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In Vitro Endothelial Differentiation of Long-Term Cultured Murine Embryonic Yolk Sac Cells Induced by Matrigel

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Key Words. *Endothelial differentiation · Yolk sac cell · Matrigel · Inhibitor · Tube formation · Angiogenesis*

ABSTRACT

The yolk sac of an early mammalian embryo contains progenitors of hematopoietic cells and vascular endothelial cells. We established a cell line, YS4, from murine embryonic yolk sac 10 years ago. The line has been successfully cultured since then. To determine whether these long-term cultured yolk sac cells still have the potential to differentiate into endothelial cells, an in vitro model of yolk sac cell differentiation into tube-forming endothelial cells was established in the present study by culturing the yolk sac cells on basement membrane proteins (Matrigel). The results indicate that upon plating onto Matrigel, YS4 cells attach quickly, align in tandem, and form a complete network of capillary structures within 12 h. By using antibodies against the known components of Matrigel in a tube formation inhibition assay, we found that extracellular matrix proteins such as laminin, collagen IV, vitronectin, and fibronectin are the most important components in the Matrigel which induce the yolk sac cells to undergo endothelial differentiation. New basement membrane proteins are also required for the endothelial differentiation process, as indicated by the fact that base membrane protein synthesis inhibitor, D609, can block the differentiation process. Furthermore, our experiments revealed the involvement of several signal transduction pathways, such as protein kinase A, C and protein tyrosine kinase in this differentiation process. *Stem Cells* *1999;17:72-81*

INTRODUCTION

Mammalian embryonic yolk sac contains hemangioblasts, progenitors that are able to differentiate into both hematopoietic cells and vascular endothelial cells through processes of hematopoiesis and vasculogenesis. Freshly isolated or short-term cultured murine embryonic yolk sac cells can be induced to differentiate into all types of hematopoietic cells [1-3], and they have been successfully used to reconstitute the hematopoietic system in sublethally irradiated mice [4]. Even long-term cultured yolk sac cells still have the potential to differentiate into some types of blood cells [5] (and our unpublished data). On the other hand, blood vessel formation has been observed from freshly isolated murine embryonic yolk sac tissue in vitro [6]. Furthermore, our previous work has shown that an established murine embryonic yolk sac cell line, YS4, which was cultured from a day 6.5 mouse embryonic yolk sac, could be induced to differentiate into endothelial cells; this was confirmed by evaluation with a broad spectrum of commonly used endothelial cell markers. The cells demonstrated active uptake of acetylated low-density lipoprotein. In vitro populations of the cells strongly expressed CD31, VCAM-1, and vitronectin receptor (αV) but were negative for ICAM-1 and major histocompatibility antigens. The cells also bound *Griffonia (Bandeiraea) simplicifolia* B4 isolectin and *Ulex europaeus* I lectin, but were not immunoreactive for Factor VIII related antigen. Polymerase chain reaction (PCR) analysis indicated that upon growing on Matrigel, these cells were positive for mRNA for tissue-type plasminogen activator (tPA), angiotensin-converting enzyme (ACE), and the endothelial-associated receptor tyrosine kinases, tie-1, tie-2, and flk-1 [7]. When coinjected subcutaneously with Matrigel into the mouse, these cells participated in the formation of new blood vessels in the Matrigel implant [8].

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In the present study, we have further investigated the potential of these long-term cultured murine embryonic yolk sac cells (up to the 69th passage) to differentiate into endothelial cells and form vascular structures when cultured in vitro on Matrigel. We also attempted to determine which components in Matrigel are crucial for these yolk sac cells to differentiate into endothelial cells and form vascular structure by utilizing several inhibitors or antibodies against the particular components of Matrigel. In addition, inhibitors of several enzymes involved in three signal transduction pathways were used to determine which signal transduction pathway(s) might be employed in triggering the process of the yolk sac cell differentiation.

Materials and Methods

Cell Culture

The murine embryonic yolk sac cell line, YS4, established in our laboratory, was cultured in alpha-minimal essential medium (α-MEM) containing 18% fetal bovine serum ([FBS], Hyclone; Logan, UT), 10% leukemia inhibitory factor (LIF) conditioned medium, 50 μg/ml gentamicin, and 0.2 mM β-mercaptoethanol at 37°C in a fully humidified atmosphere of 5% CO_2. Culture flasks or petri dishes were coated with 0.1% gelatin for at least 20 min before cell inoculation.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Assay of YS4 Cells for Specific Markers of Endothelial Origin

1×10^6 YS4 cells were grown in gelatin-coated six-well plates for 16 h. Total RNA was prepared using TRIzol reagent (Promega; Madison, WI) according to the manufacturer's directions. If any DNA contamination was present in the RNA samples, it was removed by DNAase I digestion. One μg total RNA was digested with one unit of DNAase I (GIBCO; Gaithersburg, MD) in a buffered 10 μl reaction volume at room temperature for 15 min. After digestion, one μl of EDTA was added, and the reaction was put into a 65°C bath for 10 min to inactivate the DNAase.

The DNAase-I-treated RNA was used to perform RT-PCR by using the Promega Access RT-PCR system. Three pairs of primers were designed to detect the expression of three endothelial cell specific genes: Flk-1, Tie 1, and Tie 2. The sequences of the primers are as follows: Flk-1: 5′CGGATCCACAGTGACCT3′ and GCAATTCCAAAGGACCAGACGTC3′; Tie 1: 5′ATGGCGAATGTGTTGTCC3′ and 5′GGTCACAAGTGCCACCATT3′; and Tie 2: 5′TTCTCATGAACTGAGGACGC3′ and 5′ CCTTCTTGATGCGGCCTT3′. HPRT gene-specific primers were used as positive controls. The concentration of these primers used was 0.5 μg/reaction.

