Distinct signal transduction pathways are utilized during the tube formation and survival phases of in vitro angiogenesis

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SUMMARY

Angiogenesis, the formation of new blood vessels from pre-existing ones, occurs during development, wound healing and cancer and involves stages that orchestrate a network of cooperative interactions. Peptide growth factors and extracellular matrix (ECM) components are two major groups of angiogenesis mediators. Among the different ECM proteins, collagens have been well-associated with in vivo angiogenesis. Using human umbilical vein endothelial cells (HUVEC) grown in 3-D collagen gels we show that: (1) HUVEC do not survive well in 3-D collagen gels due to rapid induction of apoptosis. (2) VEGF, a potent in vivo angiogenic factor, fails to induce tube formation. (3) PMA was effective in inducing tube formation and survival in HUVEC dispersed in 3-D collagen gels, activating MAP kinase, phosphoinositide 3-OH kinase (PI-3-kinase) and Akt/PKB (protein kinase B) pathways. (4) VEGF was effective in preventing PMA-induced tube-like structure regression after PMA-withdrawal by (5) activating the mitogen activated protein kinase (MAPK), rather than the Akt/PKB, signaling pathway.

Key words: Apoptosis, Endothelium, Signal transduction, Survival, VEGF

INTRODUCTION

The ability of endothelial cells to form capillary tubes is a specialized function of this cell type. Understanding the mechanisms responsible for the control of capillary tube formation, as well as regression, are of importance considering the central role of angiogenesis in development, inflammation, repair and cancer (Hanahan and Folkman, 1996; Beck and D'Amore, 1997; Bussolino et al., 1997). In recent years, considerable progress has been made identifying angiogenic regulators. Two major groups of mediators are various growth factors (see Beck and D'Amore, 1997; Risau, 1997; Pepper, 1997, for recent reviews) and ECM components (Bischoff, 1997; Strombald and Cheresh, 1997). Interactions between vascular endothelial cells and the ECM occur during the multiple steps of angiogenesis. Both matrix degradation by matrix metalloproteinase (MMP) and matrix deposition take place during the different phases of angiogenesis. Among the different ECM components, collagens have been associated with in vivo angiogenesis (Strombald and Cheresh, 1996). Inhibition of collagen deposition or collagen triple-helix formation prevented angiogenesis (Ingber, 1991) and de novo synthesis of collagen was required for endothelial tube-like structure formation in vitro and vessel formation in vivo (Haralabopoulos et al., 1994). In addition, collagen type I α1-chain gene knockout resulted in embryonic lethality due in part to rupture of blood vessels (Lohler et al., 1984).

A number of collagen-based in vitro angiogenic model systems have been described. These include aortic explants placed in type I collagen gels (Nicosia and Ottinetti, 1990), growing endothelial cells on top of (Montesano and Orci, 1985, 1987), between two layers (Montesano et al., 1983) or in (Madri et al., 1988; Gamble et al., 1993; Goto et al., 1993; Davis and Camarillo, 1996) type I collagen gels. Both microvascular and macrovascular endothelial cells have been shown to grow on the surface of collagen gels without infiltrating the underlying matrix (Schor, 1980; Delvos et al., 1982), but invade and form extensive network of capillary-like tubular structures upon exposure to phorbol myristate acetate (PMA, Montesano and Orci, 1985, 1987). When embedded in 3-D collagen gels, bovine capillary endothelial (BCE) cells responded to vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) by proliferation and cord formation (Goto et al., 1993). In addition, PMA has been shown to be a potent morphogen for endothelial cells suspended within 3-D collagen matrices, eliciting the formation of highly developed lumen-containing tube-like structures (Davis and Camarillo, 1996). Mechanism(s) responsible for this PMA effect remain elusive. It has been hypothesized, however, that PMA triggers in vitro phenomena mimicking angiogenic processes in vivo.

Using human umbilical vein endothelial cells (HUVEC) grown in 3-D collagen gels we found that HUVEC do not survive well in these gels. Moreover, VEGF failed to induce morphological changes or to rescue the cells from apoptosis. However, PMA treatment induced endothelial tube-like

structure formation (which closely mimics in vivo capillary formation) and was found to be PKC-, MAPK-, and PI 3 kinase-dependent. Surprisingly, VEGF was effective in preventing tube-like structure regression after PMA-withdrawal by activating the mitogen-activated protein kinase (MAPK)-, rather than the Akt/PKB-signaling pathway.

MATERIALS AND METHODS

Antibodies

Polyclonal antibodies to Flt-1 (vascular endothelial growth factor receptor-1 (VEGFR-1)), Flk-1 (vascular endothelial growth factor receptor-2 (VEGFR-2/KDR)) and extracellular-signal-regulated kinase-2 (ERK-2) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Polyclonal anti-phospho-MAPK, anti-Akt/PKB and phospho-Akt/PKB were from New England BioLabs (Beverly, MA). Anti-VE cadherin was kindly provided by Robert Wysolmerski (St Louis Health Science Ctr., St Louis, MO). Polyclonal antibody to occludin was purchased from Zymed (South San Francisco, CA) and monoclonal anti-collagen IV antibody was purchased from Chemicon (Temecula, CA) or prepared as described (Madri and Furthmayr, 1980). A polyclonal antibody directed against platelet endothelial cell adhesion molecule-1 (PECAM-1) was prepared as described (Lu et al., 1996). Monoclonal antibodies to actin (Sigma, St Louis, MO), poly (ADP-ribose) polymerase (PARP), clone C-2-10 (Pharmingen, San Diego, CA), and polyclonal antibodies to ICE and Ced3 homolog-1 (Ich-1; Upstate Biotechnology, Lake Placid, NY) were also used.

Cells

Human umbilical vein endothelial cells (HUVEC) were obtained from Jordan Pober (Yale Medical School) and were cultured in gelatin-coated flasks in M199 medium supplemented with 20% heat-inactivated fetal bovine serum, 50 µg/ml endothelial cell growth supplement (ECGS), 50 µg/ml heparin, 10 mM Hepes, 2 mM L-glutamine and antibiotics. Cells from passage 3-7 were used for 3-D cultures.

Three-dimensional cultures

3-D cultures of HUVEC were made as previously described (Madri et al., 1988). Briefly, a measured amount of collagen (2.5 mg/ml, type I, from bovine skin) with 1/10 volume of 10× DMEM was neutralized with sterile 1 M NaOH and kept on ice. Cultured HUVEC were added to the collagen suspension to a final concentration of two million cells/ml collagen. 250 µl of the collagen/cells suspension was added to 12 mm Millicell-HA (Millipore products division, Bedford MA) filter chambers set into the wells of a Costar 24-well tissue culture dish (Costar Corp., Cambridge MA) and left at room temperature for 15 minutes to permit collagen polymerization. Following gel formation, 1 ml of medium was added per well, allowing the cells to be fed from both the top and bottom of the collagen gel. Alternatively, the collagen/cells suspension was applied directly on top of 35 mm tissue culture plastic dishes as drop cultures (0.1 ml/drop). Cultures were fed every day. PMA (16 nM), VEGF (20 ng/ ml, R&D Systems, Minneapolis MN) or bFGF (2.5 ng/ml, R&D) were added daily as indicated in the text and Figure legends.

