

Embryonic Stem Cell-Derived Embryoid Bodies Development in Collagen Gels Recapitulates Sprouting Angiogenesis

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SUMMARY: The formation of new blood vessels proceeds by both vasculogenesis and angiogenesis. The development of models, which fully recapitulate spatio-temporal events involved during these processes, are crucial to fully understand their mechanisms of regulation. In vitro differentiation of murine embryonic stem (ES) cells has been shown to be a useful tool to investigate factors and genes potentially involved in vasculogenesis (Hirashima et al, 1999; Risau et al, 1988; Vittet et al, 1996; Wang et al, 1992; Wartenberg et al, 1998). We asked here whether this model system can also recapitulate angiogenesis, which may offer new means to study mechanisms involved in this process. ES-derived embryoid bodies (EBs) obtained after 11 days of differentiation, in which a primitive vascular network had formed, were then subcultured into a type I collagen matrix. In the presence of angiogenic growth factors, EBs rapidly developed branching pseudopods. Whole mount immunostainings with a PECAM antibody revealed that more than 75% EBs displayed, within a few days, a large number of endothelial outgrowths that can give tube-like structures with concomitant differentiation of α -smooth muscle actin positive cells, thus evoking sprouting angiogenesis. High expression levels of flk1 (VEGFR2), flt1 (VEGFR1), tie-1, and tie-2 are also found, indicating that budding endothelial cells displayed an angiogenic phenotype. The endothelial sprouting response was specifically induced by angiogenic factors with a major contribution of vascular endothelial growth factor (VEGF). Known angiostatic agents, such as platelet factor 4 (PF4), angiostatin, and endostatin inhibited the formation of endothelial sprouts induced by angiogenic factors. Moreover, consistent with the in vivo phenotype, VE-cadherin deficient EBs failed to develop angiogenesis in this model. ES cell differentiation can then recapitulate, in addition to vasculogenesis, the early stages of sprouting angiogenesis. This model system, in which genetic modifications can be easily introduced, may be of particular interest to investigate unsolved questions and molecular mechanisms involved in blood vessel formation. (*Lab Invest* 2001, 81:1669–1681).

Vascularization of organs and tissues proceeds by two related but distinct processes: vasculogenesis and angiogenesis (Risau, 1997). In vasculogenesis, primitive blood vessels develop from angioblast precursor cells, which differentiate and assemble into cord-like vascular structures that further connect into a primary network. Angiogenesis corresponds to the formation of new blood vessels from the pre-existing blood vasculature by the sprouting, splitting, and remodeling of the vascular network. Angiogenesis is a complex process, which results from an ordered set of events involving endothelial cell activation, growth, migration, and capillary morphogenesis. Both vasculogenesis and angiogenesis are involved in the development of a functional vascular system in the embryo (Flamme et al, 1997), but also contribute to postnatal blood vessel formation (Asahara et al, 1997, 1999;

Kalka et al, 2000). In the adult, a neovascularization response occurs in a variety of physiological and pathological settings, including wound healing, recovery from myocardial infarction, inflammation-related diseases, solid tumor growth, and tumor metastasis. Numerous studies have characterized cellular and molecular factors important to vascular formation and development (Beck and D'Amore, 1997; Risau, 1997). Although intensively studied, the molecular mechanisms involved in the regulation of both vasculogenic and angiogenic responses are still not completely understood. Therefore, the development of models, which fully recapitulate spatio-temporal events involved during blood vessel formation and which exhibit a great potential for genetic investigations, appears crucial to elucidate and fully understand the regulation of these processes.

Embryonic stem (ES) cell model has been reported to be a particularly interesting tool to investigate molecular mechanisms and genes involved in mammalian development (Rathjen et al, 1998). ES cells are pluripotent cells derived from the early mouse embryo that can be propagated stably in the undifferentiated state in vitro. Since they retain the ability to differentiate into all cell types found in an embryonic and adult

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mouse in vivo, ES cells have been most extensively used to analyze gene function in development via gene targeting and homologous recombination, by generating mutant mice (Rathjen et al, 1998). ES cell lines also provide the ability to directly probe gene function during in vitro differentiation of ES cells themselves. Indeed, ES cells differentiating in vitro form embryo-like aggregates, called the embryoid bodies (EBs), and develop many cell types including hematopoietic cells, neuronal cells, cardiomyocytes and muscle cells, epithelial cells, and cells of the endothelial lineage (Rathjen et al, 1998). Several studies have indicated the occurrence of vasculogenesis in ES-derived EBs (Hirashima et al, 1999; Risau et al, 1988; Vittet et al, 1996; Wang et al, 1992; Wartenberg et al, 1998). Furthermore, we have previously established that the endothelial development within ES-derived EBs, as defined by the onset of specific gene expression, follows an ordered sequence of events that recapitulates murine vasculogenesis in vivo and leads to the formation of vascular structures evoking a primitive vascular network (Vittet et al, 1996). Other experiments, performed by plating EBs onto gelatinized dishes, showed the formation of branching vascular structures indicative of extensive vascular morphogenesis from EBs (Bloch et al, 1997).

We asked here whether the ES/EB model system can further recapitulate events of the angiogenic process. Because angiogenesis involves endothelial cell migration and proliferation within a collagen-rich extracellular matrix (Strömbald and Cheresh, 1996), we investigated maturation of ES-derived EBs containing capillary-like structures into a type I collagen gel, in the absence or in the presence of angiogenic and angiostatic agents, to see whether sprouting angiogenesis can develop from the primitive vascular network seen in 11-day-old, ES-derived EBs. Our findings show that 11-day-old EBs embedded into collagen gels rapidly developed, in the presence of angiogenic factors, a network of branching endothelial outgrowths that displays an angiogenic phenotype. In agreement with angiogenesis regulation mechanisms, vascular sprouting was specifically induced by known angiogenic factors and was inhibited by several angiostatic agents. Moreover, consistent with in vivo and in vitro experiments showing a vascular endothelial cadherin (VE-cadherin) role in angiogenesis and vascular remodeling, we also observed that *VE-cadherin*^{-/-} ES-derived EBs failed to develop endothelial sprouts within this three dimensional model. These observations indicate that the ES cell system can also be used as a model for angiogenesis and that the ES/EB model provides a unique in vitro system to gain further insights into molecular and cellular signaling mechanisms that proceed during the process of blood vessel formation.

