

METHODS

Animals

ICR and C57BL/6 mice were purchased from Japan SLC. The pK14-apelin-pA plasmid was generated by inserting the coding region of mouse apelin cDNA into the pK14-pA plasmid.³⁶ We generated K14-apelin transgenic mice according to standard methods.³⁷ We identified transgenic offspring by PCR of tail genomic DNA using forward (5'-GCC TGT GGG TGA TGA AAG CC-3') and reverse (5'-GGT CCA GTC CTC GAA GTT CT-3') primers. Three independent K14-apelin TG lines were backcrossed with wt C57BL/6 mice. Generation of apelin^{-/-} mice was described previously.²⁴ Animals were housed in environmentally controlled rooms of the animal experimentation facility at Osaka University. All experiments were carried out under the guidelines of Osaka University Committee for animal and recombinant DNA experiments and were approved by the Osaka University institutional review board.

Quantitative real-time PCR analysis

Total RNA was extracted from cells and tissues using the RNeasy Plus Mini kits (QIAGEN) and transcribed into cDNA using Exscript RT Reagent kits (Takara) according to the manufacturer's protocol. Primers for APJ, apelin, and GAPDH were previously described.²⁴ Real-time PCR analysis was performed by Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen). The levels of PCR products were monitored with Mx3000P QPCR System (Stratagene). The baseline and threshold were adjusted according to the manufacturer's instructions. The relative abundance of transcripts were normalized using either the expression level of GAPDH mRNA or CD31 mRNA.

Western blot analysis

The human VE-cadherin expression vector was a kind gift of Dr. Fukuhara (NCVC, Osaka, Japan). Lipofectamine Plus reagent (Invitrogen Life Technologies) was used to transfect cells

with this plasmid. Cell surface and cytoplasmic VE-cadherin expression by HUVECs was analyzed using a Mem-Per Reagent kit (Thermo) according to the manufacturer's protocol. Immunoblotting was performed to detect expression of VE-cadherin, N-cadherin, apelin, VEGF and GAPDH. Total cell lysates (2 µg total protein) were heated for 2 min at 95°C and then loaded onto SDS-polyacrylamide gels. Proteins were electrophoretically transferred onto polyvinylidene difluoride membranes, blocked with 5% nonfat dry milk, and subsequently incubated with anti-VE-cadherin, -N-cadherin (BD Bioscience), -apelin²⁷ (a gift from Takeda Pharmaceutical Company), -VEGF (Santa Cruz Biotechnology) or -GAPDH Abs (CHEMICON) according to the manufacturer's protocol. Proteins were detected with horseradish peroxidase-conjugated anti-mouse, anti-rabbit (DAKO), or anti-rat secondary Ab (Biosource) and ECL reagents (Amersham). In all experiments, the blots were scanned with an imaging densitometer LAS-3000 mini (Fujifilm). Predetermined molecular weight standards were used as markers. Protein concentration was measured by the protein assay dye reagent (BioRad) with BSA as a standard.

Immunostaining and lectin staining

Sections from back skin and ears of K14-apelin Tg and wt mice were stained with anti-mouse CD31 antibody (Pharmingen), anti-mouse APJ antibody²⁴ or anti-mouse apelin antibody²⁴. The procedure for tissue preparation and staining was as previously reported.³⁸ HUVECs (Kurabo) were cultured in Humedia EG2 as described.²⁴ HUVECs were grown to confluence, serum starved, and stimulated with various factors. The cells were then fixed with 4% paraformaldehyde and permeabilized by 0.1% Triton X-100, labeled with anti-VE-cadherin antibody (BD Bioscience) and anti-p120-catenin antibody (Cosmobio), and visualized with AlexaFluor 488-conjugated goat anti-rat IgG and AlexaFluor 546-conjugated goat anti-mouse IgG (Molecular Probes). F-actin was stained with TRITC-conjugated Phalloidin (Sigma). Nuclear staining was performed using Hoechst 33258 (Sigma) or TOPRO3 (Invitrogen).