Chemicals

Z-Val-Ala-Asp-CH$_2$F (ZVAD), calphostin C (Cal. C), Ro 318220, wortmanin, PD 98059, rapamycin and SB 203580 were purchased from Calbiochem (La Jolla, CA). They were prepared as stock solutions (ZVAD, Cal.C, wortmanin, Ro 318220 and SB 203580 were dissolved in DMSO; PD 98059 and rapamycin in ethanol) and the equivalent volume of the vehicle control was always run in parallel.

No toxic effect nor inhibition of tube formation were ever detected. PMA (Sigma) was kept as a 16 mM stock in ethanol and a 1:1,000 dilution into sterile water was made daily from which a 1:1,000 dilution was made into the culture medium to give a final concentration of 16 nM.

Histology and immunohistochemistry

3-D cultures were fixed with 4% paraformaldehyde in PBS overnight, dehydrated in a series of 50-100% ethanol, cleared in xylene and embedded in paraffin. Sections (5 µm) were cut and mounted onto slides, deparaffinized, rehydrated and stained with haematoxylin-eosin. For immunofluorescence staining, dewaxed sections were washed in PBS and incubated for 30 minutes in PBS containing 10% normal goat serum. Sections were then incubated with primary antibodies for 1 hour, following by incubation with FITC/TRITC-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA) for 45 minutes. All incubations were at 37°C and in between the slides were washed 3× 10 minutes each time with PBS. Nuclei were counterstained with propidium iodide (PI, Molecular Probes, Eugene, OR) or 4,6-diamidino-2-phenylindole (DAPI, Sigma) during the last wash. For collagen IV staining, sections were pretreated with 1% trypsin (15 minutes at room temperature).

Confocal microscopy

Confocal microscopy was performed using an Olympus IX70 inverted fluorescence microscope outfitted with the Olympus Fluoview Argon/Krypton scanning laser system and Fluoview image analysis software (Olympus, Melville, NY).

Electron microscopy

Transmission electron microscopy was performed using standard methods as described (Madri and Williams, 1983).

Apoptosis studies

For programmed cell death (apoptosis) studies, dewaxed slides were pretreated with proteinase K (25 µg/ml, 5 minutes, room temperature) and DNA fragmentation was assayed by the TUNEL reaction, according to the manufacturer's instructions (Boehringer Mannheim, Indianapolis, IL). The nuclei were counterstained with PI or DAPI and apoptotic cells were counted as a percentage of the total nuclei in 7-10 independent fields. The mean value of two independent experiments was considered as the percentage cells undergoing apoptosis.

Total cell lysate preparation and protein blotting

3-D cultures were pretreated with 1 mM orthovanadate for 10 minutes at 37°C. The collagen pellets were then collected, briefly centrifuged and lysed with 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% NP-40, 0.5% deoxycholate, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM sodium orthovanadate. For PARP detection, 4 M urea was added to the same extraction buffer and samples were sonicated for 10 seconds, on ice. Total cellular protein concentration was determined by the BCA assay (Pierce, Rockford, IL), according to the manufacturer's instructions, and 20 µg were fractionated on SDS-polyacrylamide gel. Proteins were transferred onto PVDF membrane (Millipore), filters were blocked with 5% nonfat dried milk in TBS and subsequently incubated with primary antibodies overnight at 4°C. Bound antibodies were detected by horseradish peroxidase-conjugated anti-rabbit or anti-mouse (Jackson) and enhanced chemiluminescence (ECL, Amersham, Arlington Heights, IL or Pierce). Filters were stripped and reprobed successfully 3 times. Similar signal intensities of ERK-2 were used not only as control for MAPK phosphorylation but also as an internal control for similar protein loading in each lane.

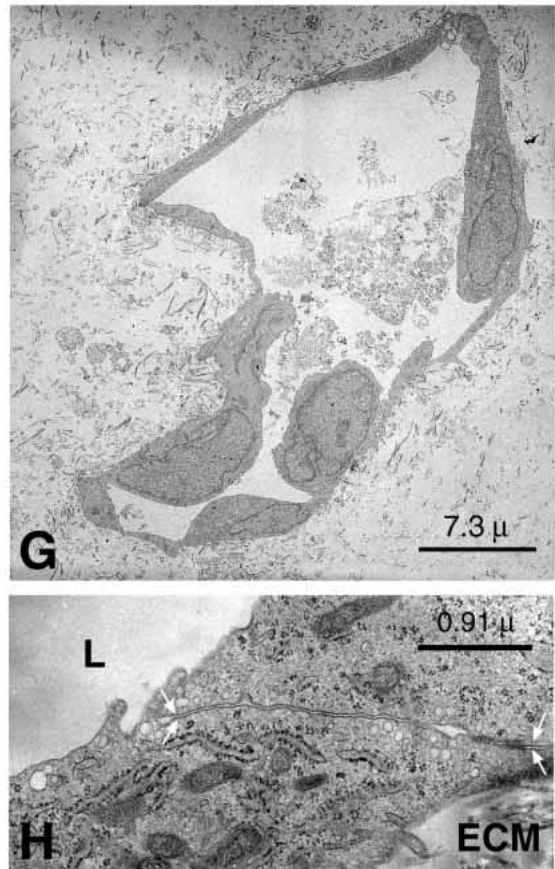All experiments were repeated at least twice with similar results.

Fig. 1. HUVEC tube formation in 3-D collagen gels. HUVEC were grown without (A), with VEGF (B, 20 ng/ml) or with PMA (16 nM, C-H). After 7 days in culture the collagen gels were formalin-fixed, paraffin embedded (A-F) or processed for electron microscopy (G,H). 5 µm sections were stained with haematoxylin-eosin (A-C) or immunofluorescently labeled for occludin (FITC) and PECAM-1 (TRITC) (D1), corresponding phase micrograph (D2); VE cadherin (FITC) (propidium iodide staining (red fluorescence) illustrates the cell nuclei) (E1); corresponding phase micrograph (E2); and Type IV collagen (TRITC) (F), and examined with an Olympus IX70 inverted confocal fluorescence microscope. Bars: 100 µm (A,B,C); 25 µm (D,E,F). Samples processed for and examined by transmission electron microscopy (TEM) (G,H), revealed multicellular, branching tube-like structures, exhibiting luminal (L) and abluminal differentiation (ECM) and junctional complexes (arrows) between cell processes. G, representative low power TEM micrograph. H, representative high power TEM micrograph.
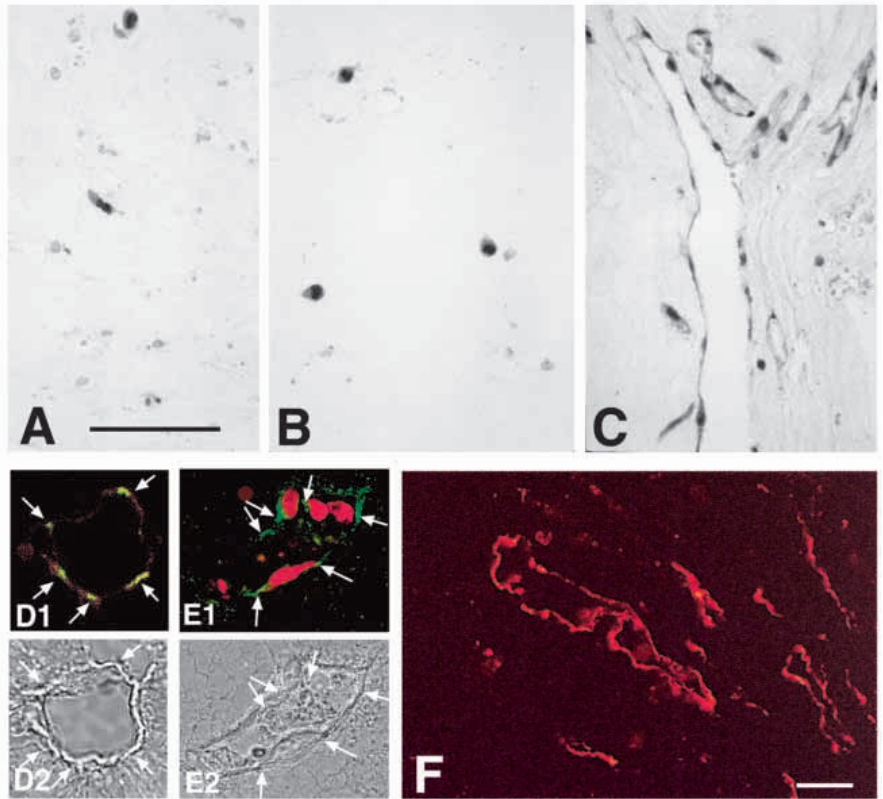
RESULTS

