

Maturation of blood vessels by haematopoietic stem cells and progenitor cells: Involvement of apelin/APJ and angiopoietin/Tie2 interactions in vessel caliber size regulation

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Summary

Apelin is a recently-isolated bioactive peptide from bovine gastric extract. The gene encodes a protein of 77 amino acids, which can generate two active polypeptides, long (42–77) and short (65–77). Both peptides ligate and activate APJ, a G protein-coupled receptor expressed in the cardiovascular and central nervous systems. Although an essential role for the apelin/APJ system in blood vessel formation has been reported in *Xenopus*, its precise function in mammals is unclear. Blood vessel tube formation is accomplished by two main mechanisms: 1) single cell hollowing, in which a lumen forms within the cytoplasm of a single endothelial cell (EC), and 2) cord hollowing in which a luminal cavity is created *de novo* between ECs in a thin cylindrical cord. Molecular control of either single cell or cord hollow-

ing has not been precisely determined. Angiopoietin-1 (Ang1) has been reported to induce enlargement of blood vessels. Apelin is produced from ECs upon activation of Tie2, a cognate receptor of Ang1, expressed on ECs. It has been suggested that apelin induces cord hollowing by promoting proliferation and aggregation/assembly of ECs. During angiogenesis, haematopoietic stem cells (HSCs) and progenitor cells (HPCs) are frequently observed in the perivascular region. They produce Ang1 and induce migration of ECs, resulting in a fine vascular network. Moreover, HSCs/HPCs can induce apelin production from ECs. Therefore, this review article posits that HSCs/HPCs regulate caliber size of blood vessels via apelin/APJ and Angiopoietin/Tie2 interactions.

Keywords

Haematopoietic stem cell, haematopoietic progenitor cells, Tie2, Angiopoietin-1, Apelin, APJ

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Blood vessel size determination

The formation of blood vessels is initiated by the assembly of endothelial cells (ECs), or EC progenitors, and their subsequent tube formation. This process is termed vasculogenesis and is followed by angiogenesis, which results in the emergence of new vessels through the sprouting and elongation from, or the remodeling of, preexisting vessels (1). In both processes, to maintain the structural stability of nascent EC tubes, mural cells (MCs) such as smooth muscle cells and pericytes are recruited around the forming tube and adhere to ECs.

Many genes and molecules involved in these processes have been identified (2–10), with vascular endothelial growth factor (VEGF) mainly playing a role in the development and tube formation of ECs. The ECs forming the tube recruit supporting MCs by releasing PDGF-BB (11). MCs subsequently adhere to

ECs resulting in the formation of a structurally stable blood vessel. It has been reported that this cell adhesion between ECs and MCs is induced when angiopoietin-1 (Ang1), produced by MCs, stimulates Tie2, a receptor tyrosine kinase on ECs (12–15). Therefore, Ang1 is involved in the maturation process of blood vessels. One of these maturation processes for blood vessel formation is adjustment of caliber size, which is very important to supply oxygen and nutrient adequately to tissues. Understanding the process of caliber size regulation is crucial for developing improved clinical approaches to treat cancer and hypoxic disease. However, the molecular mechanisms of blood vessel caliber size determination are not yet clearly understood.

Tube formation is a fundamental mechanism for organ and tissue generation in most major organs, such as the lung and kidney, as well as the vasculature. The molecular mechanisms involved in tube generation in general are not perfectly under-