Results

Day 11-Embryoid Bodies Embedded into Collagen Form Endothelial Outgrowths

Several studies have outlined that ES-derived EBs constitute a suitable in vitro model to study vasculo-

genesis (Bloch et al, 1997; Hirashima et al, 1999; Risau et al, 1988; Vittet et al, 1996; Wang et al, 1992; Wartenberg et al, 1998). In a previous work, we showed that at least 75% of Day 11 ES-derived EBs exhibit vascular structures, evoking a primitive vascular plexus resulting from vasculogenesis, when differentiation was performed in a semi-solid methylcellulose medium in the presence of vascular growth factors (GFs) (Vittet et al, 1996). To discover whether angiogenesis may develop from this primitive network, Day 11 EBs were collected and further cultured into a type I collagen matrix under the same culture conditions, in the presence of a cocktail of angiogenic factors (VEGF, FGF2, EPO, IL6) at optimal concentrations. Type I-three dimensional collagen gel has been used because it is the major constituent of the pericapillary connective tissue in vivo, which play an important role during angiogenesis via endothelial cell surface interactions (Strömbald and Cheresh, 1996). EBs were observed to rapidly undergo shape change when subjected to 3D-culture in these conditions. Morphological observations revealed that EBs rapidly develop an extensive network of cellular outgrowths when embedded into the collagen gel (Fig. 1, A, B, and C). This cellular reorganization could occur from both EBs that have undergone previous endothelial differ-

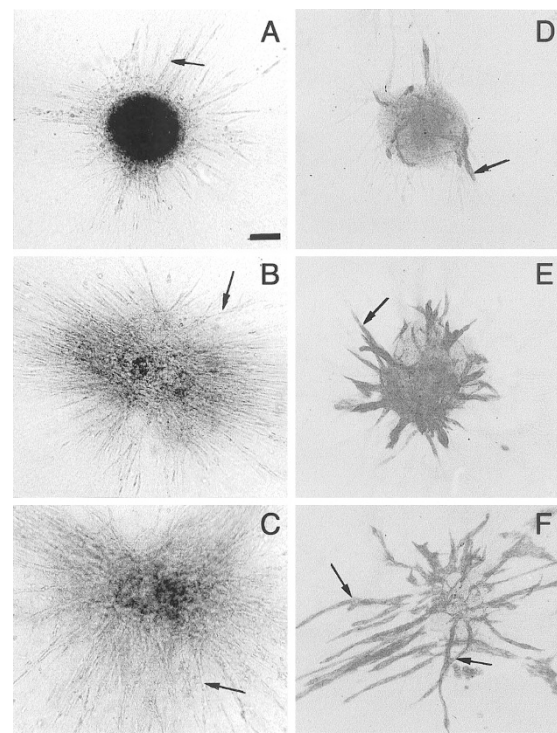


Figure 1.

Vascular morphogenesis from 11-day-old embryoid bodies (EBs) embedded into type I collagen gel in the presence of angiogenic growth factors (GFs cocktail). A, B, and C, Morphological analysis of maturation of 11-day-old EBs, 24, 48, and 72 hours after secondary culture into collagen, respectively. Arrows indicate budding outgrowths invading the collagen matrix. D, E, and F, Corresponding representative platelet endothelial cell adhesion molecule (PECAM) whole mount immunostainings of 11-day-old EBs embedded into collagen. Arrows point to expanding PECAM positive endothelial outgrowths, which further connect into a network. Scale bar: 100 μ m for all panels.

entiation (classified as vascular) and from EBs devoid of endothelial cells (classified as nonvascular), as assessed by the platelet endothelial cell adhesion molecule (PECAM) immunoreactivity of the EBs that was previously demonstrated to match with endothelial cells (Vittet et al, 1996). Whole mount immunostainings with an anti-PECAM antibody bring the evidence for numerous endothelial sprouts, which emerge from the initial primitive endothelial structures within the vascular EBs, then branch, and give rise to a complex network after 48 to 72 hours of secondary culture into collagen (Fig. 1, D, E, and F). Several outgrowths are not stained by the PECAM antibody

indicating that nonendothelial cells can also form sprouts in the collagen matrix, as previously described by others (Montesano et al, 1997; Uyttendaele et al, 1998). The cellular nature of these cells remains to be determined. Nevertheless, near 50% of sprouts seem to be true endothelial outgrowths and Figure 2A illustrates a characteristic branched PECAM-positive endothelial sprout end at a high magnification by differential interference contrast microscopy. It shows that the interconnected endothelial pseudopods are typically blunt-ended and are either formed by individual cells aligned end-to-end or, in many instances, constituted by the assembly of several endothelial cells.

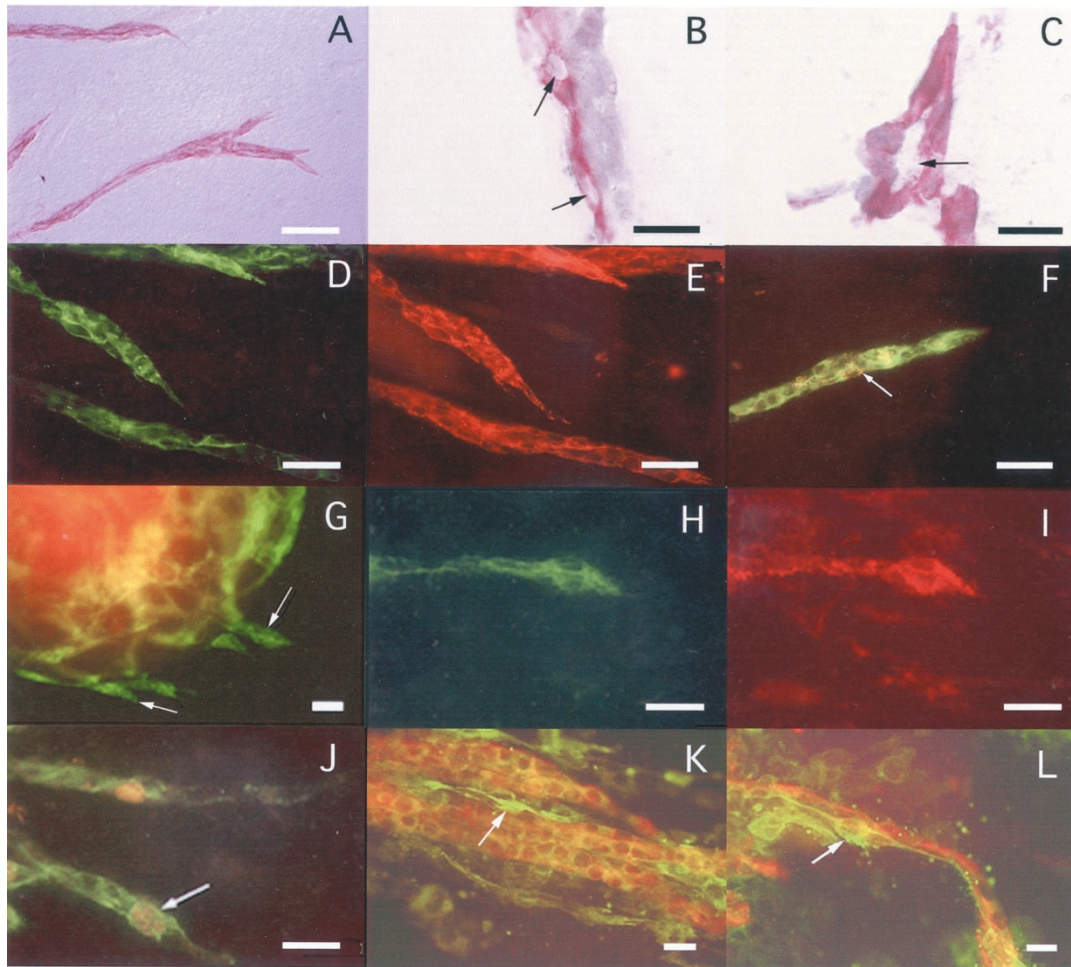


Figure 2.