Internalization analysis of VE-cadherin was performed as previously reported.³⁹ Briefly, cells were incubated in DMEM with anti-VE-cadherin antibody at 4°C for 1 hr. Antibody uptake was induced for 30 min at 37°C in serum-free medium or in the presence of VEGF and/or apelin. Cells were either fixed or subjected to a mild acid wash (2 mM PBS-glycine [pH 2.0], 15 min) in order to remove plasma membrane-bound antibodies. Lectin staining was performed as previously reported⁴⁰ using FITC-isolectin (Vector Lab). Samples were visualized using conventional microscopy (with a DM5500B equipped with HCX PL FLVOTAR 5/0.15 and HCX PL FLVOTAR 10/0.15 dry objective lenses (Leica)) or confocal microscopy (TCS/SP5 equipped with HC PLAN APO 20/0.70 and HCXPL APO 40/1.25-0.75 oil objective lenses (Leica)). Images were acquired with a DFC 500 digital camera (Leica) and processed with the Leica application suite (Leica) and Adobe Photoshop CS3 software (Adobe systems). All images shown are representative of 3–5 independent experiments.

Plasma Extravasation

To determine vascular permeability, a Miles assay was performed in mice as previously reported.⁴¹ Mice (ICR, C57 BL/6, K14 apelin Tg, or apelin^{-/-}) were anesthetized and shaved. After 2 to 3 days, mice were anesthetized again and intravenously injected with 150 µl of 1% Evans blue dye. After 15 minutes, intradermal injection of factors as follows was performed: 15 µL of VEGF (PeproTech EC Ltd), histamine (Wako), recombinant apelin peptide (Bachem AG) at different concentrations, or PBS as a negative control. The dye was eluted from the dissected samples with formamide at 56°C, and the optical density was measured by spectrophotometry (Biotrak II, Amersham Biosciences) at 620 nm.

Flow cytometric analysis

Hindlimb muscle tissue cells were pretreated with Fc-Blocker (Pharmingen) and stained with FITC-conjugated anti-CD45 mAb and PE-conjugated anti-CD31 mAb (Pharmingen).

Procedures for cell preparation and staining were as previously reported.⁴⁰ The stained cells were analyzed by FACS Calibur (Becton Dickinson) and sorted using a JSAN flow cytometer (Bay Bioscience). Dead cells were excluded from the analyses using the 2-dimensional profile of forward vs side scatter.

Hindlimb ischemia model and injection of plasmids.

Seven-week-old male Apelin KO or C57BL/6 mice underwent surgery to induce unilateral hind limb ischemia as previously described.⁴⁰ Briefly, following anesthesia by isoflurane inhalation, the left femoral artery was exposed under a stereomicroscope. The artery was then ligated both proximally and distally using 6-0 silk sutures, and the ligated vessels were resected between the ligatures without damaging the femoral nerve. Sham operations involved skin incision without femoral artery ligation. The mice were then injected with pCAG-LacZ, pCAG-VEGF and/or pCAG-apelin plasmid using GenomOne-Neo transfection reagent (Ishihara Sangyo) into the quadriceps muscle of the ischemic limb according to the manufacturer's protocol. Rate of blood flow was measured 2 weeks after surgery by means of a laser Doppler blood flow meter (Moor LDI2-IR; Moor Instruments) in both the ischemic and nonischemic hind limbs of the same animal. The blood flow values were expressed as the ratio of ischemic to nonischemic hind limb perfusion. Capillary number and size in the gastrocnemius muscle was assessed by immunohistochemistry as described above using rat anti-mouse CD31 antibody (PharMingen).

Statistical Analysis

All data were displayed as the mean \pm SD and were analyzed by repeated-measures two-way ANOVA or Student *t* test using Statview software (Abacus Concepts). A probability value of less than 0.05 was considered statistically significant.

RESULTS

Transgenic overexpression of apelin induces larger blood vessels

To ascertain the functional significance of apelin for blood vessel formation *in vivo*, we generated transgenic mice on the C57BL/6 background (K14-apelin Tg mice) expressing apelin in basal epidermal keratinocytes under the transcriptional control of the K14 promoter (Fig.1A). Real-time PCR analysis of skin mRNA obtained from wild-type (wt) and from K14-apelin mice 3 days after birth revealed that the transgene was incorporated into 8 founder mice (data not shown). Three independent transgenic mouse lines, #1 #5 and #6, were chosen for analysis because of their high level expression of apelin mRNA (Fig. 1B).

No major phenotypic differences were observed between these lines and transgenic offspring were born in normal Mendelian ratios. Induced apelin mRNA was translated into protein and expressed specifically in the dermal layer of dorsal skin in K14-Apelin Tg mice (Fig. 1C). Lectin staining revealed that capillaries, but not arteriola and venula in the dermis were larger than in wt mice (Fig. 1D, E). However, vascular density was similar (Fig. 1F). Angiopoietin-1 (Ang1) overexpression in the skin induces formation of large blood vessels without affecting the thickness of ear skin.¹⁷ The same was true for the K14-apelin Tg mice (data not shown).