VEGF fails to induce HUVEC tube formation in 3-D collagen gels

When embedded in type I collagen gels, HUVEC failed to differentiate and organize into tube-like structures, developed pyknotic nuclei (Fig. 1A) and were marked by a high apoptotic index (see below). Moreover, VEGF, a potent in vivo angiogenic factor, failed to induce morphological changes in HUVEC (Fig. 1B) or to rescue the cells from programmed cell death as evidenced by the development of pyknotic nuclei (see below). bFGF, with or without VEGF, similarly had no effect (not shown). In striking contrast, PMA was found to be a potent morphogen for HUVEC in 3-D collagen cultures. PMA induced highly developed and differentiated tube-like structures, detected by light (Fig. 1C), as well as by transmission electron microscopy (Fig. 1G,H) and elicited a low apoptotic index (see below). HUVEC expressed PECAM-1 (Fig. 1D, rhodamine label) and PMA-induced HUVEC tube formation was found to involve a 3- to 4-fold increase in platelet-endothelial cell adhesion molecule (PECAM)-1 gene expression and a transient induction of MMP-2 activity; both paralleled tube formation (not shown). To further assess the maturity of these tube-like structures we assessed the formation of junctional-complexes and tight-junctions. Vascular endothelial (VE)-cadherin expression was induced 10-fold, as judged by western blot analysis (not shown). Anti-VE-cadherin staining of HUVEC tube structures clearly revealed a specific staining at cell-cell junctions (arrows, Fig. 1E), while electron microscopy (Fig. 1G,H,) and occludin staining (arrows, Fig. 1D) indicated the formation of tight junctions as evidenced by the appearance of occasional punctate structures exhibiting intense fluorescence. Moreover, HUVEC tube-like structures deposited their own basement membrane, as
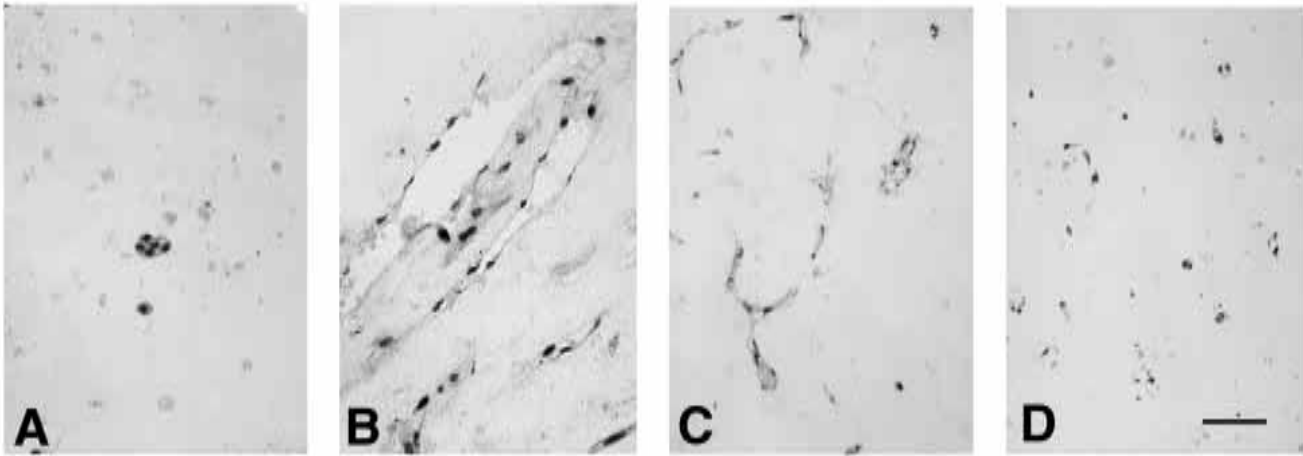
Fig. 2. PMA-induced HUVEC tube formation is PKC-dependent. HUVEC were grown without (A), with PMA (B) or with PMA and PKC inhibitors calphostin C (50 nM, C) and Ro 318220 (100 nM, D). After 7 days in culture the collagen gels were processed for light microscopy and 5 µm sections were stained with haematoxylin-eosin. Note the robust tube formation in the presence of PMA (B) while untreated cultures (A), the Cal.C (C) and Ro (D) treated cultures display single and groups of pyknotic cells. Bar, 25 µm.

judged by staining for collagen IV in abluminal linear patterns (Fig. 1F). Therefore, PMA induces mature, differentiated tube-like structures similar to those observed in in situ situations, yet such maturity would not prevent tube-like structure regression after PMA withdrawal (see below).

Signaling pathways responsible for PMA-induced tube formation

As protein kinase C (PKC) isoforms are the main target for phorbol esters, we first assessed the ability of specific PKC-inhibitors to inhibit PMA-induced HUVEC tube formation. Calphostin C, an inhibitor which interacts with the regulatory domain of PKC and competes with PMA or diacylglycerol binding sites, as well as Ro 318220, significantly inhibited PMA-induced tube formation (Fig. 2) as well as PMA-induced PECAM-1 expression (not shown). As would be expected, PKC phosphorylates and activates both MAPK isoforms (Fig. 3A, p-MAPK) more than 5-fold. This activation was drastically

