Adrenomedullin induces lymphangiogenesis and ameliorates secondary lymphoedema

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Aims Adrenomedullin (AM) is a multifunctional peptide hormone that plays a significant role in vasodilation and angiogenesis. Lymphoedema is a common but refractory disorder that is difficult to be treated with conventional therapy. We therefore investigated whether AM promotes lymphangiogenesis and improves lymphoedema.

Methods and results The effects of AM on lymphatic endothelial cells (LEC) were investigated. AM promoted proliferation, migration, and network formation of cultured human lymphatic microvascular endothelial cells (HLMVEC). AM increased intracellular cyclic adenosine monophosphate (cAMP) level in HLMVEC. The cell proliferation induced by AM was inhibited by a cAMP antagonist and mitogen-activated protein kinase kinase (MEK) inhibitors. Phosphorylated extracellular signal-regulated kinase (ERK) in HLMVEC was increased by AM. Continuous administration of AM (0.05 μg/kg/min) to BALB/c mice with tail lymphoedema resulted in a decrease in lymphoedema thickness. AM treatment increased the number of lymphatic vessels and blood vessels in the injury site.

Conclusion AM promoted LEC proliferation at least in part through the cAMP/MEK/ERK pathway, and infusion of AM induced lymphangiogenesis and improved lymphoedema in mice.

KEYWORDS
Adrenomedullin; Lymphangiogenesis; Lymphoedema

1. Introduction

Lymphatic system plays an important role in the maintenance of tissue fluid homeostasis, and damage of lymphatic vessels or surgical removal of lymph nodes often triggers the development of lymphoedema. Despite substantial advances in surgical and conservative techniques, the therapeutic options for this disease are limited. Recently, studies have suggested that vascular endothelial growth factor (VEGF)-C, VEGF-D or hepatocyte growth factor stimulates lymphangiogenesis and promotes oedema recovery in animal models of lymphoedema. The proliferation and migration of lymphatic endothelial cells (LEC) induced by these factors are dependent on activation of extracellular signal-regulated kinase (ERK) and Akt.

Adrenomedullin (AM) is a vasodilator and diuretic peptide that was originally isolated from pheochromocytoma cells. Earlier studies have shown that AM has protective effects on the cardiovascular system. In particular, AM has angiogenic properties through activation of ERK and Akt in vascular endothelial cells. Interestingly, a recent study has shown that the AM peptide is widely expressed in breast cancer and the degree of expression is associated with axillary lymph node metastasis. Endogenous AM is necessary for murine lymphatic vascular development during embryogenesis. These findings raise the possibility that AM may play an important role in lymphangiogenesis. However, whether AM promotes lymphangiogenesis and improves lymphoedema remains unknown.

Therefore, the present study was performed to (i) investigate whether AM promotes proliferation, migration, and network formation of cultured LEC in vitro, and elucidate the underlying molecular mechanisms and (ii) investigate whether AM promotes lymphangiogenesis and improves lymphoedema in a mouse model of tail lymphoedema in vivo.

2. Methods

2.1 Cell culture

Human umbilical vein endothelial cells (HUVEC) and human lymphatic microvascular endothelial cells (HLMVEC) were purchased from Lonza (Basel, Switzerland), and expanded in medium (EBM-2, Lonza) with growth supplements (EGM-2MV, SingleQuots, Lonza). HUVEC and HLMVEC were cultured on collagen I-coated dishes.

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2.2 Characterization of cultured lymphatic endothelial cells

To confirm the purity of expanded HLMVEC, we stained these cells with a LEC-specific marker Prox1. The cells were stained with anti-Prox1 antibody (Acris, Hidenhausen, Germany) followed by Alexa Fluor 488-conjugated secondary antibody (Invitrogen, Carlsbad, CA, USA), and counterstained with 4',6-diamidino-2-phenylindole (Dojindo, Kumamoto, Japan). The images were obtained with a fluorescence microscope (BZ-9000, Keyence, Osaka, Japan).

