ATP-binding cassette, subfamily F</td>
</tr>
<tr>
<td>−1.5</td>
<td>Dynamin 1</td>
</tr>
</tbody>
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**Fig. 2.** The influence of PCM on in vitro tubule formation. (a) Photomicrograph showing (i) the ability of HMEC-1 to form angiogenic cell-to-cell connective structures between individual cells when incubated in wells coated with the synthetic basement membrane Matrigel. The arrows in the micrograph indicated the structures identified as an angiogenic tube. The inhibitory action of PCM on HMEC-1 branch formation can be observed in (ii). (b) Graph showing the effect of PCM on HMEC-1 cell-to-cell tube formation on Matrigel. All data points represent the mean of three individual experiments; error bars indicate standard error of the mean. PCM significantly reduced the number of tubes formed ($p=0.0041$) compared to serum-free medium. There was no significant effect of ECCM, although significant differences between ECCM and PCM responses were observed ($p=0.04$).
Therefore, in order to confirm the microarray analysis, PAI-1 levels were assessed by Northern blot analysis to evaluate increased expression of PAI-1 mRNA in an independent assay. Fig. 3a shows a representative Northern blot. PAI-1 mRNA was detected in cells exposed to both PCM and ECCM treated control endothelial cells. After correction for 18S loading, densitometric analysis revealed that the addition of PCM or ECCM each caused induction of PAI-1 mRNA expression between 1–24 h, but the greatest induction by PCM compared to ECCM occurred between 2–6 h; this is consistent with the relative expression of PAI-1 observed in the microarray at 6 h (Fig. 3b).

Although we had detected increased transcriptional activity of the PAI-1 gene in endothelial cells following exposure to PCM, it was essential to evaluate whether this was translated at the protein level; this is of particular interest as PAI-1 protein is known to be labile at 37 °C. Interestingly, we observed that PAI-1 protein was undetectable immediately following exposure of endothelial cells to PCM, however, PAI-1 protein levels rose progressively from 8–24 h (Fig. 3c and d). This increase in protein is consistent with the kinetics of mRNA induction. PAI-1 protein could not be detected in ECCM treated control endothelial cells, even though PAI-1 mRNA was detected throughout the timecourse (Fig. 3a and b). This suggests that, in addition to factor(s) in PCM which increase transcription, there are additional factors which influence PAI-1 translation or protein stability that are not present in ECCM.

3.5. Influence of PAI-1 expression on HMEC-1 migration

Since we had shown that PAI-1 mRNA and protein levels are induced in endothelial cells in response to PCM, we now wanted to mimic these effects by directly overexpressing PAI-1 in endothelial cells. Firstly, we tested the migratory response of endothelial cells to varying concentrations of recombinant PAI-1 protein (Fig. 4a). At the low PAI-1 concentration of 1 ng ml\(^{-1}\) wound closure occurred more rapidly than in ECCM controls (\(p<0.05\)) indicating that at this concentration PAI-1 is pro-migratory. Conversely, at high concentrations (1000–10,000 ng ml\(^{-1}\)) the wound size is larger than the ECCM control (\(p<0.05\)), indicating anti-migratory effects. This bell-shaped response is consistent with the published literature [17]. We then further evaluated these effects by assessing the full time course for wound closure after exposure to low (1 ng ml\(^{-1}\)) and high (1000 ng ml\(^{-1}\)) concentrations of recombinant PAI-1 (Fig. 4b). As expected, low concentrations of PAI-1, accelerated wound closure, showing a significant difference from ECCM at 3 h and reaching maximum differences by 7 h (\(p<0.05\)). At later times, however, the rate of wound closure was not significantly different from controls. A high concentration of PAI-1 (1000 ng ml\(^{-1}\)), lead to a reduced rate of wound closure reaching maximum differences to that of the ECCM control by 5 h (\(p<0.05\)) indicating anti-migratory effects; but again this effect was lost at later times. This was obviously due to loss of recombinant protein activity since reapplication of PAI-1 (1000 ng ml\(^{-1}\)) at 7 h (Fig. 4c) inhibited wound closure with prolonged effects very similar to that observed for PCM (Fig. 1b).