Fig. 3. PMA activates both, the MAPK and PI 3 kinase pathways. (A) HUVEC were grown in 3-D collagen gels without (Con.), with PMA (16 nM, PMA) or with PMA and calphostin C (Cal.C, 50 nM) or Ro 318220 (Ro., 100 nM). After 3 days in culture total cell lysates were analyzed by western blotting for MAPK phosphorylation (p-MAPK) and, after stripping and reprobing, for total ERK-2. Numbers beneath the upper panel represent fold-changes compared to control (Con.). (B) HUVEC were grown without (Con.), with PMA (PMA), with VEGF (20 ng/ml, VEGF) or with PMA and wortmanin (25 nM, Wort). After 3 days in culture total cell lysates were analyzed for Akt/PKB phosphorylation (p-Akt). Similar protein loading was confirmed by reprobing with anti-actin antibodies. Numbers beneath the upper panel represent fold-changes compared to control (Con.). (C) HUVEC were grown without (φ), with PMA (PMA) or with PMA and the PI 3 kinase inhibitor wortmanin (Wort.), or with PMA and the MAPK inhibitor, PD 98059 (PD), at the indicated concentrations. After 3 days in culture MAPK phosphorylation (p-MAPK) and expression (ERK-2) were analyzed as in A. Note induction of MAPK and Akt/PKB phosphorylation by PMA and inhibition of both signaling mediators by wortmanin.
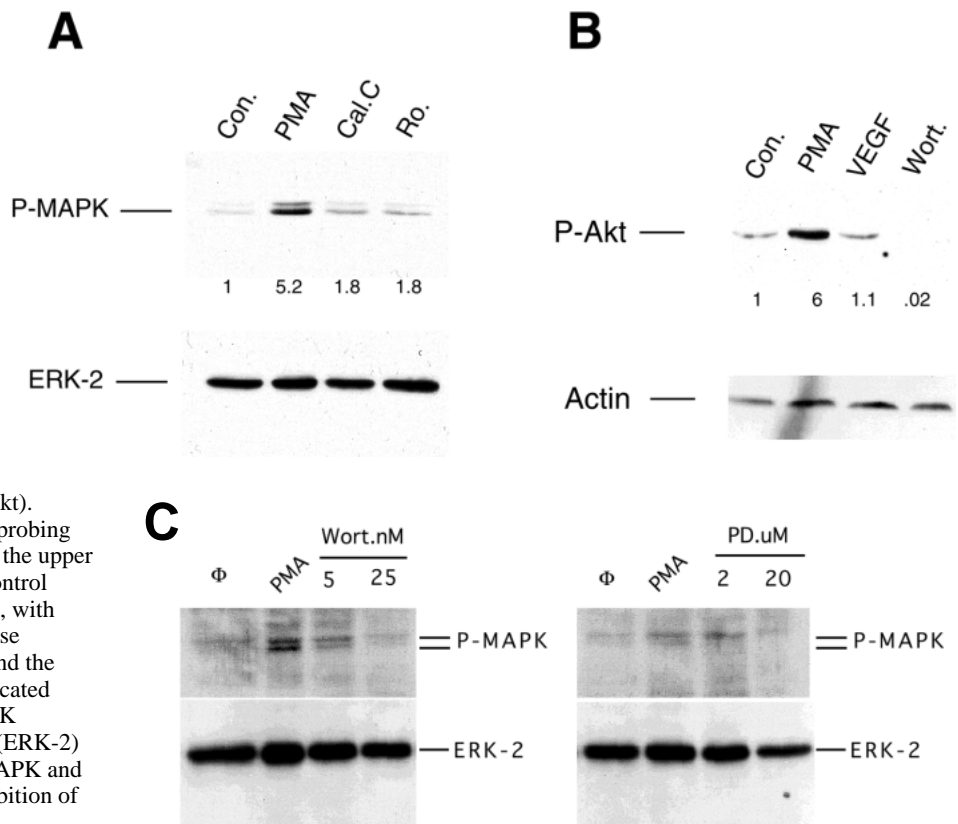
inhibited by the PKC inhibitors (Fig. 3A). Moreover, MAPK phosphorylation (Fig. 3C), as well as tube formation (Fig. 4C-D), was significantly inhibited by PD 98059, a specific inhibitor of MAP kinase kinase (MEK), the only enzyme responsible for MAPK activation (Seger and Krebs, 1995), all in a dose-responsive manner. No inhibitory effect was detected with SB 203580 (not shown), a specific inhibitor for the p38 MAPK homologue stimulated by cellular stress (Cuenda et al., 1995; Alessi, 1997). PMA-induced HUVEC tube-like structures formation is, therefore, PKC- and MAPK-dependent.

It was recently reported, however, that Ro 318220 is a potent inhibitor not only for PKC isoforms but also for RSK2 (90 kDa ribosomal S6 kinase-2) and p70 S6 kinase (ribosomal protein S6 kinase); the latter is considered to be downstream of the PI-3 kinase pathway (Ming et al., 1994; Pullen and Thomas, 1997). In order to evaluate the possible involvement of additional signaling pathways initiated by PMA, we first examined phosphorylation of Akt/PKB, an immediate substrate for PI-3 kinase. Phosphospecific antibodies revealed a 6-fold induction of Akt/PKB serine 473 phosphorylation by PMA (Fig. 3B). This induction is mediated by PI-3 kinase since wortmanin, a specific PI-3 kinase inhibitor, completely blocked Akt/PKB activation. Moreover, wortmanin is a potent inhibitor of PMA-induced HUVEC tube formation (Fig. 4E-F). These data suggest a pivotal role for PI-3 kinase in endothelial cell tube formation. VEGF had no effect on Akt/PKB (Fig. 3B) or MAPK activation (see Fig. 9).

Thus far, we identified two parallel signaling pathways involved in PMA-induced tube formation: the MAPK- and the PI-3 kinase-pathway. Although these two pathways run in parallel, they may merge at certain points, one of which, depending on the specific inducer and cell type (Duckworth and Cantley, 1997), might be the MAPK. This, indeed, was found to be the case: wortmanin, which specifically inhibits PI-3 kinase, resulted a complete, dose responsive, inhibition of PMA-induced MAPK activation (Fig. 3C). Yet, other downstream components of the PI-3 kinase pathway are crucial and independent of the MAPK pathway: rapamycin, a specific p70 S6 kinase inhibitor (Chung et al., 1992; Alessi, 1997), was found to be a potent inhibitor of PMA-induced tube formation (Fig. 4G-H). Therefore, activation of both pathways is required to initiate HUVEC tube formation. Only the MAPK pathway, however, seems to be necessary to maintain the tube-like structures after PMA withdrawal (see below).
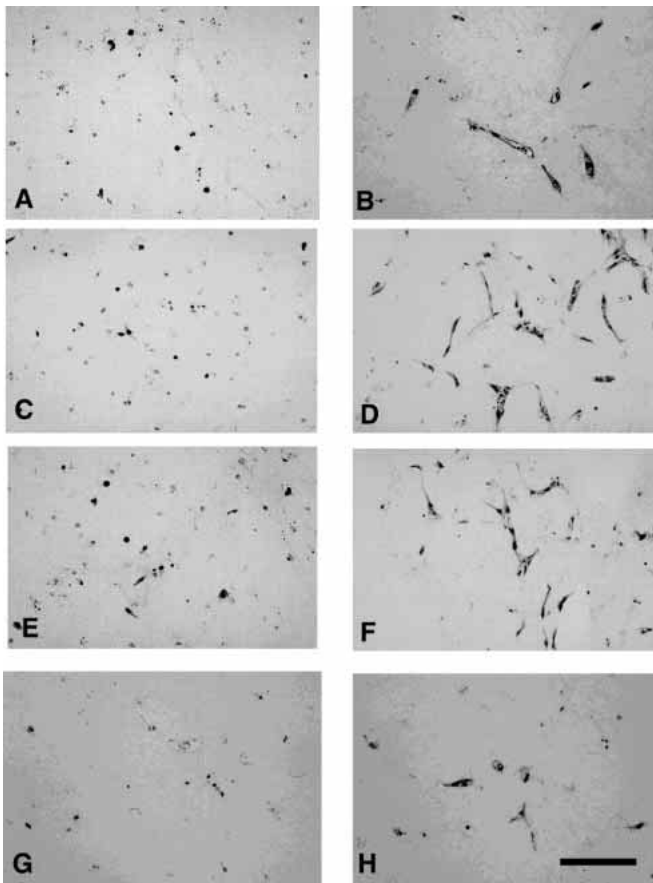
Three-dimensional collagen gels induce HUVEC programmed cell death