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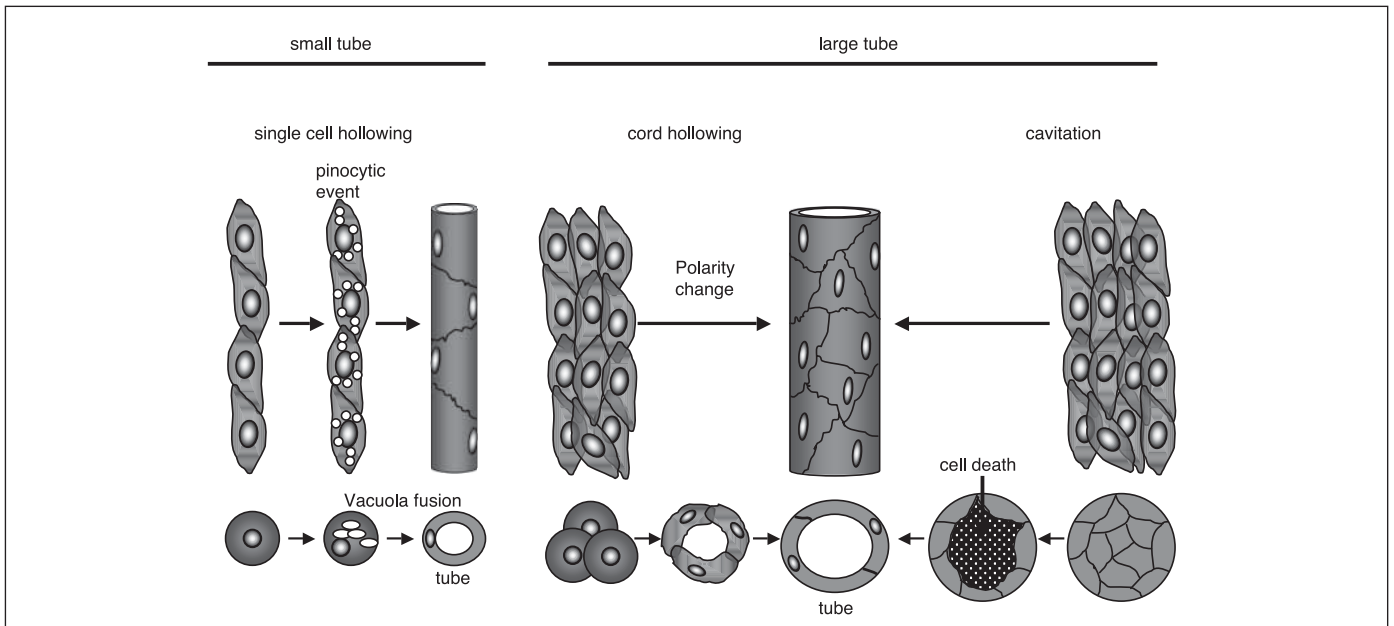


Figure 1: Tube formation in the vascular system. Schematic representation of tube formation observed in the vascular system. In single-cell hollowing, several vacuoles generated in the cytoplasm of an endothelial cell (EC) fuse with each other by pinocytotic events, forming a tube within the cell. This then connects with the tube in an adjacent EC, resulting in formation of a narrow capillary tube. On the other hand, ag-

gregated ECs change their polarity into either apical or basal orientation and gradually form tubes, resulting in the generation of enlarged blood vessels (cord hollowing). When ECs in the center of the aggregate are eliminated by apoptosis or differentiation into another lineage (i.e. haematopoietic cells), a tube will be formed (cavitation).

stood; however, anatomical observations of tube morphogenesis by epithelial cells have been well-described and show that tube development can occur in many different ways (16). Based on previous observations of tube formation in general, tubes in the vasculature might be generated by the following steps (Fig. 1). 1) single-cell hollowing: a lumen forms within the cytoplasm of a single EC; 2) cord hollowing: a luminal cavity is created *de novo* between ECs in a thin cylindrical cord; 3) cavitation: the central cells of a column composed of assembled ECs or endothelial progenitors are eliminated, forming a luminal cavity (this process may be less likely in angiogenesis, but has been observed in blood islands of the yolk sac); and 4) Wrapping or intussusception: an EC sheet invaginates and curls until the edges of the invaginating region meet and seal.

Recently, Kamei et al. (17) clearly demonstrated activity of the single-cell hollowing system for blood vessel formation in Zebrafish. Their in-vivo imaging technique showed that intracellular and intercellular fusion of endothelial vacuoles drives vascular lumen formation. Folkman and Haudenschild (18) described „longitudinal vacuoles“ that „appeared to be extruded and connected from one cell to the next“ in EC culture experiments *in vitro*. Therefore, single-cell hollowing may be able to construct capillaries of narrow caliber. However, the size of a single EC is limited, so single-cell hollowing cannot give rise to larger vessels. Thus, the cord hollowing system is required for constructing larger vessels. Identification of molecules utilised in cord hollowing but not in single-cell hollowing would therefore lead to a better understanding of how blood vessel caliber size is determined.