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Fig. 3. Expression of PAI-1 in endothelial cells following exposure to PCM: Representative Northern blot of total RNA (a) and Western blot of protein (c) isolated from HMEC-1 exposed to PCM or ECCM over a 24 h period. PAI-1 mRNA and protein levels are indicated as well as 18S or actin loading controls. Graphs show densitometric analysis of mRNA bands (b) and protein bands (d). Data are expressed as fold induction of PAI-1 mRNA induction after correction for loading controls.
These data were further supported in experiments to assess the effects of transient overexpression of PAI-1 using a mammalian expression vector. Transfection efficiency was evaluated by counting the number of GFP positive cells (expressed, in concert with PAI-1) in five random fields of view. A transfection efficiency of 8% was achieved. The effects of transient overexpression on endothelial cell migration were equally impressive. Treatment of cells with pIRES-PAI-1 significantly inhibited endothelial cell migration, as the relative wound size remained large, after correction for the effects of empty pIRES vector and lipofectin (Fig. 4d). Interestingly, when the effects of transfection of the PAI-1 construct were compared with the effects achieved by PCM, there was no statistically significant difference between the two. If these data are compared to the dose response using purified PAI-1 protein (Fig. 4a), the effect is consistent with a concentration of 1000 ng ml$^{-1}$ PAI-1 protein present in both the transfected and PCM treated endothelial cells.

3.6. Effect of PAI-1 protein repression on HMEC-1 tubule formation and endothelial cell migration.

We have demonstrated that PCM induces PAI-1 levels in endothelial cells and that high concentrations of recombinant PAI-1 and transient transfection of a PAI-1 expression vector can mimic these effects. In order to confirm a role for PAI-1, up-regulated in endothelial cells by PCM, leading to anti-angiogenic responses, we tested the ability of a PAI-1 antibody to inhibit the reduction in PCM mediated tubule formation (Fig. 5a) and endothelial cell migration (Fig. 5b). At low antibody concentrations (1 and 3 ng ml$^{-1}$) there was no significant inhibition of the effect of PCM on angiogenic branching. Antibody concentrations $\geq 6$ ng ml$^{-1}$ showed
complete ablation of the inhibitory influence of PCM with levels of branching that were not significantly different from the serum-free control conditions ($p=0.69$).

We also investigated the effect of a PAI-1 inhibitory antibody (7.5 ng ml$^{-1}$) on the PCM-mediated delay in wound closure using the HMEC-1 wound scrape assay (Fig. 5b). The antibody exposure completely inhibited the effect of PCM at early time points (up to 7 h) but beyond that time the rate of wound closure was not significantly different. A non-specific, control IgG antibody did not affect the rate of wound closure in ECCM or PCM treated cells.

4. Discussion

There is growing evidence that pericytes perform numerous functions within microcapillaries, and can inhibit endothelial cell proliferation when co-cultured in vitro [2]. More recently we have demonstrated that inhibition of endothelial cell growth by pericytes is not cell contact dependent [3], and this is consistent with the hypothesis that pericycle-induced growth inhibition may be mediated by a soluble growth factor(s), possibly TGF-$\beta_1$ [18]. Using a wound migration model, we have shown that PCM exerts a dramatic inhibitory influence on endothelial cell migration in vitro. As degradation of the basal lamina and extracellular matrix is a key step in neovascularisation in vivo [19] we further investigated the influence of PCM on endothelial cell migration and vessel assembly. Endothelial cells cultured on Matrigel$^\text{TM}$ and exposed to PCM were found to generate significantly fewer angiogenic branches over an 18 h period than controls exposed to ECCM. Collectively these findings suggest that PCM contains a bioactive element(s) that controls both endothelial cell migration and tubule formation in vitro.

We have also shown that PCM directly modulated expression of iNOS and ET-1, genes known to control vasoactivity, providing further evidence of pericyte regulation of vessel tone [3]. These findings show that pericytes induce alterations in endothelial gene expression by the generation of soluble, diffusible factors in a non-contact dependent manner. In order to further elucidate these genetic changes, microarray analysis was carried out. The gene showing the greatest level of differential expression was PAI-1. This is a particularly interesting finding as PAI-1 is the
principal inhibitor of urokinase type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA), and as such is thought to play an important role in the regulation of extracellular matrix remodelling [20]. We confirmed the induction of PAI-1 at both the mRNA and protein levels by Northern and Western blot, respectively. Interestingly, we showed that although PAI-1 is induced in a time dependent manner at both mRNA and protein levels by PCM, PAI-1 protein is absent from control endothelial cells. This information is of particular significance, as it is known that PAI-1 is a labile protein that only becomes functionally stabilized by high affinity binding within the N-terminal region of vitronectin [21].