Induction of Tube Formation by YS4 Cells on Matrigel

Matrigel (Becton Dickinson; San Jose, CA) was thawed at 4°C overnight, and wells in a 24-well plate were coated with 250 μl of liquid Matrigel per well. The coated wells were then incubated at 37°C for 30 min, and 1×10^5 YS4 cells were seeded into each well and incubated for 24 h. During this period, the morphologic changes of the cells were observed and recorded under a microscope.

Preparation of Reconstituted Collagen I Gel

Gels of reconstituted collagen I fibers were prepared as described previously [9]. Seven volumes of cold collagen I (3.3 mg/ml, Boehringer Mannheim; Indianapolis, IN) were quickly mixed with one volume of 10 × MEM and two volumes of sodium bicarbonate (11.76 mg/ml) in a sterile tube kept on ice to prevent immediate gelation. Laminin, fibronectin, collagen IV, vitronectin (ICN; Costa Mesa, CA), and some growth factors such as bFGF and VEGF (Sigma; St. Louis, MO), were then added quickly into the mixture. The cold mixture was dispensed into 12-well culture plates at 0.5 ml/well and allowed to gel for 10 min at 37°C before the plating of cells.

Fluorescence Activated Cell Sorter (FACS) Analysis of Surface Markers of YS4 Cells Grown on Matrigel

YS4 cells were seeded at a density of 5×10^6 into T-75 flasks coated with 2.5 ml of Matrigel or 10 ml of gelatin, respectively, and incubated for 4 h. Cells were then washed twice with phosphate-buffered saline (PBS) and gently scraped off into PBS with cell scrapers. After the cells were passed through a 40-μm cell strainer and the concentration determined, the cells were resuspended in staining buffer (PBS, 0.1% bovine serum albumin [BSA], 0.1% sodium azide), washed once with staining buffer, aliquoted into several groups, and stained with different antibodies conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE) (PharMingen; San Diego, CA) for 30 min on ice. The unbound antibodies were removed by washing the cells twice with staining buffer, after which the cells were resuspended in 0.5 ml of staining buffer for FACS analysis.

Tube Formation Inhibition Assay

All of the inhibition assays were performed in wells of a 24-well plate coated with Matrigel for 30 min at 37°C. Test reagents were added at the time of plating the cells. The test reagents used in this study are listed in Table 1. The inhibitory effects of the test reagents on tube formation by yolk sac cells on Matrigel were observed under a microscope and recorded photographically. For quantitation of tube formation, the total length of the tubes formed in a unit area was measured. For each test, five randomly chosen areas were measured and averaged. Inhibitory effects were determined by comparing the

Table 1. List of reagents used in the inhibition assay

Name	Source
Antibodies	
Anti-laminin antibody	ICN; Costa Mesa, CA
Anti-collagen IV antibody	ICN; Costa Mesa, CA
Anti-fibronectin antibody	ICN; Costa Mesa, CA
Anti-vitronectin antibody	ICN; Costa Mesa, CA
Anti-integrin β1 antibody	ICN; Costa Mesa, CA
Anti-tumor necrosis factor α (TNF α) antibody	ICN; Costa Mesa, CA
Anti-basic fibroblast growth factor (bFGF) antibody	ICN; Costa Mesa, CA
Anti-transforming growth factor β (TGFβ) antibody	ICN; Costa Mesa, CA
Tyrosine kinase inhibitors	
Genistein	ALEXIS; San Diego, CA
Piceatannol	ALEXIS; San Diego, CA
Tyrphostin 47	ALEXIS; San Diego, CA
Staurosporine	ALEXIS; San Diego, CA
Protein kinase C inhibitors	
Polymyxin B sulfate	
Trifluoperazine	ALEXIS; San Diego, CA
Staurosporine	ALEXIS; San Diego, CA
Base membrane synthesis inhibitor	
D609	Sigma; St. Louis, MO

values of experimental wells with those of positive controls (yolk cells on Matrigel without addition of any inhibitor) and negative controls (yolk sac cell on gelatin). The data were presented as the ratio of averaged tube length of experimental group over that of positive control. Designations of +++, ++, + and – were used to represent the ratios between 0.7 and 1, 0.7 and 0.4, 0.4 and 0.1, and <0.1, respectively. For pretreatment experiments, cells were first grown on gelatin-coated wells and treated with test reagents at concentrations that effectively inhibit tube formation on Matrigel for different periods of time. They were then transferred to Matrigel-coated wells for observation of the effect of pretreatment by various reagents on tube formation. To determine whether the inhibitory effects of some inhibitors on tube formation by YS4 cells on Matrigel were reversible, cells were first cultured for up to 12 h on Matrigel in the presence of test reagents at concentrations that can inhibit tube formation on Matrigel. Then these test reagents were removed and replaced with fresh yolk sac medium, and the cells were cultured an additional 12 h. The ability of the cells to form capillary structures after removal of the reagents was evaluated as above.

RESULTS

Long-Term Cultured Yolk Sac Cells Express Endothelial Cell Markers

To test the endothelial potential of long-term cultured yolk sac cells, expression of three endothelial-specific genes, Flk-1, Tie-1, and Tie-2 were determined by RT-PCR. As shown in Figure 1, Tie-2 and Flk-1 genes were expressed in the YS4 cells; however, Tie-1 was not expressed in the YS4 cells. This result suggests that YS4 are endothelial progenitor cells, but not matured endothelial cells.