To confirm apelin receptor APJ expression, we compared APJ levels in ECs from skin in neonates and adults. APJ was highly expressed in vascular ECs of neonatal skin, but was barely detectable in adult skin (Fig. 1G). This finding is consistent with our previous report that APJ expression is observed in ECs primarily when angiogenesis is taking place.²⁷ These results indicate that apelin can induce non-leaky larger blood vessels *in vivo*.

Endogenous apelin is required for recovery of hindlimb perfusion after induction of ischemia

We previously reported the temporal expression of APJ in ECs during angiogenesis in embryos.²⁴ To determine whether APJ is expressed in a cell type-specific manner in ECs

under hypoxia, occlusion of the femoral artery was used to induce ischemia in collateral vessels of the hindlimb. As with VEGF induction, we found that expression of *APJ* as well as *apelin* mRNA was significantly increased by hypoxia in this model (**Supplementary Fig. 1A-C**). *APJ* induction was observed beginning at day 2 after ischemia and then gradually attenuating. *Apelin* expression was slightly slower than *APJ* expression (**Supplementary Fig. 1A,B**). These expression profiles are consistent with our previous finding that VEGF induces *APJ* expression on ECs during the initial step of angiogenesis and that Ang1 subsequently induces *apelin* expression on blood vessel maturation.²⁴ Accumulation of *apelin* mRNA was increased when ECs were stimulated with Ang1 under hypoxic conditions compared with normoxic conditions (**Supplementary Fig. 1D**). This might be effected by Tie2 upregulation in ECs under hypoxia, as previously reported.⁴²

To determine whether *APJ* expression in the ischemic muscle is by ECs, CD31 expression was also assessed (**Fig. 2A, Supplementary Fig. 1E**). In the steady state (sham-operated muscle), *APJ* expression was weak in CD31⁺ ECs; however, consistent with our previous findings that ECs start to express *APJ* upon stimulation with VEGF,²⁴ we observed that most CD31⁺ ECs of newly-developing vessels in the hypoxic region strongly expressed *APJ* after induction of ischemia. We sorted CD31⁺CD45⁻ECs or CD31⁻non-ECs from ischemic muscle and confirmed specific expression of *APJ* and *apelin* in ECs under hypoxia (**Fig. 2B,C**). These results therefore suggest the involvement of the *apelin/APJ* system in collateral vessel formation during the process of recovery from ischemic states.

To determine the requirement for endogenous *apelin* in recovery from the ischemic state, hindlimb ischemia was induced in *apelin*^{-/-} mice and wt mice. After 2 weeks, severe necrosis of the toes was observed in *apelin*^{-/-} but not wt mice (**Fig. 2D**). Delayed revascularization in the *apelin*^{-/-} mice was assessed by laser Doppler-monitored blood flow measurements (**Fig. 2 E,F**). Thus, we found that *APJ* expression is induced after ischemic treatment and endogenous *apelin* is required for functional recovery.

To exclude the possibility that apelin deficiency leads to attenuation of hematopoietic cell recruitment in the ischemic hindlimb muscle which then enhances angiogenesis, we investigated the frequency of CD45⁺ and APJ⁺ hematopoietic cells there. However, no major differences in the number of recruited CD45⁺ hematopoietic cells between wt and apelin^{-/-} mice were found, and we failed to detect APJ-positive cells among the CD45⁺ hematopoietic cells in either (data not shown). This suggests a direct effect of apelin on ECs.

Apelin gene transfer promotes development of larger vessels in the hindlimb ischemia model

Although apelin in wt mice is upregulated in ECs of ischemic muscle (**Fig. 2C**), additional apelin induction may improve blood vessel formation. Therefore, we next evaluated the effect of apelin gene transfer in this hindlimb ischemia model and compared apelin induction alone with both apelin and VEGF together. Mouse apelin, human VEGF-165 (VEGF) and β -galactosidase (β -gal) expression plasmids were constructed (**Supplementary Fig. 2A**). Protein expression was confirmed by Western blotting and immunohistochemical analysis 24hrs after gene transfer to hindlimb muscle (**Supplementary Fig. 2B,C**). Necrotic toe was observed in mice receiving control LacZ gene therapy, but was reduced by VEGF or apelin gene transfer. Moreover, such necrosis was essentially undetectable in mice receiving both VEGF and apelin gene therapy (**Fig. 3A**). Efficiency of revascularization was assessed by laser Doppler-monitored blood flow measurements (**Fig. 3 B,C**). Gene therapy with either apelin or VEGF alone improved blood flow, but a combination of both was most effective.