Characterization of EB endothelial sprouting. A, Hoffman modulation contrast (DIC) photograph of a branching PECAM-positive endothelial sprout formed after 48 hours of secondary culture in the presence of the GF mixture, into type I collagen matrix. B and C, Hematoxylin counterstained cross-sections of PECAM-positive sprouts that reveal the formation of vacuoles (arrows in B) and tube-like structures (arrow in C). D to F, 11-day-old EBs cultured for 3 days into collagen gel and in the presence of angiogenic growth factors were processed for indirect immunofluorescence analysis with antibodies directed against endothelial antigens. Comparative distribution of PECAM (D) and vascular endothelial cadherin (VE-cadherin) (E), and of PECAM and von Willebrand factor (vWF) (F) by double labeling experiments. F, the arrow points to representative punctuated rice, grain-like staining pattern typical of vWF expression. G, Endothelial sprouts visualization by PECAM immunofluorescent staining (green) after 24 hours of secondary culture into collagen gels. White arrows indicate small PECAM positive sprouts originating from an 11-day-old EB. H, I, Detection of Flk-1 expression in endothelial outgrowths from 11-day-old EBs embedded into collagen gel. Eleven-day-old EBs cultured for 2 days into type I collagen matrix in the presence of the cocktail of angiogenic GFs were analyzed by immunofluorescent double labeling with PECAM (H) and Flk-1 (I) antibodies. J, Analysis of 5-Bromo-2'-deoxyuridine (BrdU) labeling of endothelial outgrowths originating from 11-day-old EBs embedded into collagen gel. EBs cultured for 2 days into the collagen matrix in the presence of angiogenic factors were processed for BrdU labeling as described in the Materials and Methods. The presence of endothelial proliferating cells was revealed by the double labeling experiment with PECAM and BrdU antibodies. The arrow is pointed to the BrdU immunoreactivity (red staining) of the nucleus of a dividing cell positive for PECAM (green staining). K and L, Double immunostaining of endothelial sprouts after 3 days of secondary culture in the presence of angiogenic factors, for PECAM and α -smooth muscle actin. Elongated α -smooth muscle positive cells (green fluorescence) can be seen (arrows) close to PECAM positive cells (red fluorescence) constituting sprouts. Scale bars for all panels: 50 μ m, with the exception of panels B, C, K and L: 25 μ m.

Identical vascular sprouting responses were obtained with two different ES cell lines: CJ7 (presented here) and R1 (data not shown). Cross sections of endothelial sprouts revealed the occasional formation of small vacuole-like structures (Fig. 2B), evoking the first steps of tube formation, and of larger spaces resembling lumen (Fig. 2C) that suggested the formation of capillary-like tubular structures, as described in Yang et al, 1999 and Yamashita et al, 2000, respectively. Although evidences for tube and lumen formation were infrequent at early stages of secondary culture into collagen gels, any long-term evaluation of tube formation or assessment of possible regression of tubes was prevented by degradation of the gels at times exceeding 72 hours. The endothelial phenotype of tube-like structures was examined by immunofluorescence studies. Analysis of the endothelial antigen expression revealed that vascular sprouts exhibit a complete endothelial phenotype as assessed by VE-cadherin and von Willebrand factor (vWF) immunoreactivity (Fig. 2, D, E, and F).

Endothelial Outgrowths Display Many Features of the Angiogenic Response

Angiogenesis is a complex process leading to the extension of the initial vasculature by formation of vessel sprouts from pre-existing vessels. This phenomenon is characterized by the growth and the migration of endothelial cells through the surrounding matrix before their organization into vascular tubes by a morphogenetic process. In addition to the whole mount results illustrated above, immunofluorescence experiments performed after 24 hours of culture in the collagen gel indicated that endothelial sprouts originated from the initial vascular structures, which cover the surface of EBs (Fig. 2G). In addition, the development of capillary-like structures seemed to be caused by enhanced proliferation of endothelial cells. Indeed, double-labeling experiments revealed that several PECAM-positive cells constituting the sprouts had incorporated 5-Bromo-2'-deoxyuridine (BrDU) in their nuclei, which clearly indicates the presence of proliferating cells (Fig. 2J).

Angiogenesis involves an interplay between angiogenic, survival, and differentiation factors and the first steps of this process have been described to be accompanied by the rapid up-regulation or high expression levels of several endothelial receptor tyrosine kinases, which represent the major signaling system involved in the regulation of the angiogenic response (Breier et al, 1997; Gale and Yancopoulos, 1999). We assessed the distribution of one of these markers, Flk-1, by immunofluorescence. As shown in Figure 2 (H and I), Flk-1 immunoreactivity was found in budding PECAM-positive endothelial sprouts. By RT-PCR analysis, we also followed *flk-1*, *flt-1*, *tie-1*, and *tie-2* gene expression pattern during EB maturation in type I collagen matrix. As expected, all transcripts were detected in 11-day-old EBs. In the absence of angiogenic factors, gene expression was down-regulated for all markers during the secondary culture in the

collagen gel. In contrast, the expression level of all these angiogenic markers remained sustained or were up-regulated in budding EBs cultured in the presence of angiogenic GFs (Fig. 3). These changes were not the result of variable representation of endothelial cells in EBs, because the percentages of PECAM-positive cells were not significantly different between the two experimental conditions, as assessed by FACS analysis of the EB contents after dissociation (data not shown).

During angiogenesis, nascent blood vessels are further stabilized by the recruitment of periendothelial cells (pericytes or smooth muscle cells), essential for vascular maturation and function (Carmeliet and Colten, 2000; Sims, 2000). Immunofluorescence experiments were performed to know whether periendothelial cells can also form in this model. Elongated α -smooth muscle actin positive cells can be found, some of them in the vicinity of endothelial cells constituting the sprouts (Fig. 2, K and L), thus indicating the concomitant differentiation of periendothelial mural cells.

Endothelial Sprouts are Specifically Induced by Angiogenic GFs

Another question we asked was whether the observed endothelial sprouting response was stimulated by angiogenic GFs. When embedded into the collagen gel, EBs were found to develop cellular outgrowths either in the absence or in the presence of angiogenic factors. However, we found after immunostaining experiments that endothelial sprouting from vascular EBs only occurred in the presence of the exogenously added angiogenic growth factor cocktail (GFs). When

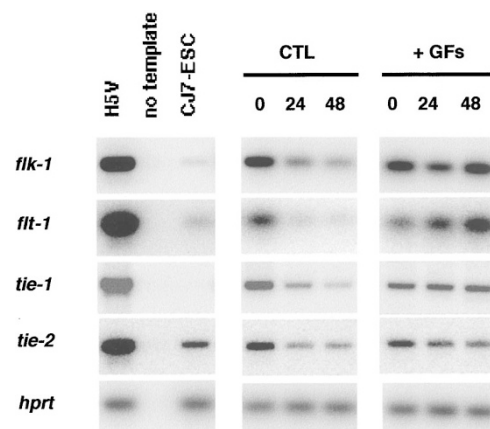


Figure 3.