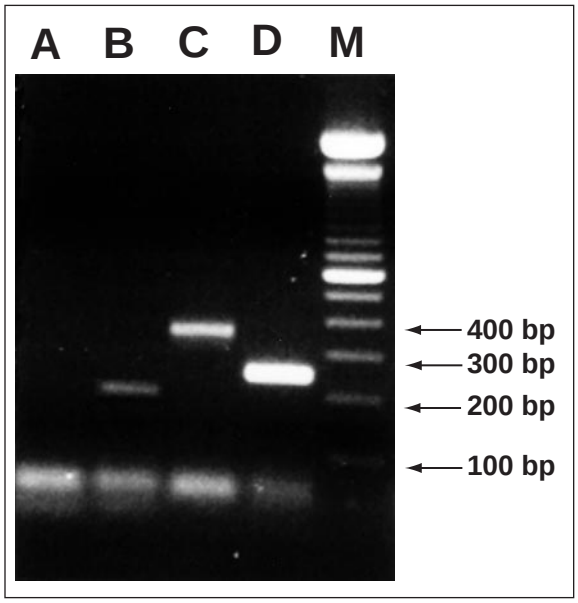

Figure 1. RT-PCR analysis of total RNA from YS4 cells for expression of endothelial specific markers, Tie-1 (A), Flk-1 (B), Tie-2 (C). *HPRT was used as positive control (D). M is 100 bp DNA ladder.*

YS4 Cells Form Capillary Structure when Cultured on Matrigel

Previous studies showed that Matrigel, and in some cases, its components, can efficiently induce endothelial cells of both human and bovine origin to form tube-like structures in vitro. Therefore, Matrigel appears to be an excellent endothelial differentiation inducer. In our tube formation assay, after seeding on Matrigel-coated wells, the YS4 cells adhere very quickly, usually within 1 h. After 3 h on Matrigel, the cells started to align in tandem. Six h later, capillary-like structures could be observed, and within 12 to 24 h, a complete extensive capillary network could be seen under the microscope (Fig. 2).

Mixture of Some Known Components of Matrigel Failed to Induce Yolk Sac Cells to Form Tube Structure

We attempted to establish conditions other than growth on Matrigel under which YS4 cells could be induced to differentiate into tube-forming endothelial cells. For this purpose, various components of Matrigel and growth factors were tested. Unfortunately, among the components we tried, only intact Matrigel is capable of inducing yolk sac cells to form tubes. All the other substrates or substrate combinations can only support cell attachment but not differentiation or tube formation.

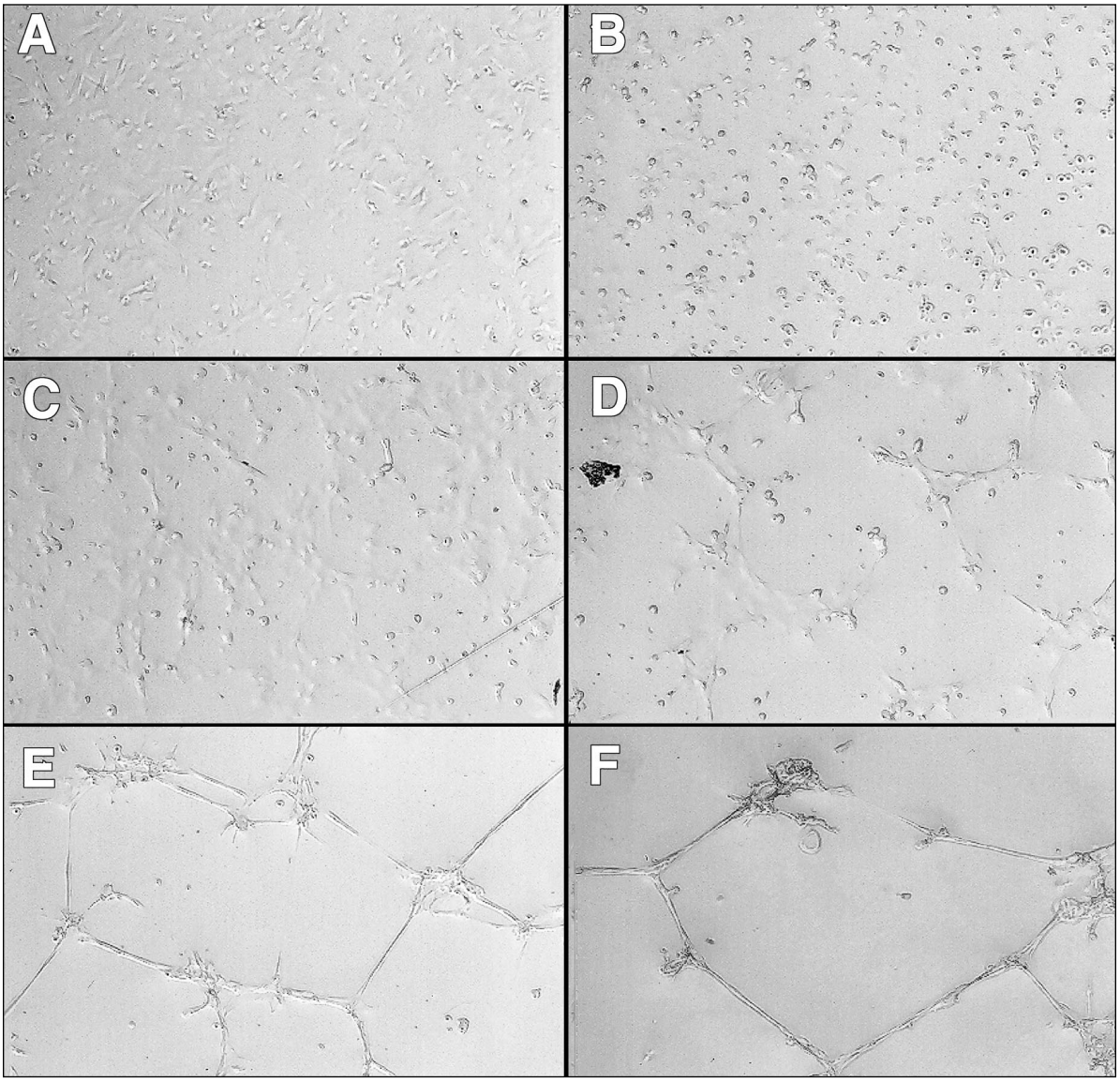

Figure 2. Time-course analysis of YS4 cells growing on Matrigel. *(A) represents YS4 cells growing on gelatin coated dish for 12 h. (B-F) are YS4 cells growing on Matrigel coated dishes for 1 h (B), 3 h (C), 6 h (D), 12 h (E) and 24 h (F).*