For cord hollowing, ECs once assembled and aggregated gradually manifest polarity, with luminal and apical regions. In-vivo experiments using zebrafish showed that the EC-derived secreted factor Egfl7 had a crucial role in proper lumen formation after aggregation of endothelial progenitors by regulating their polarity (19). Although the mechanism by which Egfl7 regulates lumen size has not yet been elucidated because of the difficulty of isolating its receptor, these findings imply that tube formation by single-cell hollowing does not occur in areas where ECs need to generate larger tubes.

Depending on the degree of tissue demand for oxygen, one EC starts to sprout from pre-existing vessels for the generation of small-sized capillaries by single-cell hollowing, but under severe hypoxia, several ECs assemble in one sprouting point from pre-existing vessels and generate larger vessels by a cord hollowing mechanism. Therefore, in the initiation of cord hollowing, several ECs/EC progenitors are required. Thus, for cord hollowing, on sensing hypoxia, ECs need to proliferate and assemble to constitute large cylinders, whereas for single-cell hollowing, proliferation is not required (Fig. 1).

Tie2 activation induces apelin in ECs

The Ang1/Tie2 and VEGF/VEGFR systems are potent regulators influencing caliber size determination in blood vessels. Transgenic overexpression of Ang1 in keratinocytes induces enlarged blood vessel formation in the dermis (20) and administration of a potent Ang1 variant was also reported to result in enlargement of blood vessels (21, 22). Therefore, knowledge of the precise molecular mechanism of Ang1/Tie2 induction of blood

vessel enlargement would facilitate our understanding of the process of caliber size determination during angiogenesis.

On the other hand, VEGFs and their cognate receptors (VEGFRs), play central roles in the proliferation of ECs under physiological conditions (23); however, in contrast to Ang1, transgenic overexpression of VEGF in keratinocytes induces formation of a greater number of blood vessels in the dermis, but these were reported to be exclusively of very small caliber (20).

Both VEGF and Ang1 are required for the process of angiogenesis. What happens when both Ang1 and VEGF are overexpressed? Double transgenic mice expressing both these factors in keratinocytes had blood vessels in the dermis larger than wild-type mice but smaller than mice transgenic for Ang1 alone (20). Therefore, the relative amounts of Ang1 and VEGF may alter the caliber size of blood vessels and molecules affected by VEGFR. Hence, Tie2 on ECs must be involved in the regulation of caliber size in blood vessels.

Genes upregulated following Ang1 binding to Tie2 on ECs have been identified by the subtraction method. In this way, the apelin gene was isolated from human umbilical venous endothelial cells (HUVECs) (24). Of many proangiogenic cytokines, such as Ang1, VEGF, bFGF, PDGF-BB, and EGF, it was found that apelin expression was upregulated in HUVECs only by Ang1 and bFGF (Table 1).

Apelin, a ligand for APJ, was recently isolated as a bioactive peptide from bovine gastric extract. The apelin gene encodes a protein of 77 amino acids, which can generate two active polypeptides: the long (42–77) and the short (65–77) forms of apelin (25–27), which both activate APJ. Apelin mRNA and protein are highly expressed in the lung and mammary gland. However, the distribution of the different molecular forms of apelin differs among tissues: apelin molecules with sizes close to apelin-36 (long forms) are major components in the lung, testis, and uterus, but both long and short (approximating apelin-13) forms are detected in the mammary gland (26).