Immunohistochemical analysis of hindlimb muscle two weeks after gene transfer revealed that VEGF alone effectively increased the number of blood vessels (**Fig. 3 D-F**), whereas apelin induced fewer but larger vessels than VEGF. Although the proportion of enlarged among total blood vessels was slightly reduced, the combination of VEGF and apelin still resulted in increased numbers of larger vessels than VEGF alone (**Fig. 3 D-F**).

Apelin suppresses VEGF-induced vascular edema

Vascular leakage resulting in tissue edema is a major problem in therapeutic angiogenesis.^{13,43} It has been reported that Ang1 induces enlarged blood vessels and inhibits VEGF-mediated hyperpermeability.¹⁷ We found that apelin expression is induced in ECs by Ang1.²⁴ Therefore, we asked whether apelin also inhibits VEGF-mediated leakiness of blood vessels. In hindlimb muscle of VEGF-treated mice, abundant edematous free spaces in the gastrocnemius muscles were observed, resulting in contraction of muscle fibers. In contrast, apelin-treated mice lacked such edema; moreover, VEGF-mediated edema was suppressed in mice receiving apelin/VEGF double gene therapy (**Fig. 4A**). We measured the circumference of the hindlimbs, which reflects the degree of angioedema, and found that femoral artery occlusion itself resulted in swollen legs. Apelin also rapidly reduced this, and inhibited VEGF-mediated swelling (**Supplementary Fig. 3**).

To study the role of apelin in vascular permeability, we applied a modified Miles vascular hyperpermeability assay *in vivo*. As shown in **Fig. 4B**, VEGF-mediated hyperpermeability was suppressed by apelin in a dose-dependent manner. Similarly, histamine-mediated vascular permeability was also suppressed by apelin (**Supplementary Fig. 4**). To ascertain the requirement for endogenous apelin, the Miles assay was performed using apelin^{-/-} mice. Vascular hyperpermeability mediated by VEGF or histamine was increased by the lack of apelin (**Fig. 5A**). In contrast, over-expression of apelin inhibited vascular leakage caused by VEGF or histamine in K14-apelin Tg mice (**Fig. 5B**). Furthermore, plasma leakage caused by topical application of mustard oil, an inflammatory agent, was enhanced in apelin^{-/-} mice and suppressed in K14-apelin Tg mice compared with wt mice (**Supplementary Fig. 5**). These data show that apelin inhibits vascular leakage caused by several different agents.

Dysfunction of lymphatic vessels and hemodynamic disorder may also cause tissue edema. To determine whether apelin directly affects vascular hyperpermeability, we tested apelin

inhibition of VEGF-mediated leakage using EC monolayers *in vitro*. We confirmed that apelin stimulation abrogates FITC-dextran leakage through EC monolayers exposed to VEGF. (**Supplementary Fig. 6**)

Apelin inhibits internalization of VE-Cadherin

To examine the mechanism of inhibition of vascular leakage, we used human umbilical vein endothelial cells (HUVECs) to investigate the role of apelin in the formation of intracellular gap and cellular junctions *in vitro*. It has been reported that VE-cadherin-forming EC-EC junctions are lost and gaps between ECs induced upon stimulation with permeability factors.^{39,44} VEGF stimulation induced stress fibers and destroyed the VE-cadherin-mediated cell-cell junction, resulting in gap formation in HUVECs (**Fig. 6A**). Apelin alone did not affect cell-cell junctions, but the effect of VEGF on gap formation was abolished by apelin pretreatment and overexpression of VE-cadherin on HUVECs mimics the effect of apelin (**Supplementary Fig. 7A,B**). Similar suppressive effects of apelin on gap formation were observed using histamine (**Supplementary Fig. 8A-C**). Time-lapse imaging of cultured ECs revealed that histamine and VEGF stimulation induced dynamic cell motility and large intercellular gap formation and showed that this was completely suppressed by pretreatment with apelin (**Supplementary Movie 1-5**). Gap formation suggests disruption of EC-EC barrier function by VEGF or histamine. Indeed, histamine rendered EC monolayers permeable to FITC-dextran. This leakage was almost completely abrogated by stimulation with apelin (**Supplementary Fig. 8D**).