FACS Analysis of Specific Surface Markers on YS4 Cells Before and After Growing on Matrigel

To determine what changes in the expression of cell surface markers may be induced by Matrigel, 5×10^6 cells were seeded into two gelatin- or Matrigel-coated T-75 flasks, respectively. After incubation for 4 h, cells were scraped off flasks. Aliquots of cells were then stained with FITC- or PE-conjugated antibodies and analyzed by FACS. As indicated in Figure 3, the expression of hematopoietic-related markers (VCAM, I-Ad, c-kit, B220, and Thy1.2) decreased dramatically after the cells were grown on Matrigel for 4 h from 22.8%, 7.79%, 6.59%, 14.75%, and 4.08% to 1.38%, 3.21%, 0.98%, 0.13%, and 1.42%, respectively. In contrast, the expression of an endothelial-cell-specific marker, CD31, increased from 0.29% to 20.73%.

Effects of Antibodies Against Components of Matrigel on Tube Formation by YS4 Cells

Matrigel contains many protein components. To understand what component(s) are necessary to stimulate yolk sac cells to differentiate and form tube-like structure, a series of antibodies against these components was used in this study. The results indicate that antibodies against extracellular matrix (laminin, fibronectin collage IV, vitronectin, and β2-integrin) showed a dose-dependent inhibitory effect. However, antibodies against soluble growth factors in Matrigel (bFGF, tumor necrosis factor-β