APJ is a G protein-coupled receptor, reportedly expressed in the cardiovascular and central nervous systems (28, 29). In the brain, APJ expression is observed in neurons (30) as well as in oligodendrocytes and astrocytes (31). In the brain, the apelin/APJ system plays a role in maintaining body fluid homeostasis and regulating the release of vasopressin from the hypothalamus (32). In the cardiovascular system, APJ is expressed in the endothelial lineage in various species of amphibians, as well as in mice and humans (29, 33, 34). In the latter two, the expression of the receptor has also been detected by immunocytochemistry in vascular smooth muscle cells and cardiomyocytes (35). Apelin/APJ function in cardiomyocytes is thought to associate with a very strong inotropic activity (36, 37). The function of apelin/APJ in the EC lineage is reported to be associated with the hypotensive activity of apelin (38), as the activation of APJ leads to nitric oxide (NO) production by the ECs (39), and this possibly plays a role in the relaxation of the smooth muscle cell.

Using morpholino antisense oligonucleotides (MO), requisite roles of the apelin/APJ system have been reported in the cardiovascular system of *Xenopus laevis* (40, 41) and Zebrafish (42). *Xenopus apelin* (*Xapelin*) was detected in the region around the presumptive blood vessels during early embryogenesis and overlapped with the expression of *Xmsr*, the *Xenopus* homolog of

Table 1: Apelin and APJ expression on HUVECs stimulated by angiogenic cytokines.

	Apelin	APJ
Ang1	↑	–
VEGF-A	–	↑
bFGF	↑	–
PDGF-BB	–	–
EGF	–	–

↑ induced, – :not induced.

Table 2: APJ expression on endothelial cells from different tissues.

E10.5 AGM	+++
E10.5 Yolk Sac	++
E10.5 head	++
E10.5 heart	+/-
adult heart	+/-
adult liver	+/-
adult tumor	+

E10.5: embryonic day 10.5, adult: 8 weeks-old. +: positive, +/-weak.

APJ. Overexpression of *Xapelin* disorganised the expression of the endothelial precursor cell marker *Xifli* at the neurula stage. Knock down of *Xapelin* or *Xmsr* induced abnormal heart morphology and attenuated the expression of *Tie2*, resulting in the disruption of blood vessel formation in the posterior cardinal vein, intersomitic vessels, and vitelline vessels. In contrast, apelin protein has been shown to induce angiogenesis in the chicken chorioallantoic membrane assay (41).

APJ expression on EC lineage cells and the phenotype of apelin-deficient mice

APJ expression is observed in the EC lineage in mammals; however, when apelin first becomes expressed by ECs and which ECs express its receptor APJ is not clear. During early embryogenesis, compared to ECs from other tissues, such as yolk sac, head region, and heart at the same stage (E10.5), ECs from the the AGM region [Aorta-Gonad-Mesonephros region followed by para-aortic splanchnopleural mesoderm (P-Sp) region at embryonic day (E) 10.5 to E11.5], in which angiogenesis is actively taking place, strongly express APJ (Table 2). However, in the adult, ECs from heart and liver express it only very weakly, but ECs of blood vessels in tumors have been reported to express APJ more strongly (43).

During early embryogenesis at E8.5–9.5, APJ is expressed on ECs sprouted from the dorsal aorta. However, it is not on ECs of the dorsal aorta constructed by vasculogenesis processes. ECs sprouted from dorsal aorta form intersomitic vessels and most express APJ at E8.5; however, APJ expression is observed on ECs in the migrating tip region of intersomitic vessels at E9.5 (24). Therefore, taken together, these expression profiles suggest that APJ is expressed by ECs during angiogenesis but not vasculogenesis. In the neonate,