When embedded in collagen, HUVEC failed to organized into tube-like structures, even in the presence of in vivo angiogenic factors including VEGF or bFGF (Fig. 1A,B, and data not shown). Moreover, in most of the cells the nuclei appeared pyknotic and/or fragmented (Fig. 1A-B), a typical indication of programmed cell death (apoptosis). We, therefore, quantitated cellular apoptosis by the TUNEL reaction, as shown in Fig. 5A. After 2 days in a 3-D collagen gel, more than 50% of the cells are in the apoptotic process. While VEGF did not rescue the cells from dying, PMA, as well as ZVAD, an inhibitor of all known caspases, drastically reduced the apoptotic index. To further confirm the death-inducing activity of 3-D collagen gels, HUVEC cell lysates were analyzed for PARP (a caspases substrate) cleavage. As shown in Fig. 5B, PARP cleavage into its 85 kDa fragment was well detected in the control, non-treated cells, as well as in the VEGF-treated cells. PARP cleavage was not detected in the PMA cultures (Fig. 5B). Thus, PMA may inhibit apoptosis inducers or induce apoptosis inhibitors. The latter seems to be the case: while the long Ich isoform (Ich$_L$) expression, which is considered to induce apoptosis (Wang et al., 1994), was not changed, the expression of the short isoform (Ich$_S$), which inhibits apoptosis (Wang et al., 1994), was induced (Fig. 5C). Therefore, PMA, under these culture conditions acts not only as a morphogen but also as a cell-survival factor. Most importantly, 3-D collagen gels induce cell death despite the presence of 20% FBS and endothelial cell growth factor (ECGF). Thus, while the ECM has been considered as a survival factor in selected culture systems in the past (Meredith et al., 1993), this does not appear to be the case in our HUVEC 3-D collagen cultures.

HUVEC tube-like structure regression after PMA withdrawal: VEGF as a survival factor

We have already demonstrated that PMA induces mature

Fig. 4. PMA-induced HUVEC tube formation is MAPK-, PI 3 kinase- and p70 S6 kinase-dependent. HUVEC were grown in 3-D collagen gels without (A), with PMA (B), or with PMA and wortmanin (25 nM, C; 5 nM, D), PD 98059 (20 μM, E; 2 μM, F) or rapamycin (200 pM, G; 20 pM, H). Haematoxylin-eosin staining of 5 μm sections was made after 2 days in culture. Note: Already at this early time point, lumen-containing structures are well detected (B) but were completely inhibited by all three inhibitors in a dose-dependent manner. Bar, 50 μm.

HUVEC tube-like structures, having tight-junction formation
(Fig. 1E,H) and basement membrane deposition (Fig. 1F). We
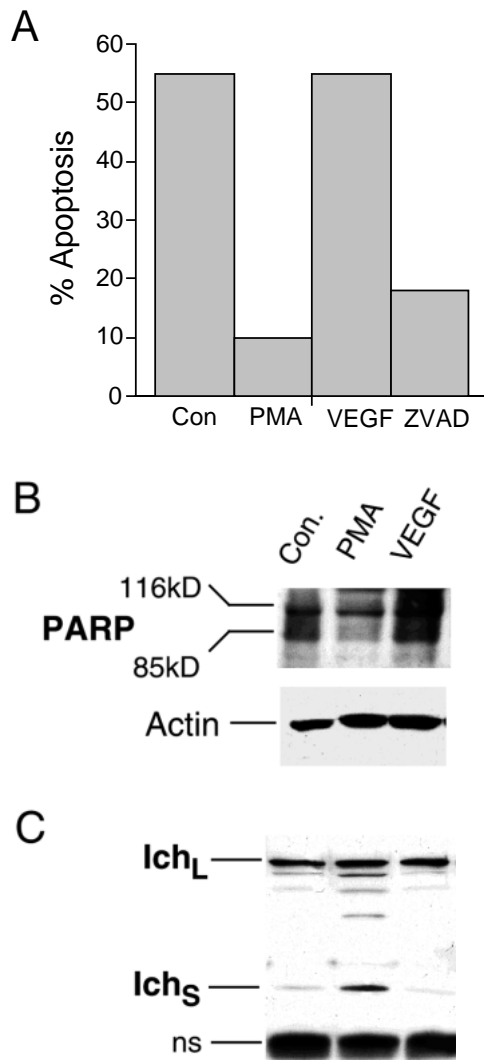then assessed the stability of these structures after PMA

withdrawal. Cells were treated with PMA for 5 days and were
then grown for an additional 3 days without PMA. PMA
withdrawal resulted in a complete regression of all the tube-
like structures (Fig. 6A). Therefore, cell-cell contact, tight
junction formation and basement membrane deposition (Fig.
1) by themselves are not sufficient to maintain tube
stabilization. A continuous positive signal must be provided in
order to maintain these tube-like structures. In an attempt to
identify factors that will substitute for PMA in maintaining
(rather than inducing) HUVEC tube-like structures, cells were
treated with PMA for 5 days and were then grown for an
additional 3 days without PMA but in the presence of VEGF
or ZVAD. Although VEGF was unable to induce tube
formation initially (Fig. 1B) or to rescue the cells from
apoptosis (Fig. 5A), it was found to prevent tube-like structure
regression after PMA withdrawal (Fig. 6C). In addition, ZVAD
was as potent as VEGF (Fig. 6D) in this regard, suggesting that
tube-like structure regression is due to apoptosis and VEGF
can be considered as a cell survival factor. This was further
confirmed by labeling cellular apoptosis by the TUNEL
method, as shown in Fig. 7. Three days after PMA withdrawal,
VEGF-treated HUVEC comprising the tube-like structures
were well attached to the underlying ECM and cellular nuclei
appeared large and elongated. Very few, if any, cells were
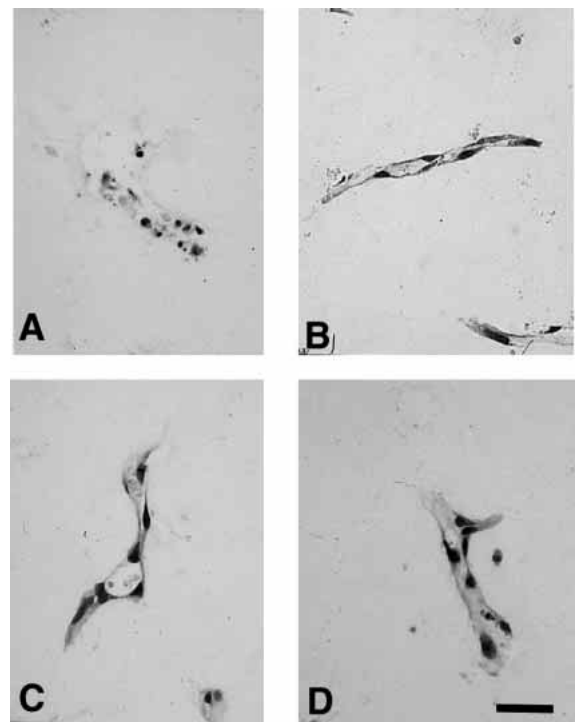TUNEL-positive (Fig. 7A,C). However, in control tubular

Fig. 5. 3-D collagen gels-induced HUVE cell death. (A) HUVEC were grown in 3-D collagen gels without (Con.) or with PMA (PMA), with VEGF (20 ng/ml, VEGF) or in the presence of ZVAD (40 μM, ZVAD). After 2 days in culture collagen gels were formalin-fixed, paraffin embedded and 5 μm sections were analyzed for apoptosis using the TUNEL assay. The percentages of TUNEL-positive cells, compared to the total cell numbers were calculated in 7-10 independent fields and the mean of two independent experiments was scored as percentage apoptosis. Note the high apoptotic index in control and VEGF treated cultures compared to the low apoptotic index in PMA and ZVAD treated cultures. (B) HUVEC were grown in 3-D collagen gels without (Con.), with PMA (PMA) or with VEGF (20 ng/ml, VEGF). After 2 days in culture total cell lysates were analyzed for PARP expression and cleavage by western blot. A similar protein loading was confirmed by stripping the same membrane and reblotting with anti-actin antibodies. Note that PARP cleavage is detected in the control and VEGF-treated cultures but not in the PMA treated cultures. (C) Parallel collagen gels from the same cultures were analyzed for Ich expression. Ich_S expression was induced by PMA, while Ich_L expression was not changed.