RT-PCR analysis of angiogenic marker expression during 11-day-old EBs maturation into type I collagen gels. RNA was extracted from 11-day-old EBs (time 0) and 24 and 48 hours after secondary culture of 11-day-old EBs into type I collagen gel in the absence (CTL) or in the presence (+GFs) of angiogenic growth factor cocktail. The expression of *flk-1*, *flt-1*, *tie-1*, and *tie-2* was examined as markers of the angiogenic phenotype. The cDNA of *hppt* was amplified to normalize for the amount of mRNA used as starting material. H5V, murine endothelial cell line (Garlanda et al, 1994) used as a positive control; CJ7-ESC, undifferentiated ES cells cultured in the presence of LIF; no template, negative control. Shown are data from a typical experiment out of two performed with CJ7 ES cells.

angiogenic factors were present, $75\% \pm 5\%$ (SD, $n = 4$) of EBs displayed endothelial cellular processes invading the collagen matrix after 48 hours of secondary culture (Fig. 4). The development of nonendothelial outgrowths also seemed to be enhanced by GFs, reflecting the pleiotropic actions of some of the compounds used in the cocktail, such as basic fibroblast growth factor (FGF2) (Bikfalvi et al, 1997). In contrast, EBs were unable to develop endothelial outgrowths and only nonendothelial outgrowths can form in the absence of GFs. EBs that developed endothelial outgrowths were classified as angiogenic, whereas EBs that have undergone endothelial differentiation, but have formed only nonendothelial outgrowths, were classified as nonangiogenic.

The sensitivity to vascular endothelial growth factor (VEGF) or FGF2 was then characterized more completely after quantification of the sprouting response by computerized image analysis. Both compounds were found to elicit dose-dependent stimulation of EBs' endothelial sprouting (Fig. 5). Nevertheless, the quantitative analysis of the sprouting revealed that FGF2 has only a minor contribution in the sprouting and that VEGF alone can account for around 80% of the response induced by the complete GFs cocktail (Fig. 5). Maximal responses were achieved with VEGF concentrations between 15 and 50 ng/ml, which was in agreement with several previously published in vitro studies (Deckers et al, 2001; Gerber et al, 1998).

Platelet Factor 4 (PF4), Angiostatin, and Endostatin Can Inhibit Vascular Sprouting Induced by Angiogenic Factors

A number of antiangiogenic agents displaying both in vitro and in vivo biological effects have been identified.

This includes various cytokines and protein fragments of larger molecules that are not themselves inhibitors of angiogenesis in their entire form (Hanahan and Folkman, 1996). To evaluate whether agents with known antiangiogenic activity were effective in the collagen-embedded EB system, PF4, angiostatin and endostatin were tested on the sprouting response induced by angiogenic factors. Representative images obtained after treatment with maximal doses of these inhibitors are illustrated in Figure 6. They showed a defective vascular sprouting response in the presence of these inhibitors. Quantitative analysis revealed that 5 $\mu\text{g/ml}$ PF4 significantly affected the onset of EB endothelial sprouting, because a 41% reduction in the number of EBs exhibiting endothelial outgrowths can be measured (Fig. 7). PF4 was also observed to significantly affect the total mean length of endothelial sprouts (Fig. 7). Although angiostatin and endostatin did not exert a significant reduction in the number of sprouting EBs, they markedly inhibited the density and length of endothelial sprouts in the collagen gel (Fig. 7). Treatment with 5 to 15 $\mu\text{g/ml}$ endostatin or with 1 to 2.5 $\mu\text{g/ml}$ angiostatin resulted in significant inhibition in the mean total, endothelial-sprout length. Conversely, all these agents did not seem to interfere with the formation of nonendothelial sprouts. These experiments have been repeated at least three times in over 50 EBs for each experiment and yielded consistent results.

VE-Cadherin Deficiency Results in Defects in Endothelial Cell Sprouting

One of the major advantages of the ES/EB system is the possibility to investigate in vitro, after the introduc-

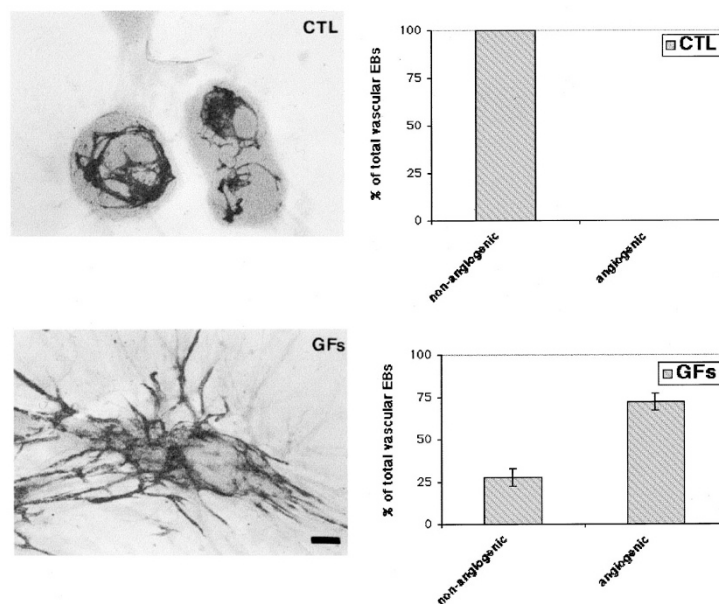


Figure 4.

Endothelial sprouting from 11-day-old EBs embedded into collagen gel is specifically induced by angiogenic growth factors. Eleven-day-old EBs were cultured for 3 days into type I collagen gel in the absence (CTL) or in the presence (GFs) of a mixture of angiogenic factors, before being processed for PECAM whole mount immunostaining to reveal endothelial cells. Nonangiogenic and angiogenic vascular phenotypes were scored among at least 100 vascular EBs in each differentiation experiment. Data are the mean \pm SD of four independent differentiation experiments. Scale bar: 100 μm .

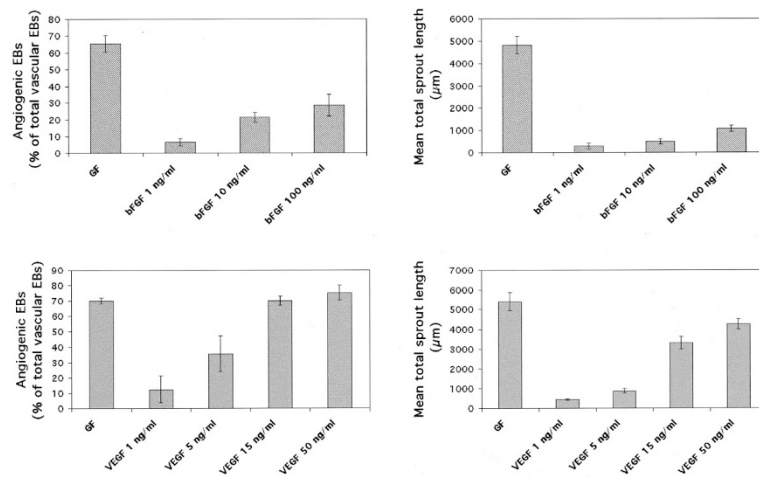


Figure 5.

Quantitative analysis of angiogenesis activators effects on endothelial sprouting. Effects of basic fibroblast growth factor (FGF2) or vascular endothelial growth factor (VEGF) on proportion of angiogenic EBs and on EB mean total length of endothelial sprouts were measured after 72 hours of collagen secondary culture. Quantitative analysis was performed on at least 30 EBs for each factor concentration. Data represent mean values for 3 independent experiments \pm se. The *left bar* indicated as a reference the mean value obtained for the complete GF angiogenic growth factor cocktail at maximal dose.