To examine the expression of AM receptor messenger ribonucleic acid (mRNA) in HLMVEC, reverse transcription polymerase chain reaction (RT–PCR) was performed. Total RNA were prepared from cultured HLMVEC using an RNeasy mini kit (Qiagen, Hilden, Germany). PCR was carried out on a thermocycler (ASTEC PC-818, Fukuoka, Japan) using the following protocol: an initial denaturation step at 95 °C for 5 min, followed by 40 cycles at 95 °C for 60 s, 60 °C for 30 s, and 72 °C for 60 s. The specific primer pairs were: calcitonin-receptor-like receptor (CRLR), 5'-CTCTCTCTATATCATCATTACCATCGG-3' and 5'-CTCTCTCTGGAATCATTCC-3'; receptor-activity-modifying proteins (RAMP) 1, 5'-GGACTGGGATGACATGG-3' and 5'-GGCTCAATACGCGTCTCA-3'; RAMP2 5'-AGTTCCAGGTAGACATGG-3' and 5'-GGAAATGGAGTAGATGAG-3'; RAMP3 5'-AGACAGGGGATGTGGGA-3' and 5'-TTCCAGCGTGGCGTGT-3'. A set of β-actin primers was used as a Control for the RT–PCR.

2.3 Cell proliferation assay

HLMVEC were cultured for 36 h in EB M-2 medium containing 5% foetal bovine serum (FBS, Invitrogen) with (10^{-7} M) or without AM (Sionogi, Osaka, Japan). The cells were stained with Diff-Quik (Sysmex Internal Reagents, Kobe, Japan), photographed (BZ-9000, Keyence), and the number of cells was counted. In addition, HLMVEC were cultured in 96-well plates (5000 cells/well) with AM (10^{-7} – 10^{-7} M) or 3', 5'-cyclic adenosine monophosphate (cAMP) (10^{-6} – 10^{-6} M) (Calbiochem, San Diego, CA, USA), a cell-permeable cAMP analogue, and cell proliferation were measured by MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium] assay (Promega, Madison, WI, USA). The reagent was added and plates were incubated for 4 h, and absorbance was measured at 490 nm (Bio-Rad, Hercules, CA, USA).

2.4 Cell migration and network formation assay

Migration assay was performed using a Transwell permeable support (Corning, NY, USA) containing a membrane insert (6.4 mm diameter, pore size 8.0 μm, collagen matrix uncoated). HLMVEC in EB M-2 with 5% FBS were added to the upper chamber at a density of 10^5 cells/cm², AM (10^{-9} – 10^{-7} M) in EB M-2 medium containing 5% FBS was added to both the lower and upper chambers or to the lower chamber only, and HLMVECs were allowed to migrate for 12 h at 37 °C. The cells remaining at the upper surface of the membrane were scraped off, and the cells on the lower side of the membrane were stained with Diff-Quik (Sysmex Internal Reagents), then the number of migrated cells was counted under a microscope (BZ-9000, Keyence).

Network formation assay was performed using Matrigel tissue culture plates (2-well plates, Becton Dickinson, San Jose, CA, USA). HLMVEC (10^5 cells/well) were seeded into each well and incubated for 18 h in EB M-2 medium containing 5% FBS with (10^{-7} M) or without AM. During this period, the morphologic changes of the cells were observed under a microscope (BZ-9000, Keyence).

2.5 Analysis of intracellular signalling

Intracellular cAMP was measured as reported previously. Briefly, following treatment of HLMVEC (2 x 10^5 cells) with various concentrations of AM for 5 min, the medium was removed and washed twice with PBS (phosphate buffered saline). The cellular extract was obtained by addition of cold 70% ethanol. Each ethanol sample was evaporated in a vacuum until dry and dissolved in radioimmunoassay (RIA) buffer. cAMP was measured using an RIA kit (cAMP assay kit, Yamasa Shoyu, Chiba, Japan). Radioactivity was measured with a gamma counter (Aloka, Tokyo, Japan).

HLMVEC (5000 cells/well in 96-well plates) were pretreated for 30 min with 3', 5'-cyclic adenosine monophosphorothioate Risperide (Rp-cAMP) (10^{-5} M, Calbiochem), an antagonist of cAMP, PD98059, and U0126 (5 x 10^{-6} and 10^{-5} M, respectively, Calbiochem), inhibitors of mitogen-activated protein kinase (MEK). The cells were further incubated for 36 h with AM (10^{-7} M) or 8-Br-cAMP (10^{-4} M) then MTS assay was performed as described previously.