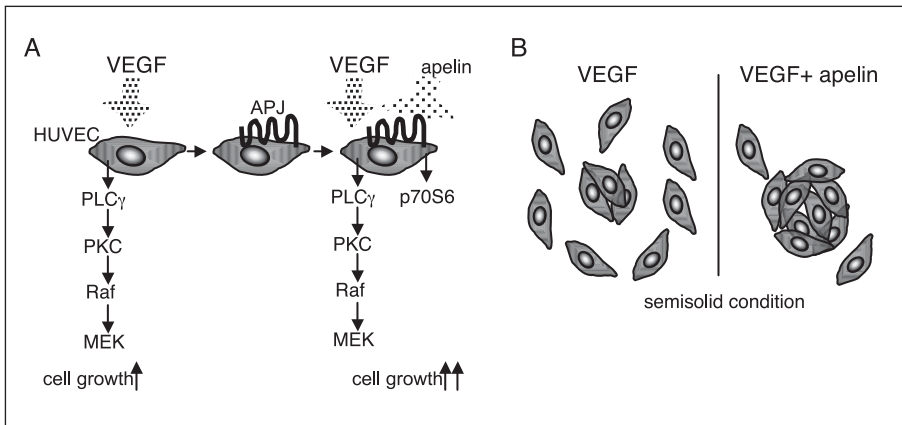


Figure 2: Effect of VEGF and apelin on proliferation and assembly of ECs. A) VEGF activates the PLC γ -PKC-Raf-1-MEK-MAP kinase pathway through its cognate receptor. VEGF also induces expression of the apelin receptor APJ on HUVECs. Apelin then activates p70S6 kinase. Therefore, VEGF and apelin coordinately enhance the proliferation of HUVECs. B) Apelin function in spheroid formation by ECs. In semisolid culture media, HUVECs pre-stimulated with VEGF generate larger spheroids in the presence of apelin than with VEGF alone.

APJ expression is observed in ECs of blood vessels in the dermis, but gradually disappears with maturity. These expression patterns strongly suggest that APJ plays a spatio-temporal role for the maturation of blood vessels by transient expression on ECs where angiogenesis is taking place. Generally, apelin-deficient mutant animals appear healthy as adults, but although body size and number of somites was similar between wild-type and apelin mutant embryos at E9.5, the caliber of intersomitic vessels was narrower in the apelin-deficient embryos (24). Moreover, the blood vessels observed in the trachea, dermis, heart and other organs were narrower than those in wild-type mice after birth. Therefore, it is suggested that apelin regulates caliber size of blood vessels.

Coordinate effect of apelin with VEGF for the proliferation of HUVECs

It is well known that VEGF induces proliferation of HUVECs. However, apelin alone is not so effective in this respect. Because APJ expression is observed in ECs during angiogenesis, it is pos-

sible that apelin cannot function in the absence of VEGF, which is upregulated during angiogenesis in response to tissue hypoxia. HUVECs do not constitutively express APJ strongly; however, it is greatly upregulated on stimulation with VEGF (Table 1). Therefore, in the presence of VEGF, HUVECs can respond to apelin effectively. Indeed, apelin alone does not affect proliferation of HUVECs, but in the presence of VEGF, it enhances their proliferation to VEGF (24).

It has been reported that VEGF-A-induced activation of the Raf-1-MEK-MAP kinase pathway mediated mainly by activation of PLC γ and subsequent stimulation of PKC (particularly PKC β) resulted in the proliferation of ECs (44, 45). Recently, it has been reported that apelin activates p70S6 kinase for cell-cycle progression (46). Therefore, VEGF and apelin may coordinately induce proliferation of HUVECs (Fig. 2).

Alone among the proangiogenic cytokines, such as Ang1, bFGF, PDGF-BB, and EGF, VEGF induces APJ expression on HUVECs (Table 1). Of course, other molecules may also affect APJ expression on ECs; however, it is very interesting that VEGF affects APJ expression, suggesting a close relationship between the APJ/apelin system and tissue hypoxia in which angiogenesis is induced.