When living HUVECs were labeled with anti-VE-cadherin antibody, internalization of VE-cadherin was induced upon stimulation with VEGF, as previously reported.⁴⁵ Only internalized VE-cadherin was visible after acid wash treatment which removes membrane-bound antibodies (**Fig. 6B,C**). However, upon pre-treatment with apelin, VEGF no longer induced internalization of VE-cadherin, and membrane-bound VE-cadherin was lost

from EC-EC junctions on acid washing (**Fig. 6B,C**). Western blot analysis of extracts of cells stimulated with apelin and/or VEGF demonstrated unaltered total cell-associated VE-cadherin, but VEGF induced the accumulation of VE-cadherin in the cytoplasmic fraction, depleting the membrane fraction. This internalization of VE-cadherin was suppressed by simultaneous addition of apelin (**Fig. 6D**). p120 catenin has been shown to act as a retention factor for VE-cadherin, preventing endocytosis of the transmembrane protein. Although loss of VE-cadherin after treatment with VEGF, impacting on the EC-EC junction, was accompanied by a loss of p120 catenin, apelin treatment together with VEGF resulted in retention of plasma membrane associated VE-cadherin and p120 catenin (**Supplementary Fig. 7C**). Therefore, we concluded that apelin stabilizes VE-cadherin on the cell surface, resulting in promotion of the formation of non-leaky blood vessels.

Nonetheless, histamine stimulation did not promote the internalization of VE-cadherin in cultured HUVEC monolayers (**Supplementary Fig. 9A,B**). This suggests that additional mechanisms contribute to the suppressive effects of apelin on gap formation.

DISCUSSION

Here we report that endogenous apelin is required for the recovery from ischemia, and that additional apelin induction together with VEGF significantly restores ischemia damage by generating enlarged blood vessels in the ischemic muscle. Interestingly, VEGF-mediated hyperpermeability is almost completely suppressed by simultaneous induction of apelin. Therefore, we concluded that apelin is one of the factors inducing enlarged and non-leaky blood vessels.

We previously reported that the apelin/APJ system spatially and temporally modulates caliber size enlargement during embryogenesis.²⁴ APJ expression in adult ECs was weak, but VEGF induced its expression on HUVECs.²⁴ Our present results also showed that APJ expression on ECs is induced in the hind limb ischemia model and this is consistent with the upregulation of VEGF in ischemic muscle, suggesting the induction of APJ expression on ECs by VEGF. Moreover, in the retina, it has been reported that strong APJ expression was observed temporally in the radial vessels from day 3 to day 12 after birth, but APJ expression on ECs was attenuated later.⁴⁶ Thus, these data strongly suggest that APJ expression is induced when angiogenesis is taking place under conditions of tissue hypoxia. In the case of apelin, we previously reported that its expression is upregulated in ECs upon stimulation with Ang1;²⁴ however, recently it has been suggested that apelin expression is induced by tissue hypoxia and this is consistent with evidence for the existence of hypoxia-inducible factor binding sites in the promoter sequences of the *apelin* gene.²⁶ A function for apelin/APJ during postnatal angiogenesis in physiological or pathological situations, especially associating with tissue hypoxia, is strongly implied by these data.

It is widely believed that maturation of blood vessels is closely related to cell contact between mural cells and ECs and that this process is induced by Ang1 produced from mural cells recruited around ECs by PDGF, resulting in initiation of cell adhesion between mural cells and ECs.⁴⁷ So far, however, it has not been established when enlargement of blood

vessels is induced for the adjustment of blood flow responding to tissue demands for oxygen and nutrients. Exchange of gas and nutrients is mainly regulated in the capillary bed. Therefore, caliber size regulation associated with gas and nutrient perfusion must be regulated at the capillary level. Generally, on adhesion between mural cells and ECs, proliferation of the latter is suppressed by angiogenic factors such as TGF β ⁴⁷ and this event finalizes angiogenesis. Because it is thought that caliber size regulation is exerted during angiogenesis, blood vessels formed by EC alone may change their caliber before contact with mural cells and cell adhesion takes place. In K14-apelin Tg mice, caliber size of arteriola and venula in the dermis was not different from WT mice. This may be because those blood vessels are already covered with mural cells during embryogenesis before activation of the K14 promoter. In this case, apelin overexpressed in the skin would not affect the ECs of those vessels in which APJ expression is attenuated. On the other hand, capillaries in the dermis of K14-apelin Tg mice were significantly enlarged compared with those of WT mice. Because mural cell covering is usually sparse in the capillaries compared with arteriola and venula and sprouting angiogenesis is actively occurring at the capillary level after birth, it is possible that apelin affects capillaries but not arteriola and venula. Taken together with the phenotype of K14-apelin tg mice and the expression of apelin and APJ on ECs under tissue hypoxia, we conclude that the target vessels of apelin are immature blood vessels at the stage before mural cell adhesion to ECs.