We then mimicked the anti-migratory effects exerted on endothelial cells by PCM, using purified PAI-1. We showed that the response of endothelial cell migration is highly dependent on the concentration of purified PAI-1 protein; low concentrations of PAI-1 (1 ng ml\(^{-1}\)) were pro-migratory, but high concentrations (>1000 ng ml\(^{-1}\)) of the protein were anti-migratory. This bell shaped curve is typical of many angiogenic molecules [17] and also suggests that the level of PAI-1 generated within endothelial cells following exposure to PCM is around 1000 ng ml\(^{-1}\). However, the effects were transient (Fig. 4b) as a result of PAI-1 protein instability. Re-application of PAI-1 at 7 h restored inhibition of migration mimicking the time course of the PCM effects (Fig. 4c). These data are further supported by transfection of a PAI-1 expression construct, where we showed that transient transfection was at least as potent as PCM at inhibiting endothelial cell migration. The results also suggest that overexpression of

![Graph showing the effect of the PAI-1 inhibitory monoclonal antibody (mAb) on the PCM-mediated inhibition of endothelial cell-to-cell tube formation. PCM significantly reduced tube formation (\(p=0.0041\)) compared to the serum-free control. These effects were inhibited at concentrations at 6 ng ml\(^{-1}\) (\(p=0.00014\)) or higher, with no significant effects observed at lower concentrations (\(p>0.05\)). Data points are the mean of three individual experiments ± standard error of the mean. (b) Effect of the PAI-1 inhibitory mAb (7.5 ng ml\(^{-1}\)) on the migration of HMEC-1 in the in vitro wound scrape assay. The PAI-1 antibody significantly inhibited PCM induced anti-migratory action between 2 and 6 h (\(p=0.014\)) and at 18 and 23 h (\(p<0.01\) and \(p=0.03\), respectively). Data points are the mean of three individual experiments ± standard error of the mean. An IgG antibody (7.5 ng ml\(^{-1}\)) was used as a control.

Fig. 5. The effect of a PAI-1 inhibitory antibody on HMEC-1 tubule formation and endothelial cell migration.
PAI-1 may exert a paracrine influence on surrounding non-transfected cells, since the transfection efficiency was only 8%. It should also be noted that TGF-β1 is known to be the main activator of PAI-1 in vivo, and when present in picomolar concentrations significantly increases the synthesis of PAI-1 mRNA and protein in endothelial cells, epithelial cells, fibroblasts and other cell types [22].

Previous research by Orlidge and D’Amore [2] showed that pericytes inhibit endothelial growth when grown in coculture; they concluded that this effect was dependent upon contact or proximity and that the mechanism of inhibition was by activation of TGF-β1. Our findings, however, strongly suggest that activation of TGF-β1 and the subsequent induction of PAI-1 in endothelial cells is effected by pericytes in a non-contact-dependent manner. PCM may therefore contain a soluble factor or factors capable of cleaving the latent TGF-β1 complex produced by endothelial cells, thereby inducing PAI-1 gene expression.

Finally, an inhibitory antibody was used to evaluate the potential involvement of PAI-1 in the inhibition of angiogenic branching and endothelial cell migration observed when HMEC-1 were treated with PCM. The results from these experiments suggest that PAI-1 induction by PCM is a significant mediator of the observed inhibitory influence on the migratory and angiogenic potential of HMEC-1. It is clear, however, that other mechanisms may be important since the inhibition by the PAI-1 antibody did not completely restore cell migration rate (Fig. 5b).

Devy et al. [17] recently carried out an aortic ring assay employing vessels from PAI-1−/− mice. Lack of PAI-1 was found to inhibit angiogenesis, demonstrating its importance in the control of plasmin-mediated proteolysis. Interestingly, addition of recombinant PAI-1 led to a bell shaped angiogenic response, similar to our own, clearly showing that PAI-1 is pro-angiogenic at physiological concentrations and anti-angiogenic at higher levels. In a recent study of proliferation in wild-type and PAI-1-deficient endothelial cells enhanced growth rates were observed in the PAI-1-deficient cells [23].

In conclusion, we have shown that pericytes produce soluble factors capable of altering gene expression in endothelial cells. Of particular note was the significant role of PAI-1; over expression of this gene had a potent inhibitory influence on endothelial cell migration and angiogenic branching. However, there may be other mediators of the PCM inhibitory response that, if identified, could lead to the discovery of other novel anti-angiogenic molecules. This is consistent with the complexity of neoangiogenesis. While we have clarified one of the downstream effectors by which PCM modulates endothelial cell migration and tubule formation, the pericyte-derived factor(s) involved remain to be characterised.

References