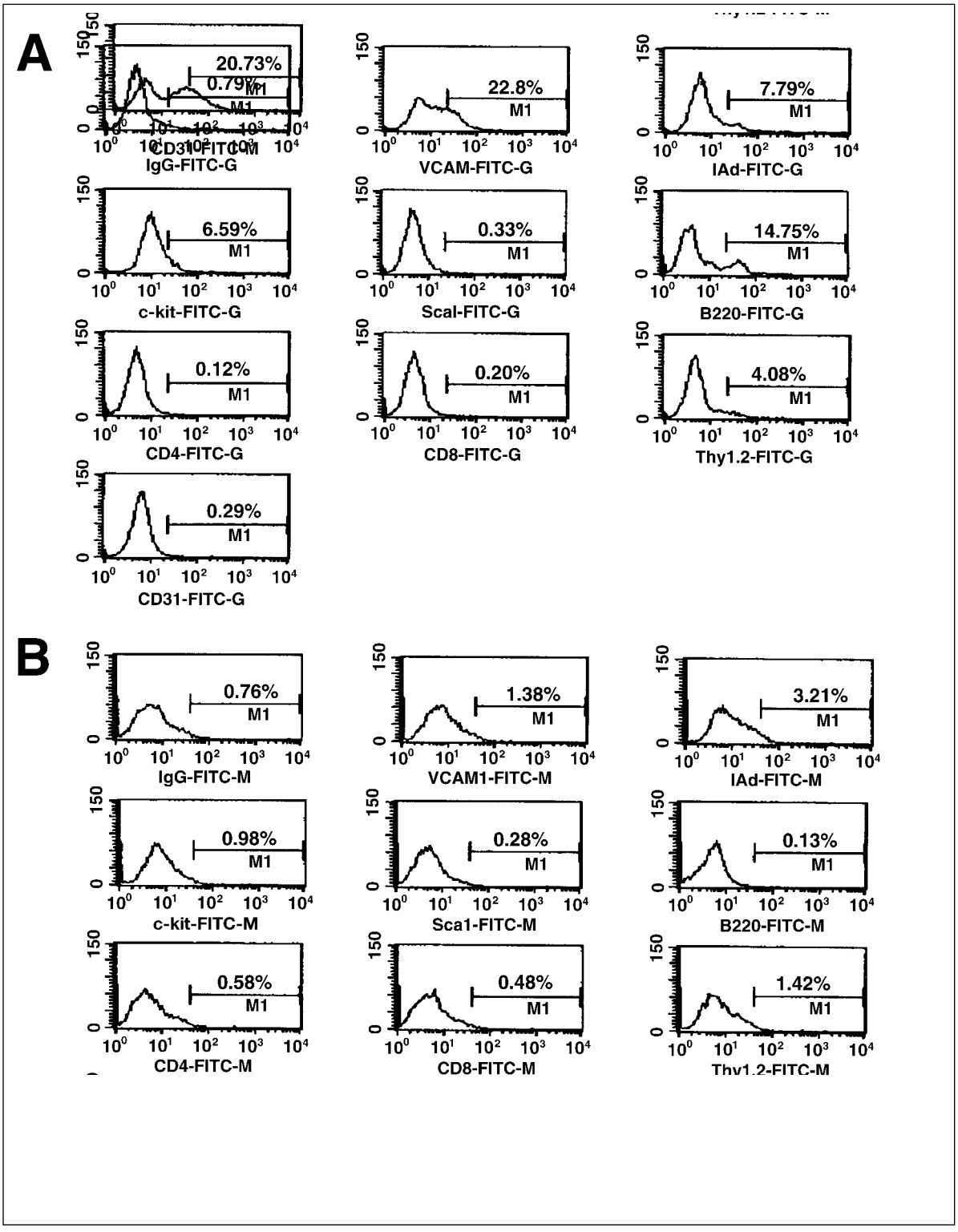

Figure 3. FACS analysis of expression of cell surface molecules on YS4 cells induced by Matrigel. *A) Surface markers on cells grown in gelatin-coated flasks for 4 h; B) Surface markers on cells grown in Matrigel coated flasks for 4 h. 5 ×10⁶ YS4 cells were seeded into T-75 flasks coated with 2.5 ml of Matrigel or 10 ml of gelatin, respectively, and incubated for 4 h. Cells were then washed twice with PBS and gently scraped off into PBS with cell scrapers. After passing through a 40 µm cell strainer and determining the concentration, the cells were resuspended in staining buffer, washed once with staining buffer, aliquoted into several groups and stained with different antibodies conjugated with FITC or PE of 30 min on ice. The unbound antibodies were removed by washing the cells twice with staining buffer after which the cells were resuspended in 0.5 ml of staining buffer or FACS analysis. M1 stands for marker number 1.*

[TGF-β], and TNF-α) did not show any inhibitory effect on tube formation.

Table 2. Basement membrane protein synthesis inhibitor, D609, inhibits yolk sac cell differentiation/tube formation

Concentration (μg/ml)	Tube formation
10	+++
25-50	++
100	+
250	–

D609 Inhibits Tube Formation by YS4 Cells on Matrigel

Tricyclodecan 9-ylxanthate (D609) is an inhibitor of basement membrane synthesis and angiogenesis and also has antitumor properties [10]. To test whether new basement membrane proteins are needed in the process of yolk sac cell differentiation and tube formation on Matrigel, D609 was tested in this system. As shown in Table 2, D609 caused a dose-dependent inhibition observable at 25 μg/ml, with a maximum effect at 250 μg/ml. This inhibition is reversible because yolk sac cells on Matrigel in the presence of D609 for 6 h formed tube-like structures after the removal of D609. Furthermore, pretreatment of YS4 cells with D609 before seeding on Matrigel has no effect on tube formation. All of the above suggests that new basement membrane proteins are required for the differentiation process.

Table 3. Effects of protein tyrosine kinase inhibitors on yolk sac cell differentiation/tube formation

Inhibitor	Specificity	Concentration	Tube formation
Lavendustin A	PTK, not PKA or PKC	20 μM 200 μM	+++ +
Lavendustin C	PTK, EGFR, pp60$^{c\text{-}src}$, Ca2+/camoldulin-dependent kinase II	20 μM 50 μM 100 μM 200 μM	+++ ++ + –
Genistein	PTK	10 μM 25 μM 50 μM 100 μM	+++ ++ + –
Piceatannol	Syk, not Lyn	100 μM 200 μM	+++ –
Tyrphostin 47	EGFR, GTPase, activity of transduction	100 μM 200 μM	+++ –

Table 4. Effects of PKC inhibitors on yolk sac cell differentiation/tube formation

Inhibitor	Specificity	Concentration	Tube formation
Bisindolyl-maleimide II	PKC, others also at high concentrations	20 μM	–
Bisindolyl-maleimide IV	PKC, PKA	20 μM 200 μM	++ –
Chelerythrine-chloride	PKC	20 μM	–
H-7dihydro-chloride	PKC, PKA, PKG, etc.	20 μM 200 μM	+ –
Polymyxin B sulfate	PKC	1,000 u/ml 2,500 u/ml 5,000 u/ml	+++ + –
Trifluoperazine	calmodulin, cAMP-gated cationic channels	1 μM 2.5 μM 10 μM 25 μM	+++ ++ + –
Staurosporine	PKC, PKA, PKG, etc.	1 nM 2.5 nM 10 nM 100 nM	+++ ++ + –

Protein Kinase Activities are Required for Tube Formation Induced by Matrigel

During angiogenesis, endothelial cells react to stimulation with finely tuned signaling responses. It has been reported that signal transduction pathways involving PKC, PKA, PTK, etc., signals play important roles in angiogenesis and the endothelial cell differentiation process [11-13]. To determine whether such signal transduction pathways are involved in the differentiation of yolk sac cells into tube-forming endothelial cells, two protein kinase signal transduction pathways (PKC and PTK) were studied by using various protein kinase inhibitors. The results indicated that both pathways are involved in the endothelial differentiation process (Tables 3 and 4). Most of the tyrosine kinase C and K inhibitors showed a dose-dependent inhibitory effect on tube formation by yolk sac cells on Matrigel. For example, after 24 h of culture, cells in 10 nM of staurosporine showed a marked decrease in tube formation as compared with control cells, and there were large numbers of monolayer cells which showed a spindle-like morphology. For cells in 100