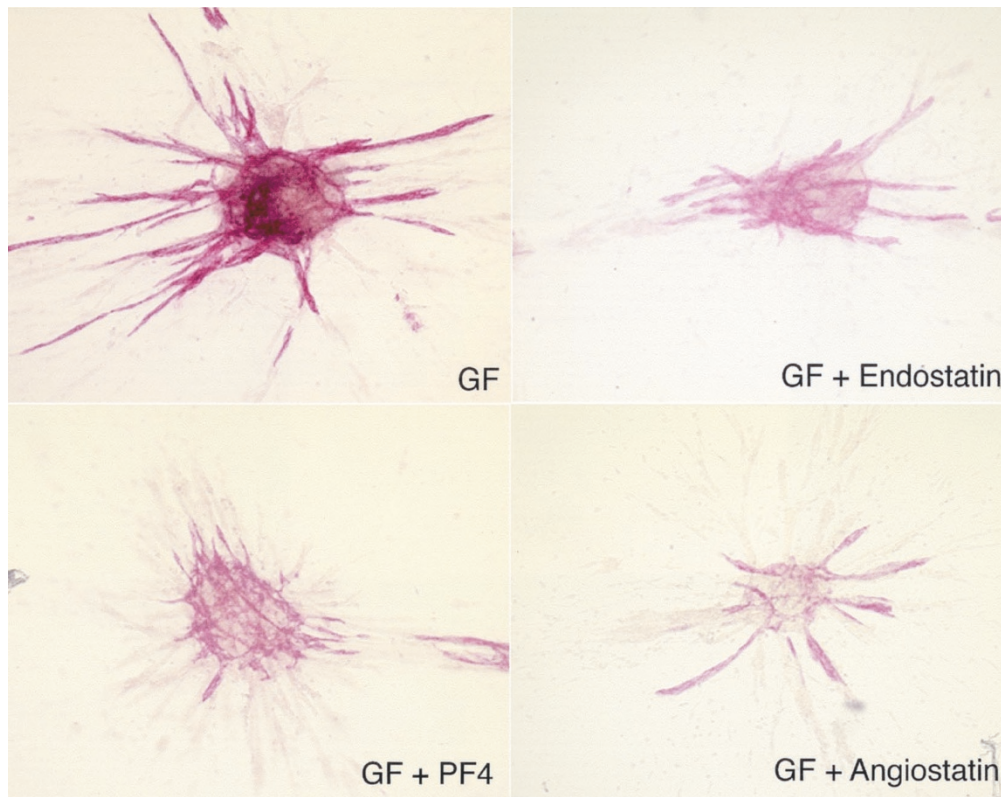


Figure 6.

Effect of angiostatic agents on endothelial sprouting induced by angiogenic GFs. Illustration of endostatin (10 μ g/ml), platelet factor 4 (PF4) (5 μ g/ml), and angiostatin (2.5 μ g/ml) effects on EB endothelial sprouting activity induced by angiogenic GFs.

tion of genetic modifications, the precise contribution of a gene to developmental processes. In addition to early vascular development, VE-cadherin has been shown to play a fundamental role in the angiogenic response, both in vitro and in vivo (Bach et al, 1998; Carmeliet et al, 1999; Gory-Fauré et al, 1999). We then examined the development in collagen gels of VE-cadherin deficient EBs originating from the initial dif-

ferentiation of two different *VE-cadherin* $-/-$ ES cell clones (Vittet et al, 1997). In agreement with our previous work (Vittet et al, 1997), we observed defective vascular structures organization in the great majority of *VE-cadherin* $-/-$ ES-derived EBs after 11 days of differentiation. When placed into collagen gels in the presence of angiogenic factors, the development of cellular outgrowths can still be observed, but VE-

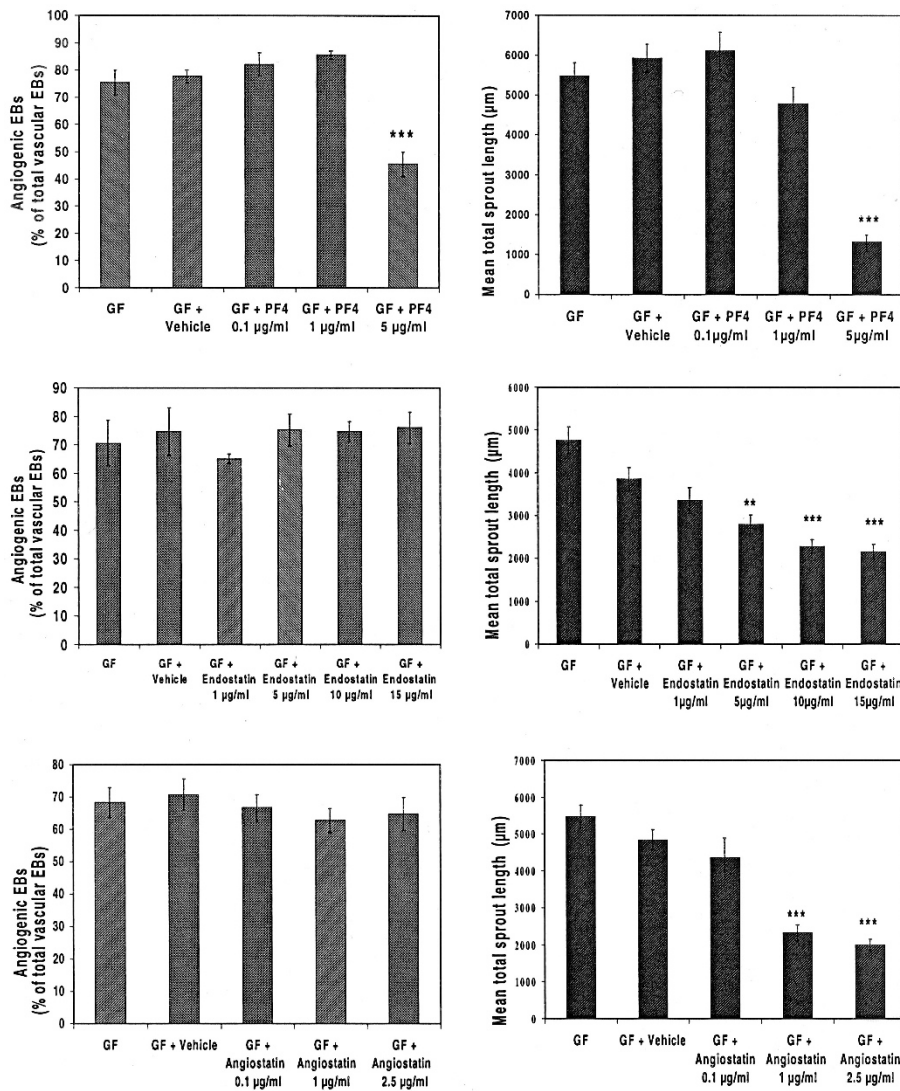


Figure 7. Quantitative analysis of angiostatic agents effects. EBs were treated with the angiogenic cocktail (GF) in the absence or in the presence of the indicated concentrations of angiogenesis inhibitors or with their corresponding vehicle. Analysis of the percentage of angiogenic EBs and of the mean total sprout length of angiogenic EBs was performed after 3 days of secondary culture into the collagen gel. Data represent mean values for 3 independent experiments ± se. ** $p < 0.01$, *** $p < 0.001$; significantly different from respective control values corresponding to the addition of the vehicle.

cadherin deficient EBs failed to form a network of branched endothelial sprouts (Fig. 8). On some occasions, some endothelial cells can migrate outside the EBs, but they remained isolated and never organize into capillary-like structures.