Fig. 6. HUVEC tube-like structure regression after PMA-withdrawal: VEGF as a survival factor. HUVEC were grown in 3-D collagen gels for 5 days with PMA and then for an additional 3 days without (A), with (B) PMA or without PMA but in the presence of VEGF (20 ng/ml, C) or ZVAD (40 μg/ml, D). 5 μm sections of formalin-fixed, paraffin-embedded collagen gels were haematoxylin-eosin stained. Note the complete regression of the tube-like structures, and nuclear fragmentation after PMA withdrawal (A) compared to the stabilization of these structures in the presence of VEGF and ZVAD. Bar, 50 μm.
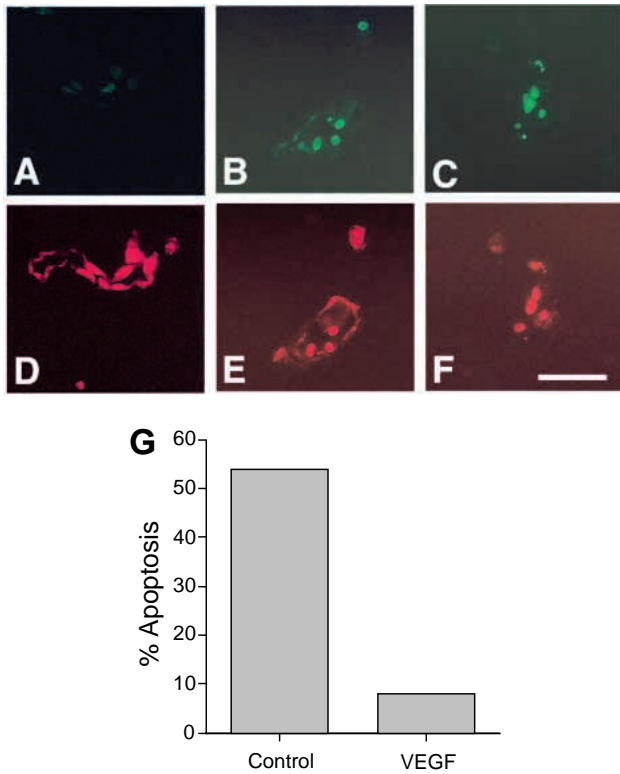
Fig. 7. Tube-like structure regression after PMA-withdrawal is due to apoptosis. HUVEC were grown in 3-D collagen gels for 5 days with PMA and then for an additional 3 days without (B-C, E-F) PMA or without PMA but in the presence of VEGF (A,D). Five micron sections were stained with TUNEL reagents (A-C) to illustrate apoptotic cells (FITC) or with propidium iodide (PI) to illustrate the structures and cells present (D-F). Note nuclear pyknosis, cellular detachment from the underlying matrix and accumulation of intensely staining TUNEL-positive cells in the lumens of the involuting structures in B and C, while the VEGF treated cultures exhibit normal nuclear morphology and no specific TUNEL labeling (A). Bar, 50 μm. (G) The percentages of TUNEL-positive cells, compared to the total cell numbers were calculated in 7-10 independent fields and the mean of two independent experiments was scored as percentage apoptosis. Note the high apoptotic index in the cultures in which PMA was withdrawn compared to the low apoptotic index in the cultures in which VEGF was added following PMA withdrawal.

structures most nuclei appeared pyknotic and were TUNEL-positive. While some cells were still attached to the underlying ECM (Fig. 7B,E) the majority were detached and accumulated in the lumen (Fig. 7C,F). VEGF significantly decreased the apoptotic index when given after PMA pretreatment (Fig. 7G), while bFGF had no rescuing effect (not shown). Interestingly, VEGF functioning as a tube-like structure survival factor (Fig. 7A,B,G) and the mechanism of regression (cell death, accumulated in the lumen, Fig. 7A,C,F) are in agreement with recently published in vivo studies (Benjamin and Keshet, 1997).

MAPK, but not Akt/PKB phosphorylation as a survival machinery

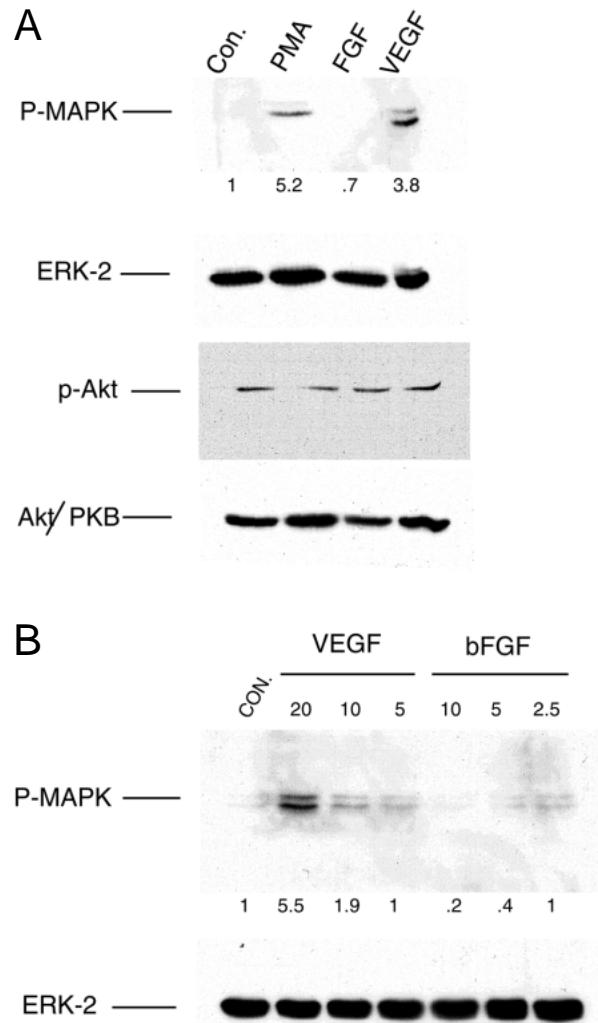The advantage of our in vitro system over in vivo model systems