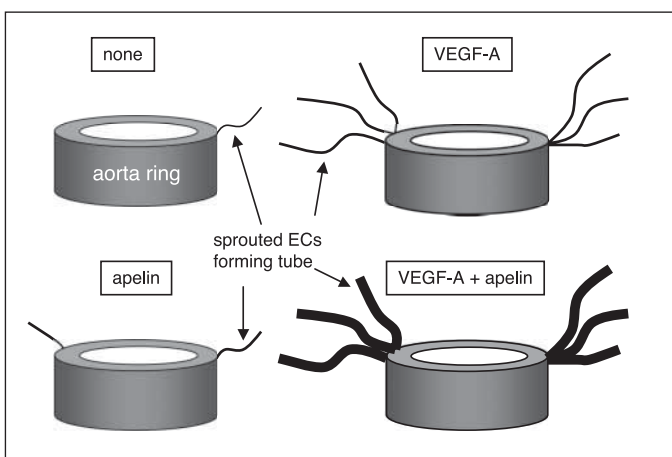
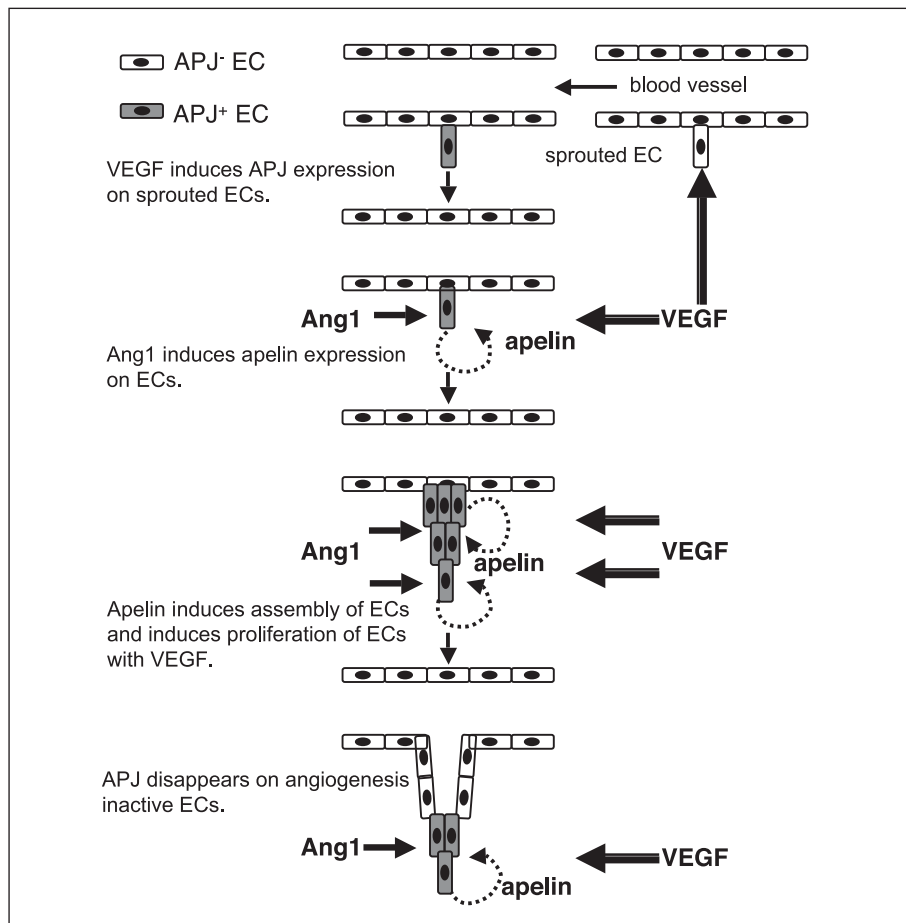


Figure 3: Apelin and VEGF together induce enlarged capillary tube formation in the aorta ring assay. Apelin alone does not induce capillary tube formation in the aorta ring assay. However, in the presence of VEGF, it induces larger capillary tubes than VEGF alone.

Apelin regulates cell assembly in spheroids

Spheroid models of cells have been widely used in tumor and embryonic stem cell studies of cellular differentiation, cell-cell interactions, and hypoxia responses, and were recently utilised to induce proliferation of neural stem cells. Based on these studies, Korff and Augustin (47) developed a spheroid culture system of ECs, such as HUVECs or bovine aortic ECs (BAECs) and showed that these three-dimensional spheroid EC models are useful for the analysis of differentiated cell function. In this culture system, ECs are suspended in culture medium containing 20% methocel, seeded into non-adhesive bacteriological dishes and cultured. Under these conditions, suspended ECs aggregate spontaneously within 4 hours to form cellular aggregates of varying size and cell number (24). Therefore, molecules affecting cell-to-cell assembly can induce larger spheroids in this culture system. When HUVECs were pretreated with VEGF for the induction of APJ and maintained in this spheroid culture system in the presence of apelin, this agent induced the formation of