Discussion

The main purpose of the present study was to determine whether ES cells can provide a useful tool to investigate angiogenesis molecular mechanisms. Different types of assay systems have been used to study angiogenesis in vitro, including collagen models, but they at best represent only part of the angiogenic process (Cockerill et al, 1995; Jain et al, 1997). Some previous reports using ES cells have mentioned the formation of an extensive and branched endothelial network from EBs plated onto gelatinized dishes

that may probably represent angiogenesis (Bloch et al, 1997; Goumans et al, 1999). Here, we characterized the EB sprouting response obtained into a type I collagen gel. Our data indicate that PECAM-positive vascular sprouts can arise from the EB pre-existing primitive vascular network when they are embedded into a type I collagen gel in the presence of angiogenic factors. The phenotypic characterization of these sprouts confirmed that they represent fully differentiated endothelial cells, because they also expressed VE-cadherin and sometimes vWF, the latter being a heterogenous marker for differential endothelial cell gene expression (Coffin et al, 1991). These endothelial sprouts appeared to further organize into tube-like structures, in parallel with the differentiation of α -smooth muscle actin positive cells. Sprouting, progressive elongation, invasion of the interstitium-like

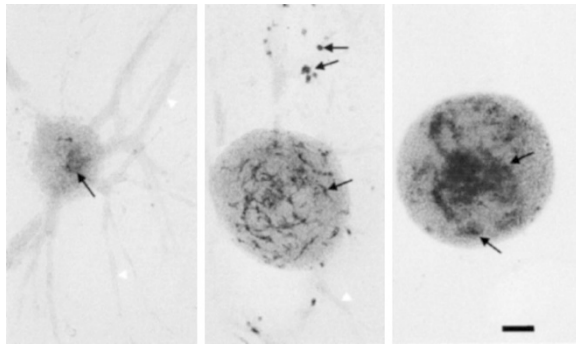


Figure 8.

Embryoid bodies lacking VE-cadherin failed to develop endothelial sprouts into collagen gel. Maturation of 11-day-old EBs resulting from the differentiation of ES cells in which the VE-cadherin gene was inactivated by gene targeting (Vittet et al, 1997) was examined after 3 days of secondary culture in the presence of the angiogenic factor cocktail. Three different VE-cadherin^{-/-} EBs, originating from two different VE-cadherin^{-/-} ES cell clones are illustrated. Whole mount immunostainings with PECAM antibody showed that endothelial cells inside the VE-cadherin-deficient EBs remained dispersed or poorly organized into vascular structures, as previously described (Vittet et al, 1997), and that the formation of a branched network of endothelial outgrowth was impaired. In some occasions, as illustrated by the EB in the center of the figure, some endothelial cells have migrated into the surrounding collagen matrix but the constitution of a sprout was defective. White arrow heads show some nonendothelial outgrowth and examples of PECAM positive cells are shown by black arrows. Scale bar: 100 μ m.

collagen substrate, cell-cell assembly, morphogenesis into tubes, and periendothelial cell differentiation are all features which recapitulate angiogenesis in vivo (Jain et al, 1997; Risau, 1997). In addition, BrDU incorporation assays supported the existence of proliferating endothelial cells, correlating with a complete angiogenic response involving proliferative and migratory responses and morphogenesis.

Moreover, endothelial cells constituting angiogenic EBs were found to display an angiogenic phenotype. Angiogenesis has been characterized to be accompanied by coordinate changes in endothelial cell morphology and gene expression (Auerbach and Auerbach, 1994; Breier et al, 1997). Indeed, during the neovascularization process, endothelial cells change their genetic program and express an angiogenic phenotype by up-regulating several endothelial receptor tyrosine kinases that represent the major signaling system involved in the regulation of the angiogenic response. They include receptors for VEGF (flk-1,flt-1), angiopoietins (tie-2), and the tie-1 receptor (Breier et al, 1997). Activation of flk-1 was reported to be associated with endothelial cell proliferation, whereas the flt-1 receptor seemed to be more involved in the regulation of cell-cell and cell-matrix interactions. Overexpression of flk-1 has been found in the endothelial cells of angiogenic vessels in several tumor types (Plate et al, 1993). On the other hand, expression of flt-1 was found associated with growing vessels in the wounds (Peters et al, 1993) and its expression level has been shown to be increased in ischemic cardiac tissue, suggesting its involvement in the growth of collateral blood vessels (Li et al, 1996). Essential functions for tie-1 and tie-2 in developmental angiogenic expansion were also revealed by genetic

studies in mice (Sato et al, 1993). Both molecules also play a role in normal and pathological angiogenesis in adults (Peters et al, 1998; Wong et al, 1997). Sustained or higher levels of expression for all these tyrosine kinase receptors are observed in angiogenic EBs, thus indicating the induction of an angiogenic phenotype when EBs are cultured in type I collagen gels in the presence of angiogenic factors.

Endothelial sprouting was specifically induced by the angiogenic growth factors exogenously added into the collagen gel. The presence of angiogenic factors was indeed required to undergo the formation of endothelial sprouts into the collagen matrix, because EBs cultured in the absence of these factors failed to develop endothelial sprouts and a marked decrease in angiogenic markers expression was observed. This requirement is consistent with other reports employing similar in vitro models, which mentioned that endothelial cells require angiogenic and/or survival signals to undergo vascular morphogenesis in three dimensional gels (Satake et al, 1998). The decrease in endothelial gene expression observed in the absence of angiogenic factors may be the result of endothelial cell apoptosis, as previously described (Kuzuya et al, 1999; Pollman et al, 1999). Whereas, in the presence of angiogenic factors, endothelial cells may survive, proliferate, and form a capillary-like endothelial network in three dimension. The angiogenic growth factor cocktail used included FGF2, VEGF, erythropoietin (EPO), and interleukin-6 (IL6), which all are known or have been considered to be potent angiogenic inducers (Bikfalvi et al, 1997; Ferrara and Davis-Smyth, 1997; Motro et al, 1990; Ribatti et al, 1999). Among these factors, VEGF has proved to exert a central role in angiogenesis regulation (Neufeld et al, 1999). In our model, a key role is also played by VEGF, because VEGF alone can induce the major part of the sprouting response. Conversely, FGF2, by itself, exhibited only a minor contribution in EB endothelial sprouting, further indicating, as shown by others, that FGF2 does not play a substantial role in angiogenic processes during development (Ortega et al, 1998; Ozaki et al, 1998).