We previously suggested that EC-EC aggregation might be an important mechanism for enlargement of blood vessels mediated by apelin during embryogenesis.²⁴ At present, it is not clear how larger blood vessels are induced by apelin during adult angiogenesis but this might be similar to the mechanism utilized in the embryo.

In addition to mere enlargement, we found that apelin induces non-leaky blood vessels. A requirement for endogenous apelin for the suppression of vascular permeability caused by factors such as VEGF, histamine and inflammatory agents was noted in apelin-deficient mice;

we confirmed this effect in K14-apelin tg mice. Tissue edema is caused by lymphatic dysfunction and hemodynamic disorder as well as vascular hyperpermeability. We observed that lymphatic vessel formation was not greatly different in wt and apelin^{-/-} mice (data not shown). It has been reported that apelin- or APJ-deficient mice have similar, normal blood pressure.⁴⁸ Although we cannot completely exclude the possibility of a contribution of apelin function to lymphatic vessel integrity and hemodynamics, its direct effects on vascular permeability were confirmed only by using in vitro endothelial monolayer assays (**Supplementary Fig. 6**). These results suggest a benefit of apelin in therapeutic angiogenesis when combined with several proangiogenic factors. VEGF has the disadvantage of promoting edema, but apelin may prevent this by suppressing vascular leakiness.¹³ Indeed, we found that apelin inhibited VEGF-mediated hyperpermeability of newly-developed blood vessels in the hindlimb ischemia model. Previous reports showed that apelin activates src-dependent PKC activation and blocks increased cAMP accumulation by reducing the intracellular Ca²⁺ concentration.^{28,49} Furthermore, apelin also induces the phosphorylation of myosin light chains in vascular smooth muscle cells.⁵⁰ From those data, one would not expect apelin to suppress vascular leakage, but rather to promote it. However, our results are completely at odds with this predicted function of apelin in vascular permeability. We are unable to explain this apparent inconsistency at present; however, suppression of VE-Cadherin internalization by apelin as shown here may surpass the signaling of gap formation by stabilizing cell-to-cell junctions. The molecular mechanism of how apelin might stabilize VE-Cadherin localization in plasma membranes is also not clear at present. It is well-known that VEGF-mediated hyperpermeability and VE-Cadherin internalization is suppressed by Ang1.^{16,17} Recently, it has been reported that this process involves the activation of RhoA by Ang1 and its subsequent association with mDia, a RhoA downstream target.⁴⁵ We found apelin induction in ECs via Tie2 activation by Ang1. Therefore, similar signaling pathways may be involved in

the suppression of VE-cadherin internalization by apelin/APJ; however, the precise molecular mechanism requires elucidation in future work.

Recently, the effectiveness of FGF1 gene therapy for patients with severe limb ischemia was demonstrated in clinical trials; apelin might also be useful in developing gene therapy for such patients in combination with FGF1 or VEGF.⁷ Especially for diabetic patients, who often suffer severe limb ischemia, it is anticipated that apelin/FGF or apelin/VEGF gene therapy will be particularly beneficial. Further studies will be required to confirm the effect of apelin together with FGF or VEGF for treating ischemia using diabetic mouse models.

As angiogenesis is controlled by molecular multistep processes,¹³ the use of a single growth factor may be insufficient to promote a functional vascular system response in therapeutic angiogenesis. Although several factors or cells can initiate the formation of neovessels by promoting proliferation and migration of ECs, stabilization of EC-EC contacts must be considered essential for producing mature vessels. As shown in the present study, our data suggest a new model for generating functional non-leaky blood vessels by regulating caliber size and permeability of newly-formed vessels by apelin in ischemic disease.

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AUTHORSHIP

Contribution: H.K. and N.T. designed and performed research, analyzed data, and wrote the paper; H.N. performed research and analyzed data.

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