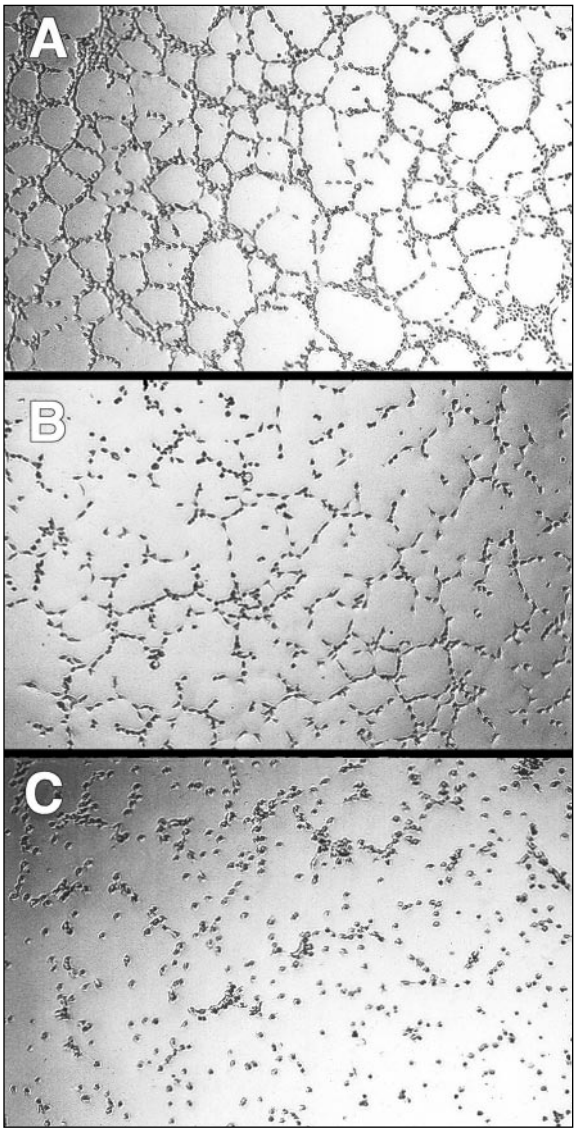

Figure 4. Inhibition of capillary structure formation of YS4 cells by staurosporine. *YS4 cells were seeded on Matrigel-coated dishes in the presence of 0 nM (A), 10 nM (B) and 100 nM (C) of staurosporine for 24 h.*

nM of staurosporine, no tubes were observed, they had many cellular processes, and they seemed more flat under microscopic evaluation (Fig. 4).

Discussion

Several tyrosine kinase messages have been identified in a population of pluripotent murine embryonic stem cells [14]. In situ hybridization analysis of one of them, the product of the Flk-1 gene, revealed that Flk-1 was expressed in the proximal-lateral embryonic mesoderm, tissue fated to become the heart. By the headfold stage, staining was confined to the endothelial cells of the heart primordia as well as to the blood islands of the visceral yolk sac and the developing allantois. Patchy, speckled staining was detected in the endothelium of all the major embryonic and extraembryonic blood vessels as they formed. During early organogenesis, expression was detected in the blood vessels of highly vascularized tissues such as brain, liver, lungs, and placenta. Since Flk-1 was expressed in early mesodermal cells prior to any morphological evidence for endothelial cell differentiation (vasculogenesis), as well as in cells that form blood vessels from preexisting ones (angiogenesis), it appears to be a very early marker of endothelial cell precursors. Another receptor tyrosine kinase, designated TEK or Tie-2, was found to be expressed in differentiating endothelial cells. A third receptor tyrosine kinase, Tie-1, was also found to be endothelial-cell-associated. All three have also been demonstrated to be very important in blood vessel formation by gene knock-out experiments [15]. Herein, the possibility that embryonic yolk sac cells are the precursors of endothelial cells is being addressed by determining the expression of these genes in the YS4 cells. Our RT-PCR results showed that these yolk sac cells expressed the endothelial cell markers Flk-1 and Tie-2, but not Tie-1 (Fig. 1), suggesting that these yolk sac cells are not mature endothelial cells but serve as the progenitors for endothelial cells, since our previous study had shown that upon growing on Matrigel, Tie-1 was expressed [7].

Early studies by researchers showed that Matrigel can induce human umbilical vein endothelial cells (HUVEC) to differentiate into tube-like structures. Studies also showed that some combinations of the Matrigel components plus certain growth factors can induce HUVEC to form tubes as well. *Kubota et al.* [16] found that endothelial cells cultured on a collagen I gel supplemented with laminin-formed tubes, while supplementation with collagen IV induced a lesser degree of tube formation. In another study [17], cultured rat hepatic sinusoidal endothelial cells were found to invade a laminin-containing collagen I gel and exhibited three-dimensional tube formation. This demonstrates the importance of laminin in inducing tube formation. By using human neonatal foreskin microvascular endothelial cells, *Jackson et al.* [18] were able to demonstrate that soluble type I collagen can induce vascular tube formation when it contacts the apical side of a confluent endothelial monolayer. In the same study, they also determined that sulfated polysaccharides such as heparin are required for collagen-induced vascular tube formation. However, in *Kuzuya's* work [19] it was found that fibroblast-conditioned medium also induced endothelial cells to elongate initially and subsequently to organize into a capillary-like structure within collagen gels. This information suggests that many of the components in Matrigel are important in the induction of endothelial cells to organize into capillary structures.

In our study, however, the case is more complicated because the cells we used are not typical endothelial cells but are early progenitors of endothelial cells, as indicated

by the RT-PCR analysis. Therefore, the induction of these embryonic yolk sac cells to form tube structures involves the differentiation of the yolk sac cells into endothelial cells as a first step, followed by the organization of the endothelial cells into tube structures. Among all the mixtures of extracellular matrix proteins and components we have tested, only Matrigel itself was found to be capable of inducing the YS4 cells to differentiate and form tubes (Fig. 2). Combinations of collagen I, collagen I gel, collagen IV, laminin, fibronectin, VEGF, bFGF, aFGF, etc., failed to achieve the induction. However, when plated on Matrigel-coated wells, the cells attached to the Matrigel within 2 h and started to align with each other. Primary capillary-like structures could be observed 4 h later, and within 12 h, a complete capillary network was established. This demonstrates not only the stem cell potential of the embryonic yolk sac cells but also their ability to organize into tube-like structures.

The fact that long-term cultured embryonic yolk sac cells can be induced to differentiate into endothelial cells is further supported by the FACS results. The expression of the endothelial-cell-related marker, CD31, increased from 0.29% to 20.73% (Fig. 3) after growing on Matrigel.