To investigate whether maturation of ES-derived EBs respond to angiostatic agents, and to test the validity of the ES/EB model for the evaluation of molecular mechanisms involved in angiogenesis inhibition, we conducted experiments with angiostatic molecules. A diverse group of angiogenesis inhibitors have been characterized and identified by their ability to inhibit endothelial cell proliferation, migration, cord formation, and to suppress both angiogenesis and tumor growth (Hanahan and Folkman, 1996). These inhibitors include various proteins, such as angiostatin, endostatin, and PF4, which can interfere with new blood vessel formation both in vitro and in vivo (Jouan et al, 1999; Kruger et al, 2000; O'Reilly et al, 1994, 1997; Tanaka et al, 1997). After addition of PF4, endostatin, or angiostatin, the formation of a branched endothelial network was markedly impaired. The active concentrations of these inhibitors were in the μ g/ml range, that is in accordance with what is known from other in vitro studies (Deckers et al, 2001; Kruger

et al, 2000; Sharpe et al, 1990; Troyanovsky et al, 2001). However, some previous published studies have also reported activity both *in vivo* and *in vitro* at much lower endostatin concentrations (Yamaguchi et al, 1999). Endostatin interaction with heparan sulfate was reported to be potentially involved in some of the endostatin effects (Dixelius et al, 2000; Sasaki et al, 1999) and glypican-1 has recently been identified as a low affinity endostatin binding site on endothelial cells (Karumanchi et al, 2001). Our results may then indicate that the observed endostatin inhibitory effect on EB endothelial sprouting may be in part dependent on endostatin binding to heparin/heparan sulfate proteoglycans. Future studies with endostatin heparin binding mutants should bring additional information. On the other hand, higher endostatin activity of recombinant endostatin was reported only when a different *in vitro* assay (endothelial cell migration) was used (Yamaguchi et al, 1999). The necessity to use higher endostatin concentrations in our assay may be the consequence of the complexity of the response occurring in a multicellular assay, which integrates multiple aspects of the angiogenic response, as observed during outgrowth of capillary structures from fetal mouse bone explants, where endostatin from the same origin as ours exhibited significant inhibitory effects at concentrations not below 1 $\mu\text{g/ml}$ (Deckers et al, 2001). The active concentrations of angiogenesis inhibitors *in vitro* are not easy to compare with those eliciting angiogenesis inhibition *in vivo*. Indeed, blood levels of endostatin or angiostatin after protein therapy have not been reported and efficient inhibition of tumor growth is achieved after daily administration of inhibitors, whereas a single addition is performed in our study at the initiation of the secondary culture in the collagen gel. Nonetheless, the active concentrations of angiostatic factors on EB endothelial sprouting are consistent with circulating levels eliciting significant angiogenesis inhibition resulting in inhibition of tumor growth, which are obtained after gene transfer (Kuo et al, 2001; Sauter et al, 2000). Then, the ability of these well-known angiogenesis inhibitors to significantly inhibit the EB angiogenic sprouting response indicated that this model could be of primary importance in investigating the angiostatic molecules' mechanisms of action that remain largely unknown (Risau, 1997). Furthermore, this model will enable us to characterize which parameters of the angiogenic response (proliferation, sprouting, branching, etc.) are targets for these angiostatic molecules. In addition, virtually nothing is known about the effects of angiostatic molecules on angioblasts differentiation. This model will also offer the opportunity to investigate this aspect.

To evaluate the potency of the ES/EB model to investigate angiogenesis molecular mechanisms, we looked at the maturation of *VE-cadherin* $-/-$ ES-derived EBs and compared it with the published *in vivo* phenotype. Deficiency in the junctional interendothelial VE-cadherin adhesion protein has been previously reported to impair sprouting and remodeling into a network of large and small branches (Carmeliet

et al, 1999). We also clearly found in our model that endothelial sprouting failed to take place in the absence of VE-cadherin. This model may then be particularly useful for the analysis of the consequences of specific gene modification.

Although *in vivo* models such as the corneal neovascularization model, the mesenteric window assay, and numerous pouch assays display a more complete portrait of angiogenesis, inflammation caused by wounding and matrix implantation makes angiogenesis sometimes difficult to assess (Jain et al, 1997). The ES/EB model system has the advantage to be devoid of interference with host responses. Moreover, it may constitute an alternative to animal experimentation, which suffers from ethical constraints. Moreover, the ES/EB system allows the study of both vasculogenesis and angiogenesis, which is a unique property conferring superiority to all other *in vitro* angiogenesis models. In addition, the differentiation of genetically modified ES cells by gain-of-function and loss-of-function analyses are also excellent alternatives and substitutes to *in vivo* studies with transgenic animals to analyze the consequences of mutations on vascular development. Finally, this model responding to both positive and negative modulators of angiogenesis can be exploited for the identification of novel signaling molecules. Because the number of new antiangiogenic agents is rapidly increasing, each of them representing promising tools in therapy, but whose molecular mechanisms of action remained largely unsolved, this model may be particularly useful to identify their cellular targets as well as differentially expressed genes during new blood vessel formation.

Materials and Methods

ES Cell Differentiation

Subconfluent CJ7-ES cells (Swiatek and Gridley, 1993) or R1-ES cells (Naguy et al, 1993) were initiated to differentiate in Iscove's medium containing glutamax (IMDM; Invitrogen, Carlsbad, California) supplemented with 1% methylcellulose (Stem Cell Technologies Inc., Vancouver, Canada), 15% fetal calf serum (Seromed, Berlin, Germany), 450 μM monothioglycerol, 10 $\mu\text{g/ml}$ insulin (Roche Diagnostics, Meylan, France), 50 U/ml penicillin, and 50 $\mu\text{g/ml}$ streptomycin. To optimize endothelial differentiation, ES cell differentiation was performed in the presence of 50 ng/ml human VEGF (Peprotech, Rocky Hill, New Jersey), 100 ng/ml human FGF2 (Peprotech), 2 U/ml mouse EPO (Roche Diagnostics) and 10 ng/ml murine IL6 (Peprotech), as described previously (Vittet et al, 1996). ES cells (1.25×10^3 cells/ml) were seeded in a final volume of 2 ml in 35-mm bacterial grade Petri dishes (Greiner, Nurtingen, Germany).

EB Maturation into Type I Collagen Gels

For secondary culture into collagen gel, EBs were collected after 11 days of differentiation by dilution of the semi-solid methylcellulose medium with IMDM

Glutamax. After centrifugation ($200\times g$, 5 minutes) and two further washes, EBs were suspended in IMDM Glutamax and mixed with the collagen culture medium at a final concentration of 50 EBs/ml. Final composition of freshly prepared collagen-based culture medium was as follows: IMDM containing 1.25 mg/ml rat tail collagen, type I (lot 900649; Becton Dickinson, Bedford, Massachusetts), 15% fetal calf serum (Seromed), 450 μM monothioglycerol, 10 $\mu\text{g}/\text{ml}$ insulin, 50 U/ml penicillin, and 50 $\mu\text{g}/\text{ml}$ streptomycin. After thorough mixing of EBs into collagen-based medium, 1.2 ml were dispensed into 35-mm bacterial Petri dishes. When tested, factors were included into the collagen gel medium at the onset of the culture. Neutralization of the acidity of the collagen solution by the medium, in combination with incubation at 37°C , promotes within 30 minutes the formation of a stable collagen gel matrix. Cultures are then incubated up to 3 days in the presence or in the absence of angiogenic and/or angiostatic agents before harvest and analysis of their vascular development.