2.6 Animal model and adrenomedullin administration

Male BALB/c mice (8–10 weeks, Japan SLC, Hamamatsu, Japan) were randomly divided into two groups: an AM treatment group and a Control group (n = 10 in each group). Tail lymphoedema was created as described previously with modification. In brief, mice were anaesthetized by intraperitoneal injection of pentobarbital sodium (30 mg/kg, Dainippon Sumitomo Pharma, Osaka, Japan). A 1.5-2 mm-wide ring of skin was removed 1 cm distal to the tail base, leaving the underlying bone, muscle and major blood vessels intact, and then the tail was wrapped with adhesive tape to protect the surgical site from interference. The AM group received continuous subcutaneous injection of recombinant human AM (Shionogi) dissolved in 0.9% saline at a rate of 0.05 μg/kg/min for 14 days from the day of operation, using an osmotic minipump (Alzet, Cupertino, CA, USA). The Control group received 0.9% saline instead of AM.

All protocols were performed in accordance with the guidelines of the Animal Care Ethics Committee of the National Cardiovascular Center Research Institute. The investigations conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.7 Measurements of plasma adrenomedullin concentration and tail lymphoedema

Twenty-four hours after the start of AM administration, blood samples were collected into ice-cooled tubes with 70 μg/mL aprotinin and 1.5 mg/mL ethylenediaminetetraacetic acid 2Na (Bayer, Leverkusen, Germany), immediately centrifuged at 4 °C, and stored at −80 °C until use. Plasma human AM level was measured using an immunoradiometric assay kit (Shionogi), as described previously. In brief, 200 μL of standard or plasma sample was placed in a polystyrene tube, and a mixture of biotinylated anti-AM and iodolabelled anti-AM was added. One bead coated with anti-biotin antibody was added (total volume = 300 μL), and the mixture was incubated at 4 °C for 20 h. After removal of the incubation mixture, the bead was washed twice with 2 mL distilled water and radioactivity was measured with a gamma counter (Aloka).

Two days after operation, the tape wrapping the injury site was removed, and tail diameter at 1 cm distal to the distal edge of the injury site was measured by a calliper. Measurement was performed every other day until the mice were sacrificed on day 16 postoperation.
2.8 Fluorescence microlymphangiography

Lymphatic vessels in the dermis of the tail were evaluated by fluorescence microlymphangiography as previously described.23 In brief, mice were anaesthetized by intraperitoneal injection of pentobarbital sodium (30 mg/kg) on day 16 postoperation. Fluorescein isothiocyanate (FITC)-dextran (70 kDa, Sigma-Aldrich) (2 mg/mL) was infused into the tip of the mouse tail. Then, images of superficial capillaries of the tail were photographed (MZ16FA, Leica Microsystems, Wetzlar, Germany).

2.9 Immunohistochemical study

On day 16 postoperation, the injury sites were removed, fixed in 4% paraformaldehyde, embedded with paraffin, and cut into 5 μm sections. For antigen retrieval, the sections were incubated with citrate buffer (DAKO, Glostrup, Denmark) at 120°C for 10 min. After treatment with protein block (DAKO), the samples were incubated with anti-lymphatic endothelial hyaluronan receptor-1 (LYVE-1) antibody (R&D Systems, Minneapolis, MN, USA), anti-Podoplanin antibody (Acris) or anti-von Willebrand factor (vWF) antibody (AB7356, Chemicon, Temecula, CA, USA) for 1 h at room temperature, and further incubated with horseradish peroxidase-conjugated antibodies (DAKO), then visualized with a DAB kit (Wako, Osaka, Japan). Cell nuclei were counterstained with haematoxylin (Muto, Tokyo, Japan).

2.10 Western blot analysis

HLMVEC were incubated for 12 h in basal medium containing 5% FBS and stimulated with (10^{-7} M) or without AM, then lysed in sample buffer with protease inhibitor (Complete, Roche, IN, USA). The lysates were loaded on 7.5% sodium dodecylsulphate-polyacrylamide gels (Bio-Rad, Hercules, CA, USA), transferred to membranes (Millipore, MA, USA) and probed with anti-ERK and anti-phosphorylated ERK (p-ERK) (Thr202/Thr204) antibodies (Cell Signaling, Boston, MA, USA) or anti-Akt and anti-phosphorylated Akt (p-Akt) (Ser473) antibodies (Cell Signaling). An anti-α-tubulin antibody (Sigma-Aldrich, St Louis, MO, USA) served as a loading control. The membranes were then incubated with horseradish peroxidase-conjugated antibodies (Cell Signaling), and visualized by enhanced chemiluminescence reaction (GE Healthcare, Piscataway, NJ, USA).