Fig. 8. MAPK, but not Akt/PKB, phosphorylation is involved in rescuing tube-like structures from regression. (A) HUVEC were grown in 3-D collagen gels for 5 days with PMA and then for an additional 3 days without (Con.), with PMA (PMA) or without PMA but in the presence of bFGF (2.5 ng/ml, bFGF) or VEGF (20 ng/ml, VEGF). Total cell lysates were analyzed for phosphorylated MAPK (p-MAPK) or, after stripping and reblotting, for total MAPK expression (ERK-2). Parallel samples were similarly analyzed for Akt/PKB phosphorylation (p-Akt) or expression (PKB). Note the increased MAPK phosphorylation in cultures treated continuously with PMA and with VEGF following PMA withdrawal compared to the similar, low, Akt/PKB phosphorylation levels in involuting (Con.) and stabilized (PMA) samples. Numbers beneath the upper panel represent fold-changes compared to control (Con.). (B) HUVEC were grown in 3-D collagen gels for 5 days with PMA and then for an additional 3 days without (Con.) PMA but in the presence of the indicated concentrations (ng/ml) of VEGF or bFGF. Total cell lysates were analyzed for MAPK phosphorylation (p-MAPK) or expression (ERK-2). Note the dose-dependent VEGF-induced MAPK phosphorylation. Numbers beneath the upper panel represent fold-changes compared to control (Con.).

is the ability to better study the signaling pathways responsible for the rescue of tube-like structures from regression. We have
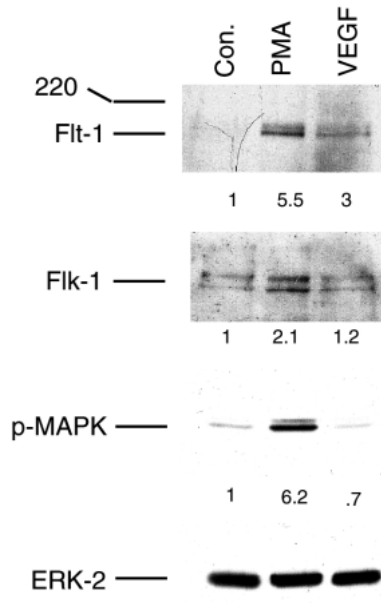
Fig. 9. PMA-induced Flt-1 expression. HUVEC were grown in 3-D collagen gels without (Con.), with PMA (PMA) or with VEGF (VEGF). After 1 day in culture total cell lysates were analyzed for Flt-1 and, after stripping and reblotting, for Flk-1 expression. Parallel samples were similarly analyzed for MAPK phosphorylation (p-MAPK) and expression (ERK-2). Note the induction of Flt-1/Flk-1 expression by PMA and the lack of VEGF-induced MAPK activation at this early time point of culture. Numbers beneath the gel panels represent fold-changes compared to control (Con.).

already demonstrated MAPK and PI-3 kinase-induced Akt/PKB as two signaling pathways involved in PMA-induced tube-like structure formation (Figs 2-4). However, only the MAPK, but not Akt/PKB, seems to be involved in rescuing these structures from regression (Fig. 8A), as low Akt/PKB phosphorylation levels were detected in control, involuting, as well as in PMA-treated, stabilized tubular structures. Moreover, VEGF, which could not activate the MAPK pathway at day 1 of culture (Fig. 9) was almost as effective as PMA after PMA pretreatment (Fig. 8A), and the response was dose-dependent (Fig. 8B). Thus, VEGF is HUVEC-effective only after PMA pretreatment. A possible explanation for this would be an induction of specific molecule(s) involved in VEGF signaling, and reasonable candidates are the VEGF receptors, Flt-1 and Flk-1. Western blot analysis of cell lysates made after one day of PMA treatment in 3-D collagen culture revealed a 5.5-fold induction of Flt-1, and a 2-fold induction of Flk-1, expression (Fig. 9). Increase in receptor levels might explain VEGF's ability to function as a survival factor only after PMA pretreatment (Fig. 8).

Thus, given our findings, we suggest a mechanism in which a continuous positive signal must be provided to the vasculature, in addition to appropriate cell-cell and cell-ECM interactions, in order to prevent vascular regression. VEGF is likely an in vivo provider of such a signal, in agreement with its expression around microvessels in areas where the endothelial cells are normally quiescent (Ferrara and Davis-Smyth, 1997; Ment et al., 1997).

DISCUSSION

Interactions between cells and the extracellular matrix (ECM) initiate a flow of information that acts to regulate many fundamental processes. These include modulation of growth and differentiation programs of many specialized cell types. Examples include: hepatocytes, mammary cells, keratinocytes, Sertoli cells, myoblasts and endothelial cells (Adams and Watt, 1993; Hay, 1993; Lin and Bissell, 1993; Roskelley et al., 1995). Moreover, it has been suggested that, in addition to regulating cell growth and differentiation, the ECM also functions as a survival factor for many cell types, including endothelial cells (Meredith et al., 1993; Scatena et al., 1998). Clearly, when embedded in 3-D collagen gels, HUVEC did not survive and went through rapid apoptosis. This is based on morphological examination (Figs 1A, 4A,C,E,G), PARP cleavage (Fig. 5A), TUNEL staining (Fig. 5C) and the ability of ZVAD, an inhibitor of apoptosis, to rescue the cells from dying (Fig. 5B). Cell death in 3-D collagen gels has been previously reported for other endothelial cells as well (Goto et al., 1993), suggesting that this phenomena is not HUVEC-specific. Angiogenesis often proceeds in a microenvironment consisting predominantly of interstitial collagens, most of which, for example in the skin, is type I collagen (Senger et al., 1997). This would suggest that 3-D collagen gel cultures have relevance to in vivo situations. However, even in a physiological, permissive 3-D collagen milieu, the endothelial cell's first choice is to die (Figs 1A,B, 5A-C; Goto et al., 1993). Thus, under these culture conditions, both collagen type I, in which the cells were initially embedded (Figs 1A, 5) and collagen type IV, subsequently deposited by the forming tubes (Figs 1F, 6, 7) do not function as a surviving environment.

In many in vitro studies apoptosis was induced by serum and/or growth factor withdrawal (Satake et al., 1998; Villaschi and Nicosia, 1993). In our studies, as well as in the studies of others (Goto et al., 1993), endothelial cell apoptosis occurred in spite of the presence of 20% FBS and ECGF. Thus, apoptotic machinery may differ from cell type to cell type and among similar cells in different microenvironments. Interestingly, Akt/PKB, but not MAPK, was implicated in cell survival when apoptosis was induced by serum starvation (Yao and Cooper, 1995; Datta et al., 1997; Dudek et al., 1997; Kennedy et al., 1997) or UV irradiation (Kulik et al., 1997). Nevertheless, activation of MAPK, rather than Akt/PKB, was noted upon rescuing 3-D collagen tube-like structures from regression (Fig. 8A). This suggests different, physiologically relevant, survival machinery being activated under different conditions. Blood vessel stabilization, regression and remodeling occurs during normal development (Alon et al., 1995) and has a great clinical importance in controlling tumor growth (Boehm et al., 1997), as well as in other pathologies, including the intraventricular hemorrhage observed in premature infants (Ment et al., 1992, 1995). Understanding the mechanisms of vessel formation, maintenance and regression has, therefore, a great basic and applicable importance. Several lines of evidence support VEGF as a vasculature survival factor: (1) in vivo, VEGF expression is detectable around microvessels where endothelial cells are normally quiescent (Ment et al., 1997) and Flt-1 mRNA is expressed in both proliferating and quiescent endothelial cells (Ferrara and Davis-Smyth, 1997). (2) VEGF expression is decreased during physiological blood-