In this study, different time-frozen aliquots of YS4 cells after passage 18 were used. Although after long-term culture (the longest of which was passage 69), the cells still have the potential to differentiate into endothelial cells upon induction, there are some changes from different time-frozen aliquots. For example, at relatively early stages (from passage 18 to 25), the cells were morphologically heterogeneous (Fig. 2A), less tumorigenic, and express a relatively high level of CD31. After passage 30, the cells became morphologically homogeneous (Fig. 4A), more tumorigenic, and express a relatively low level of CD 31. The cells do not express hematopoietic stem cell marker CD34.

Since the combination of several extracellular membrane (ECM) proteins and growth factors failed to induce the YS4 cells to differentiate into tube-like structures, we then set out to determine what components in the Matrigel are important in the induction by using an indirect inhibition of tube formation assay. Our results showed that antibodies against laminin, collagen IV, fibronectin, vitronectin, integrin β1 receptor had a dose-dependent inhibitory effect on tube formation by YS4 cells grown on Matrigel, while antibodies against bFGF, TNF-α, and TGF-β had no effect, even at higher concentrations. This indicates that laminin, collagen IV, fibronectin, and vitronectin are very important components in the Matrigel that enable YS4 cells to differentiate, while growth factors such as bFGF, TNF-α, TGF-β may not play such an important role in this process. The data also demonstrate that yolk sac cells must recognize several components of Matrigel and interact with them through laminin and collagen receptors (integrins), since antibody against integrins abolishes tube formation.

During angiogenesis, endothelial cells respond to stimulation with an orchestrated series of signaling responses. The process of angiogenesis involves signals from both soluble and insoluble factors [20]. One effect of soluble factors is to provide the initial stimulus for endothelial cells to secrete basement membrane degrading proteases. The endothelial cells subsequently migrate into the underlying stroma, where they proliferate, align with one another, and form into new vessels. The interaction of endothelial cells with insoluble factors, such as basement membrane proteins, may dominate the later phases of this process [21]. This may explain why, in our system, antibodies to soluble factors such as bFGF, TGF-β, and TNF-α had no inhibitory effect on tube formation by cells on Matrigel. Since, in our system, the platform (Matrigel) for tube formation was already in place, soluble factors such as bFGF, TGF-β and TNF-α are not so critical in this system. In contrast, the ECM proteins present in the Matrigel were critical and dominated the differentiation process, as demonstrated by the fact that antibodies to ECM proteins significantly blocked tube formation by yolk sac cells on Matrigel.

Another key element in the morphologic differentiation of the yolk sac cells on Matrigel is the requirement of new base membrane protein synthesis. In the present study, we investigated the role of de novo basement membrane synthesis during the formation of tube-like structures by using D609, a reagent that has previously been shown to inhibit basement membrane synthesis both in vivo and in vitro [10]. D609 was also shown to inhibit angiogenesis and tumor growth [22]. Endothelial cells have been shown to synthesize basement membrane collagen in vitro [23]. This newly synthesized basement membrane is essential for tube formation in our Matrigel system, since D609 prevented tube formation in a dose-dependent fashion. The presence of antibody to type IV collagen probably prevents tube formation because it prevents the interaction and deposition of newly synthesized basement membrane collagen with preexisting basement membrane collagen. On the whole, our results indicate that although yolk sac cells on Matrigel are in contact with a collagen-rich substrate, the synthesis and perhaps the deposition of new collagen is likely essential for tube formation.

Studies by *Haralabopoulos et al.* [10] using HUVEC support this hypothesis. Several signaling pathways have been implicated in the differentiation of endothelial cells into vessels or tubes, both in vivo and in vitro. For example, PKC was suggested as an important positive regulator of angiogenesis by studies showing that tumor-promoting phorbol esters, which activate PKC, stimulate angiogenesis both in vitro and in vivo [24, 25], and downregulation of PKC by prolonged treatment with tumor-promoting phorbol esters or

inhibition of protein kinases prevents angiogenesis [12]. Here, we attempted to determine the signal pathways involved in the yolk sac cell differentiation into tube-like structures in our Matrigel system by using a wide range of inhibitors to protein kinases, such as PKC inhibitors and tyrosine kinase inhibitors. The results revealed the importance of PKC and PTK involvement in the differentiation process of the embryonic yolk sac cells, since several PKC and PTK inhibitors were shown to prevent tube formation by yolk sac cells on Matrigel. Among those PKC inhibitors tested (Table 4), staurosporine demonstrated the most potent inhibitory effect in tube formation assay. At 100 nM, staurosporine totally abolished tube formation with the phenotypic appearance of monolayer cells. Since staurosporine also affects other protein kinases such as PKA and PKG, etc., it is possible that the signal pathway acts through PKA or PKG. Tyrosine kinases are important regulators in the developmental process of an animal with regard to cell growth, proliferation, and differentiation. Since many receptors for the growth factors that are involved in the differentiation of endothelial cells to form blood vessels are themselves tyrosine kinase receptors, such as receptors for VEGF, and there also are many such growth factors in the Matrigel, we speculate that the tyrosine kinase signal transduction pathways may likely be involved in the process of yolk sac cell differentiation into tube-like structures. Our study using inhibitors to tyrosine kinase to prevent tube formation by the yolk sac cells supports this belief. Different inhibitors to PTK prevented tube formation by yolk sac cells on Matrigel to different degrees (Table 3). One of the PTK inhibitors, genistein, has been extensively investigated in these studies. Tube formation assays demonstrated that genistein at different concentrations resulted in different responses in yolk sac cells. At a dose of <10 μM, genistein has no inhibitory effect on the ability of yolk sac cells to form tubes on Matrigel, while at a dose of 25 μM, it reduced tube formation to some extent. At a dose of 50 μM, genistein significantly decreased tube formation, and at doses of 100 to 150 μM, it almost totally abolished tube formation, with only a small degree of cellular alignment apparent. At doses of >200 μM, no tube formation, no alignment of cells at all, or any change in the phenotype of the cells was observed.