HLMVEC were stimulated with (10^{-7} M) or without AM for 12 h, then lysed in sample buffer. Tail tissue of the injury site was obtained on day 8 postoperation and also lysed in sample buffer. Concentrations of VEGF-C and VEGF-A in each lysate were measured by western blot analysis. An anti-VEGF-C (H-190, Santa Cruz Biotech, Santa Cruz, CA, USA) antibody or an anti-VEGF-A (VEGF147, Santa Cruz, for HLMVEC lysate; AB1442, Chemicon, for tail tissue lysate) antibody was used as the probe. A rabbit polyclonal antibody against α-tubulin (Abcam, Cambridge, MA, USA) was used as an internal control.
2.11 Statistical analysis

All data are expressed as mean ± SE. Comparisons of parameters among the groups were made by one-way ANOVA (analysis of variance), followed by Newman–Keul’s test. Comparisons of parameters between two groups were made by Student’s t-test. Values of P < 0.05 were considered statistically significant.

3. Results

3.1 Expression of calcitonin-receptor-like receptor and receptor-activity-modifying proteins mRNA in lymphatic endothelial cells

To determine whether LEC express AM receptors, we analysed commercially available HLMVEC which were positive for lymphatic maker Prox1 (Figure 1A). HLMVEC expressed CRLR, RAMP1, RAMP2, and RAMP3 mRNA (Figure 1B).

3.2 Enhancement of proliferation, migration, and network formation in cultured lymphatic endothelial cells by adrenomedullin

AM (10^{-7} M) significantly increased the number of cultured HLMVEC (Figure 2A). MTS assay also demonstrated that AM enhanced proliferation of cultured HLMVEC in a dose-dependent manner (Figure 2B). The number of migrated cells was significantly increased by AM (10^{-9}–10^{-7} M) when it was added to the lower chamber only (Figure 2C). This result indicates that AM is chemotactic for LEC. In addition, a marked increase in network formation was observed in the AM (10^{-7} M) group as compared with the Control group (Figure 2D). These results suggest that AM promotes proliferation, migration, and network formation of LEC.

3.3 Activation of cAMP/MEK/ERK pathway in lymphatic endothelial cells by adrenomedullin

We examined whether AM increases intracellular cAMP, the major second messenger of AM, in cultured HLMVEC. AM dose-dependently increased the cAMP level in these cells (Figure 3A). Cell proliferation induced by AM was inhibited by a cAMP antagonist, Rp-cAMP (Figure 3B). In addition, a cAMP analogue, 8-Br-cAMP induced proliferation of HLMVEC in a dose-dependent manner (Figure 3C).

ERK and Akt are known to regulate cell proliferation and these signalling pathways were reported to function downstream of the AM/cAMP pathway. Therefore, we investigated the activity (phosphorylation) of ERK and Akt in cultured LEC after stimulation with AM. The level of p-ERK in HLMVEC was significantly increased as early as 5 min after the start of AM (10^{-7} M) stimulation (Figure 4A). In contrast, the level of p-Akt in HLMVEC was not markedly increased by AM (Figure 4B). In addition, the cell proliferation induced by AM or 8-Br-cAMP was significantly attenuated by MEK inhibitors, PD98059, and U0126 (Figure 4C and D). These results suggest that AM-induced cell proliferation is mediated at least in part by the cAMP/MEK/ERK pathway.

3.4 Improvement of mouse tail lymphoedema by adrenomedullin

To evaluate the effect of AM on lymphoedema, a mouse model of tail lymphoedema was developed in BALB/c mice, and human AM was administered to the mice at a rate of 0.05 µg/kg/min (Figure 5A). Plasma human AM level was 5.4 ± 0.9 fm in AM-treated mice, whereas human AM was not detected in Control mice. The tail became oedematous after the surgical procedure and this change peaked on day 8 postoperation then gradually recovered in the AM and Control groups. However, AM treatment promoted the recovery of the injury site (Figure 5B). A significant difference in tail thickness was observed on days 14 and 16 postoperation (Figure 5C). These results suggest that AM improves secondary lymphoedema of the tail in mice.