Figure 4: Apelin is involved in the regulation of blood vessel caliber size. Endothelial sprouts from pre-existing vessels express APJ following stimulation with VEGF. During angiogenesis, when the Tie2 agonist, Ang1, stimulates ECs sprouted from vessels, apelin expression is induced in these cells. VEGF and apelin coordinately enhance proliferation and assembly of ECs, resulting in the formation of larger tubes. VEGF continuously stimulates ECs during angiogenesis; however, once VEGF expression is reduced in the foci, APJ expression is down-regulated in ECs and caliber size regulation is finalised.



larger spheroids than VEGF alone (Fig. 2). Induction of APJ on Ba/F3 hematopoietic cells (pro-B lymphocyte cell line), also facilitated their aggregation upon stimulation with apelin (Kidoya and Takakura unpublished data). These data indicate that apelin acts on cell-to-cell aggregation or assembly.

When ECs are cultured on Matrigel, a solid gel of basement membrane proteins, they rapidly align and form hollow tube-like structures. Grant et al. (48) first reported this effect of Matrigel. In the original study, the authors reported that tube formation is a multi-step process induced by laminin and that laminin-derived synthetic peptides can induce single-cell hollowing. However, in a similar culture system, Kamei et al. (17) induced cord hollowing to create enlarged tubes. Therefore, this culture system can be utilised to examine whether a certain molecule regulates capillary caliber size. HUVECs cultured on Matrigels in the presence of apelin generate larger tube-like structures than when they are cultured in the presence of VEGF (24). Therefore, this indicates that apelin is involved in cord hollowing.

Apelin induces formation of large tubes in the aorta ring assay *ex vivo*

The aorta ring assay, first reported by Nicosia and Madri in 1987 (49), can be employed to explore the roles of angiogenesis-related molecules *ex vivo*. This report described the utilization of rat aorta

"rings" as explants. Several subsequent studies modified this method; now most researchers culture aorta rings in a three-dimensional (3-D) extracellular matrix, such as type I collagen or Matrigel. Under these culture conditions, ring explants generate capillary-like endothelial sprouts *in vitro*. Thus, this culture system mimics sprouting angiogenesis from pre-existing blood vessels.

Upon addition of proangiogenic factors or anti-angiogenic factors, formation of capillary-like tubes is affected (Fig. 3). In the absence of growth factors, very small numbers and very short capillary-like structures are observed. However, upon addition of VEGF, capillary-like tubes radially sprout from the aorta ring. In this 3-D system, apelin alone does not induce abundant capillary-like tube formation, but in the presence of VEGF, the caliber size of the capillary tube is enlarged by apelin. In culture there is of course no blood flow through capillary-like tubes, showing that the effect of apelin on capillary enlargement is independent of blood flow.

Apelin acts as a potent caliber size regulator by inducing cord hollowing

Given the expression of APJ on ECs and the function of apelin, the role of this molecule in inducing enlarged blood vessels by promoting proliferation and cell-to-cell aggregation/assembly may be as follows (Fig. 4). Upon stimulation by VEGF, ECs