Angiogenesis Inhibitors

The Kringle 1–4 (K1–4) angiostatin was prepared by digestion of Glu-human plasminogen (Glu-HPg) with porcine elastase as previously described (Cao et al, 1996). Briefly, 3 mg elastase was incubated at room temperature with 400 mg of HPg in 50 mM Tris-HCl, pH 8.0 overnight with shaking. The reaction was terminated by the addition of diisopropyl fluorophosphate (DFP) to a final concentration of 1 mM. The mixture was rocked for an additional 30 minutes at room temperature and dialyzed overnight against 50 mM Tris-HCl, pH 8.0. The proteolytically cleaved K1–4 angiostatin was purified using a lysine-Sepharose 4B column ($2.5 \times 15\text{ cm}$) (Amersham Pharmacia Biotech,

Orsay, France) equilibrated with 50 mM Tris-HCl, pH 8.0. Angiostatin was eluted with the Tris buffer containing 200-mM Σ -aminocaproic acid, pH 8.0. The eluted samples were then dialyzed overnight against 20 mM Tris-HCl, pH 5.0 and were applied to a BioRad Mono-S column equilibrated with the same buffer. After dialysis, K1–4 angiostatin (Biorad Laboratories, Hercules, California) was separated from K1–3 using a heparin-Sepharose column ($5\text{ cm} \times 10\text{ cm}$) (Sigma Instruments, Monterey, California) pre-equilibrated with 20 mM Tris-HCl buffer, pH 8.0. The K1–3 fragment was eluted with 250 mM KCl and K1–4 was recovered from the flow-through fraction. The purified kringle fragments were analyzed on SDS-gels followed by staining with Coomassie blue.

Either recombinant (Peptotech) and purified (Diagnostica Stago, Asnières, France) human PF4 were used. Purified soluble recombinant human endostatin derived from the EBNA 293 embryonic kidney cell line (Yamaguchi et al, 1999) was provided by Dr. Bjorn Olsen (Harvard Medical School, Boston, Massachusetts).

Immunofluorescence Analysis, Whole Mount Immunocytochemistry and BrdU Labeling

Collagen gels containing EBs were collected onto glass slides, then dehydrated in seconds using a nylon linen and absorbent filter cards. Indirect immunofluorescence experiments were performed as described (Vittet et al, 1996, 1997) with: rat monoclonal anti-mouse PECAM antibody (clone MEC-13.3) (Vecchi et al, 1994), rat monoclonal anti-mouse Flk-1 antibody (clone AVAS12; provided by Dr. S-I. Nishikawa, Department of Molecular Genetics, Kyoto University, Kyoto, Japan) (Nishikawa et al, 1998), rabbit polyclonal anti-human vWF (Dako S.A., Trappes, France), and rat monoclonal anti-mouse VE-cadherin antibody (clone 11D4.1; Pharmingen, San Diego, California).

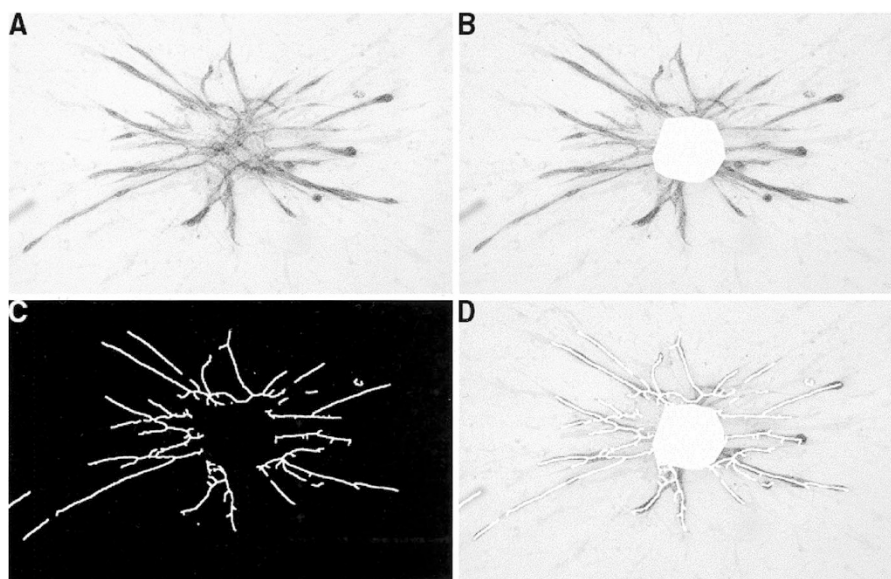


Figure 9.

Image processing for quantification of endothelial sprouting. A, Image of PECAM staining captured by CCD camera. B, Image A from which the area corresponding to the body mass is subtracted. C, Skeletonized image of the PECAM staining of image B. D, Superposition of image C and image A.

For whole-mount PECAM staining, EBs embedded into collagen gels were fixed in methanol-dimethylsulfoxide (4:1) overnight at 4° C and stained with rat monoclonal anti-mouse PECAM antibody (clone MEC-13.3) as previously described (Vittet et al, 1997). Dehydrated collagen gels, prepared as outlined above, were then mounted in aqueous medium onto glass slides before microscope examination.

For the detection of proliferating cells, the gels were covered, at Day 2 of EB secondary culture, with 1 ml IMDM-based medium containing 50 μ M BrdU (Sigma, St. Quentin Fallavier, France) for 6 hours. After 4 washes with PBS, collagen gels were turned out onto glass slides and dehydrated. Fixation was done with 4% paraformaldehyde for 20 minutes at room temperature. Dividing endothelial cells were then revealed by double immunofluorescence using monoclonal anti-mouse PECAM antibody (MEC-13.3) and monoclonal anti-BrdU antibody (Sigma), as described (Deloulme et al, 1991).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

EBs were collected after digestion of the collagen matrix by 30 minutes treatment at 37° C with a PBS solution containing 0.2% collagenase B (Roche Diagnostics). Semiquantitative RT-PCR was performed as previously described (Vittet et al, 1996). After prior treatment with RQ1 DNase to eliminate any contaminating genomic DNA, 2 μ g of total RNA was reverse-transcribed. The equivalent of 200 ng reverse-transcribed RNA was used for PCR experiments. All PCR analyses were performed using exponential amplification conditions. Annealing temperature and PCR cycle numbers were: 55° C, 27 cycles; 55° C, 30 cycles; 60° C, 32 cycles; 55° C, 30 cycles, and 57° C, 32 cycles for *flk-1*, *hprt*, *flt-1*, *tie-1*, and *tie-2*, respectively. Reaction products were gel electrophoresed, transferred onto nylon membrane (Hybond N+; Amersham Pharmacia Biotech, Les Ulis, France), and hybridized with their respective [³²P]-5'-end-labeled internal oligonucleotide probe according to standard protocols (Sambrook et al, 1989). Hybridized filters were visualized and signals quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale, California).

Oligonucleotide primers and probes to analyze *flk-1*, *tie-2* and *hprt* gene expression were described in Vittet et al, 1996. For *flt-1* gene expression, the primers were those described by Choi et al, 1998, and the probe was: 5'-AGAAGCCCCGCCTAGACA-3'. For *tie-1* gene expression, the primers were as follows 5'-TCTCTATGTGCACAACAGCC-3' (sense), 5'-ACACACATTCGCCATCAT-3' (antisense), and the probe was 5'-TGACGGGCGTTTTCAACTGC-3'.

Computer-Assisted Morphometric Analysis of Vascular Sprouting

For quantitation of endothelial sprouting from the EBs, EBs embedded into collagen gels were immunostained

with anti-PECAM antibodies by the whole mount procedure. Images were captured with a Spot computer-supported digital camera. After masking the EB area, the resulting image comprising endothelial sprouts alone was binarized to black/white and skeletonized (Fig. 9). Measurement of the total length of endothelial sprouts was achieved by using the visiolab@2000 software (BIOCOM, Les Ulis, France). The results were subjected to statistical analysis by the paired Student's *t* test; the level of significance was set as *p* < 0.05.

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