3.5 Promotion of lymphangiogenesis and angiogenesis by adrenomedullin

Lymphatic flow in the tail was evaluated by fluorescence microlymphangiography on day 16 postoperation.
After infusion of FITC-dextran, fluorescence was soon observed in the distal part of the injury site. Although slow drainage of FITC-dextran was seen in Control group, rapid movement of fluorescence to the proximal part of the injury site was observed in the AM group. Lymphatic vessels in the injury site were revealed by staining with anti-LYVE-1 antibody or anti-Podoplanin antibody. Blood vessels in the injury site were visualized by staining with anti-vWF antibody (Figure 6B). A large number of LYVE-1-, Podoplanin-, and vWF-positive vessels were observed on day 16 postoperation. The density of lymphatic vessels and blood vessels in the injury site was higher in the AM group than in the Control group. We determined the expression of VEGF-C and VEGF-A in the injury site after AM administration. Western blot analysis demonstrated that expression of VEGF-C or VEGF-A was not notably affected by AM (0.05 μg/kg/min) administration. In vitro study also demonstrated that the expression of VEGF-C or VEGF-A in HLMVEC was not significantly changed by AM (10⁻⁷ M) stimulation. These results suggest that AM directly promotes lymphangiogenesis and angiogenesis in a mouse model of tail lymphoedema.

4. Discussion

In this study, we demonstrated that (i) AM augmented proliferation and migration of LEC, and that the proliferation induced by AM was mediated at least in part by the cAMP/MEK/ERK pathway and (ii) administration of AM promoted lymphangiogenesis and improved mouse secondary lymphoedema.

AM has a variety of biological effects including angiogenic properties. However, whether AM induces lymphangiogenesis and what is the underlying mechanism responsible for the process remain unknown. In the present study, HLMVEC expressed CRLR, RAMP1, RAMP2, and RAMP3 mRNA. This suggests that AM could stimulate LEC through a complex of CRLR and one of the three RAMPs. Treatment of cultured HLMVEC with AM enhanced cell proliferation. Migration assay and network formation assay also demonstrated that AM enhanced cell migration and network formation. The process of lymphangiogenesis is known to include proliferation and migration of LEC. Therefore, AM-induced lymphangiogenesis is mediated by promotion of proliferation and migration of LEC.

Earlier studies have shown that cAMP plays an important role in proliferation of vascular endothelial cells. In the present study, AM increased intracellular cAMP level in HLMVEC, and the proliferation induced by AM was inhibited by a cAMP antagonist Rp-cAMP. ERK in cultured LEC was markedly activated by treatment with AM. In addition, AM- or 8-Br-cAMP-induced cell proliferation was attenuated by MEK inhibitors, PD98059 and U0126. These results suggest that AM-induced cell proliferation is mediated by cAMP and at least in part through its downstream MEK/ERK pathway. In fact, cAMP-dependent ERK activation has been shown in the proliferation of HUVEC following adenosine receptor stimulation.

We demonstrated the therapeutic potential of AM for lymphoedema using a mouse model of tail lymphoedema. The mouse tail exhibits a highly regular hexagonal network of lymphatic vessels in the skin. Therefore, depletion of circumferential skin in the tail obstructs lymphatic flow, resulting in acute lymphoedema, so this system could serve as a model of secondary lymphoedema to examine lymphangiogenesis in vivo. Mature AM peptide consists of 52...
amino acids in humans and 50 amino acids in the mouse. Mouse AM is 88% identical to human AM. Administration of human AM to the mouse exerts biological effects in vivo. In the present study, continuous administration of human recombinant AM at a rate of 0.05 μg/kg/min promoted recovery of the injury site and promoted a decrease in tail thickness. The number of LYVE-1- or Podoplanin-positive lymphatic vessels and vWF-positive blood vessels in the injury site were significantly increased in AM-treated mice. These findings suggest that AM improves lymphoedema and induces both lymphangiogenesis and angiogenesis. The mechanism of accelerated healing of the tail injury in AM-treated mice is unclear. However, previous studies showed that angiogenesis as well as lymphangiogenesis is crucial in the wound-healing process. Therefore, the accelerated healing of the tail injury may be explained in part by increased blood vessels and lymphatic vessels in AM-treated mice.

VEGF-C and VEGF-A are key factors in lymphangiogenesis and angiogenesis. However, in the present study, AM did not affect the expression of VEGF-C or VEGF-A in the tail tissue or in cultured HLMVEC. These results suggest that AM may directly stimulate LEC and endothelial cells to promote lymphangiogenesis and angiogenesis in our animal model.

In conclusion, AM promoted LEC proliferation at least in part through the cAMP/MEK/ERK pathway. Administration of AM improved secondary lymphoedema, and promoted lymphangiogenesis and angiogenesis. Thus, administration of AM may be a novel therapeutic strategy for patient with lymphoedema.

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