In summary, besides having the potential to differentiate into hematopoietic cells, long-term cultured murine embryonic yolk sac cells also have the potential to differentiate into vascular endothelial cells and form tube-like structures under the proper conditions. In order to stimulate the differentiation process of yolk sac cells, it seems that every extracellular matrix component of Matrigel is necessary, but not the associated soluble growth factors. Furthermore, because almost all the commonly used inhibitors tested in this system functioned properly, our in vitro system may serve as an in vitro testing system for potential antiangiogenic factors.

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REFERENCES

1 Liu CP, Auerbach R. In vitro development of murine T cells from prethymic and preliver embryonic yolk sac hematopoietic stem cells. Development 1991;113:1315-1323.

2 Liu CP, Auerbach R. Ontogeny of murine T cells: thymus-regulated development of T cell receptor-bearing cells derived from embryonic yolk sac. Eur J Immunol 1991;21:1849-1855.

3 Toles JF, Chui DHK, Belbeck LW et al. Hemopoietic stem cells in murine embryonic yolk sac and peripheral blood. Proc Natl Acad Sci USA 1989;86:7456-7459.

4 Huang H, Auerbach R. Identification and characterization of hematopoietic stem cells from the yolk sac of the early mouse embryo. Proc Natl Acad Sci USA 1993;90:10110-10114.

5 Ji H, Yu XZ, Wagner TE. A long-term culture system for the expansion of hematopoietic stem cells from embryonic yolk sac with the capacity to seed erythroid and lymphoid development in vitro and to reconstitute the lymphoid compartment in severe combined immunodeficient mice. Artif Organs 1996;20:1093-1109.

6 Palis J, McGrath KE, Kingsley PD. Initiation of hematopoiesis and vasculogenesis in murine yolk sac explant. Blood 1995;86:156-163.

7 Barut BA, Antczak M, Cioffi L et al. Isolation and characterization of endothelial cell lines from murine yolk sac. Blood 1994;84(suppl 1):74.

8 Wei YZ, Quertermous T, Wagner TE. Directed endothelial differentiation of cultured embryonic yolk sac cells in vivo provides a novel cell-based system for gene therapy. STEM CELLS 1995;13:541-547.

9 Greenburg G, Hay ED. Epithelia suspended in collagen gels can lose polarity and express characteristics of migrating mesenchymal cells. J Cell Biol 1982;95:333-339.

10 Haralabopoulos GC, Grant DS, Kleinman HK et al. Inhibitors of basement membrane collagen synthesis prevent endothelial cell alignment in Matrigel in vitro and angiogenesis in vivo. Lab Invest 1994;71:575-582.

11 Slevin M, Krupinski J, Kumar S et al. Angiogenic oligosaccharides of hyaluronan induce protein tyrosine kinase activity in

endothelial cells and activate a cytoplasmic signal transduction pathway resulting in proliferation. Lab Invest 1998;78:987-1003

12 Wright PS, Cross-Doersen D, Miller JA et al. Inhibition of angiogenesis in vitro and in ovo with an inhibitor of cellular protein kinases, MDL 27032. J Cell Physiol 1992;152:448-457.

13 Kohn EC, Alessandro R, Spoonster J et al. Angiogenesis: role of calcium-mediated signal transduction. Proc Natl Acad Sci USA 1995;92:1307-1311.

14 Yamaguchi TP, Dumont DJ, Conion RA et al. flk-1, an flt-related tyrosine kinase, is an early marker for endothelial cell precursors. Development 1993;118:489-498.

15 Sato TN, Tozawa Y, Deutsch U et al. Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation. Nature 1995;376:70-74.

16 Kubota Y, Kleinman HK, Martin GR et al. Role of laminin and basement membrane in the morphological differentiation of human endothelial cells into capillary-like structures. J Cell Biol 1988;107:1589-1598.

17 Shakado S, Sakisaka S, Woguchi K et al. Effects of extracellular matrices on tube formation of cultured rat hepatic sinusoidal endothelial cells. Hepatology 1995;22:969-973.

18 Jackson CJ, Giles I, Knop A et al. Sulfated polysaccharides are required for collagen-induced vascular tube formation. Exp Cell Res 1994;215:294-302.

19 Kuzuya MK. Induction of endothelial cell differentiation in vitro by fibroblast-derived soluble factors. Exp Cell Res 1994;215:310-318.

20 Ingber DE, Folkman J. How does extracellular matrix control capillary morphogenesis? Cell 1989;58:803-805.

21 Grant DS, Tashiro KI, Segui-Real B et al. Two different laminin domains mediate the differentiation of human endothelial cells into capillary-like structures in vitro. Cell 1989;58:933-943.

22 Maragoudakis ME, Sarmonica M, Panoutsacopoulou M. Inhibition of basement membrane biosynthesis prevents angiogenesis. J Pharm Exp Ther 1988;244:729-733.

23 Madri JA, Pratt BM, Tucker AM. Phenotypic modulation of endothelial cells by transforming growth factor depends upon the composition and organization of the extracellular matrix. J Cell Biol 1988;106:1375-1384.

24 Montesano R, Orci L. Tumor-promoting phorbol esters induce angiogenesis in vitro. Cell 1985;42:469-477.

25 Morris PB, Hida T, Blackshear PJ et al. Tumor-promoting phorbol esters induce angiogenesis in vivo. Am J Physiol 1988;254:C318-C322.