vessel involution (Takahashi et al., 1994). (3) VEGF-withdrawal by means of hyperoxia (Alon et al., 1995), anti-VEGF antibodies (Yuan et al., 1996) or conditional switching (Benjamin and Keshet, 1997) all resulted in vascular regression. Our culture model permits investigations into the signaling pathways and mechanisms of action of soluble factors functioning as survival factors under controlled in vitro conditions and, thus, complements the in vivo models already established. In our culture conditions, VEGF could only rescue preformed tube-like structures from regression (Figs 1B, 6, 7B), most likely due to increases in Flt-1 expression (Fig. 9) and MAPK activation (Fig. 8). The ability of the antiangiogenic factor, the 16 kDa fragment of prolactin, to inhibit VEGF-induced MAPK activation (D'Angelo et al., 1995) supports the importance of this pathway and might suggest a common mechanism for other inhibitors of angiogenesis (Good et al., 1990; O'Reilly et al., 1994, 1997). Although VEGF and bFGF have strong angiogenic activities, no rescuing capabilities or MAPK phosphorylation were detected for bFGF at concentrations of 1-10 ng/ml (Fig. 8, and data not shown). This may suggest different roles for growth factors once the vasculature is already established. The ability of bFGF to control vascular tone (Zhou et al., 1998) may be one such activity on the mature vessels. Taken together, these findings strongly suggests that VEGF is required not only to induce vascular proliferation but also for the maintenance of vessels, continuously providing a positive survival signal. This activity should be taken into consideration when one designs anti-VEGF-based therapies.

The molecular mechanisms of endothelial cell survival are not as well understood as those involved in angiogenesis, but may involve changes in the balance between apoptosis inducers and inhibitors. The induction of Ich_s expression (Fig. 5B), an inhibitor of apoptosis, may support such a mechanism. A more detailed study of the relevant caspases, apoptosis inducers and inhibitors is currently underway.

As would be expected, PMA-induced tube-like structure formation was significantly inhibited by the PKC inhibitors Ro 318220 and calphostin C (Fig. 2). The latter was also shown to inhibit PMA- and VEGF-induced angiogenesis in the chick CAM assay (Friedlander et al., 1995), suggesting similarities in PMA and VEGF signaling pathways. Indeed, phospholipase C (PLC)-gamma, a mediator of PKC activation, was found to be rapidly tyrosine-phosphorylated by, and associated with, Flk-1, followed by MAPK activation (Takahashi and Shibuya, 1997). In this regard, PMA may mimic endogenous VEGF activity. Unexpectedly, PMA also activated the PI 3 kinase pathway. Wortmanin, a specific inhibitor of PI 3 kinase (Ui et al., 1995), inhibited tube formation (Fig. 4C-D), PMA-induced Akt/PKB (Fig. 3B) and MAPK (Fig. 3C) phosphorylation. Phorbol dibutyrate-activated p21^{ras} was reported in T-cells (Downward et al., 1990) and Arbiser et al. (1997) have recently shown that H-ras is capable of activating the angiogenic switch, and that wortmanin partially inhibited ras-induced tumor angiogenesis. Since PI 3 kinase has been implicated as a direct target of ras (Rodrigues-Viciana et al., 1994), it is tempting to suggest a scenario by which PMA-induced PKC isoforms activate simultaneously both the MAP- and PI 3-kinase pathways. Inhibition of one of them, for example by wortmanin (Fig. 3C-D), by the MEK inhibitor PD 98059 (Figs 3C, 4E-F) or by the p70 S6 kinase inhibitor rapamycin (Fig. 4G-H),
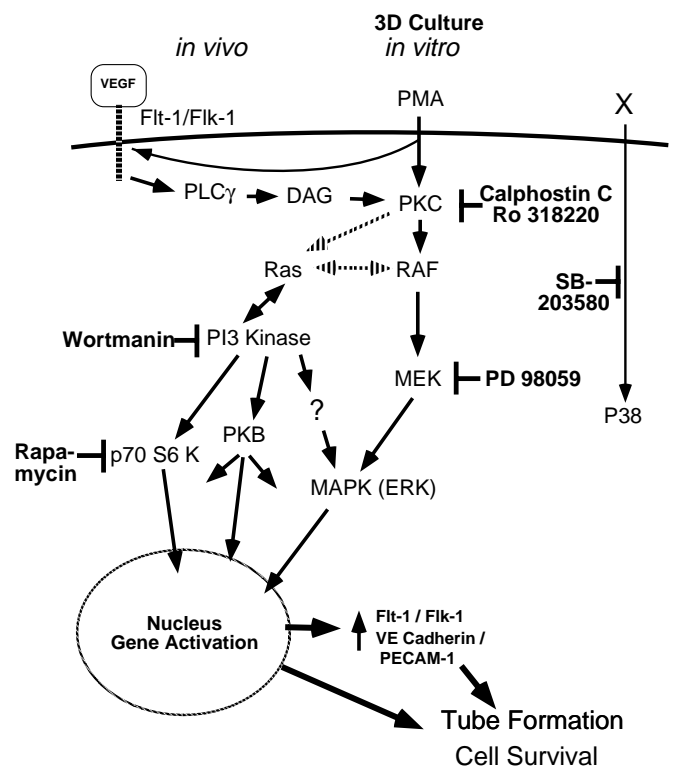

Fig. 10. Scheme representing putative signaling pathways activated by PMA and VEGF, leading to tube formation. In vivo activation of the VEGF receptors (Flk-1 = VEGFR1 and Flt-1 = VEGFR2) results in vasculogenesis and angiogenesis. Signaling is thought to occur via PKC activation and the MAPK (ERK) and PI-3-kinase signaling pathways, leading to gene activation and ultimately tube formation. In our in vitro tissue culture model VEGF$_{165}$ stimulation is insufficient to elicit an angiogenic response leading to tube formation. PMA stimulation of PKC and the subsequent activation of the MAPK (ERK) and PI-3-kinase pathways leads to gene activation including Flk-1 (VEGFR1) and Flt-1 (VEGFR2) expression and ultimately in vitro tube formation. Only after PMA induced Flk-1 and Flt-1 expression is VEGF$_{165}$ able to function as a survival factor, which has its effect via activation of the MAPK pathway, but not the Akt/PKB signaling pathway.

however, is sufficient to block tube formation completely (Figs 4 and 10). In terms of concentration (20-200 pM), rapamycin was the most potent inhibitor of PMA-induced tube-like structure formation (Fig. 4G-H). p70 S6 kinase, the target for rapamycin, plays a role in protein translation initiation (Pullen and Thomas, 1997; Thomas and Hall, 1997), indicating the necessity of de novo protein synthesis for tube formation, in agreement with the initial observation of Montesano and Orci (1985). It is not entirely clear, however, what is the precise role of PI 3-kinase during the maintenance phase of preformed tube-like structures. Akt/PKB phosphorylation on serine 473 was similar in control, involuting tubes, and in stabilized, PMA-treated ones (Figs 6, 8A). Since serine 473 phosphorylation correlates with Akt/PKB activity (Alessi et al., 1996), it is unlikely to play a role in tube survival after PMA withdrawal. However, PI 3-kinase pathway mediators are mostly unknown. For example, the enzyme responsible for Akt/PKB serine 473 phosphorylation has not yet been

identified, although new pieces of the puzzle are being discovered (Burnett et al., 1998; Gingras et al., 1998). Therefore, components other than Akt/PKB may play a role in the maintenance phase of angiogenesis as well. Additional studies utilizing specific inhibitors of the PI 3 kinase pathway may further elucidate the roles of this pathway in the processes of tube formation, maintenance and involution. Identification of specific, new proteins involved in endothelial tube regression/survival pathways will be necessary if there is to be continued development and therapeutic use of novel agents in the control of angiogenesis.

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