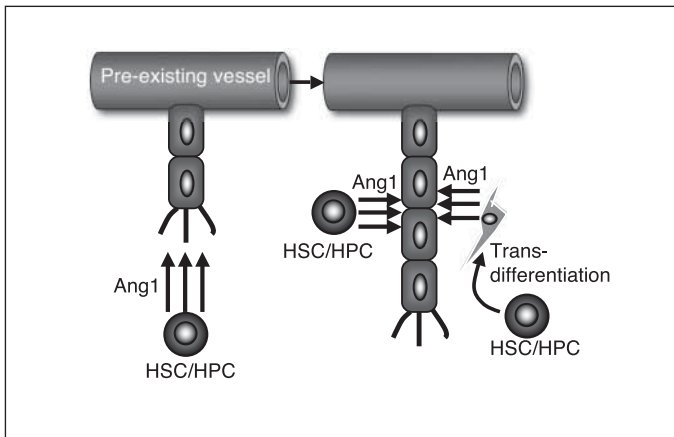


Figure 5: Function of HSCs/HPCs and production of Ang1. Haematopoietic stem cells (HSCs) and progenitor cells (HPCs) migrate into avascular regions and produce Ang1, which then induces chemotaxis of ECs and determines the migration direction of EC sprouting. Therefore, in this case, HSCs/HPCs act as proangiogenic accessory cell components. Moreover, HSCs/HPCs located at perivascular regions differentiate into mural-like cells. Both HSCs/HPCs and such mural-like cells produce Ang1 and then induce apelin expression in ECs to regulate vascular diameter.

sprouted from pre-existing vessels express APJ. Subsequently, Ang1 or bFGF stimulates such sprouted ECs to express apelin. In the presence of both VEGF and apelin, EC proliferation is enhanced more than in the presence of VEGF alone. They then adhere and form contacts with each other through junctional proteins, and construct enlarged blood vessels. Upon stimulation of APJ by apelin, junctional proteins such as Claudin-5 and VE-Cadherin are upregulated in HUVECs (24). When VEGF ceases to affect ECs, APJ expression is lost, and the modification of caliber size is finalised. As described above (Fig. 1), the single-cell hollowing system generates narrow capillaries and the cord hollowing system is responsible for the production of larger blood vessels. Therefore, apelin may function in the later, cord hollowing system and be involved in the size determination of blood vessels during angiogenesis.

Haematopoietic stem cells are candidate sources of Ang1 for the production of apelin during angiogenesis

Ang1 is usually produced from MCs in cells composing blood vessels (50). However, haematopoietic stem cells (HSCs) and

progenitor cells (HPCs) also produce Ang1 (51). HSCs/HPCs migrate into avascular areas before ECs, so Ang1 from these cells can induce angiogenesis by promoting EC chemotaxis (51). Moreover, HSCs/HPCs induce the enlargement of blood vessels observed in the fibrous cap surrounding tumors (52) and Ang1 from HSCs/HPCs in embryos, as well as adults, facilitates structural stability of newly developed blood vessels as a physiological function during angiogenesis (53). Indeed, AML1/RUNX1 mutant embryos that lack HSCs have unstable blood vessels which frequently rupture (53–55). These findings support the notion that HSCs play an important role in structural stabilization of blood vessels. HSCs/HPCs are suggested to give rise to MCs (53, 56), which are a major source of Ang1. Therefore, it is possible that Ang1 from the HSC/HPC population, frequently observed in ischemic regions, and from MCs differentiated from HSCs/HPCs, is the source of Tie2 activation during angiogenesis (Fig. 5). Ang1 produced in this manner then induces the production of apelin from ECs.

Conclusions

Control of blood vessel caliber changes is an important mechanism influencing blood pressure and flow, especially for larger vessels, and is a fundamental event for supplying oxygen and nutrients in smaller vessels. The apelin/APJ system may be involved in the size-sensing mechanism of blood vessels. Knocking out the apelin gene suggests that molecular cues other than apelin can rescue narrow caliber size blood vessels by compensational upregulation, because in the early stage of embryogenesis the narrow caliber of intersomitic vessels, observed in apelin mutant embryos, was rescued in the later stage (24). To further clarify the size-sensing mechanism of blood vessels, isolation of upregulated molecules responsible for such compensation of blood vessel caliber in apelin mutant embryos will be required.

Recently, therapeutic angiogenesis using genes or cytokines such as VEGF, HGF, etc. and cells from bone marrow or peripheral blood has been applied to the clinical management of ischemic patients (57). For the development of ideal therapeutic angiogenesis modulators, molecules controlling the caliber size of newly developing blood vessel would be the preferred choice. Apelin could be one candidate for such a modulator.

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